UNIVERSIDAD DE GRANADA
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NEW INSIGHTS ON THE USE OF MAGNETIC PARTICLES FOR LAKE RESTORATION: TOXICITY ASSESSMENT AND EVALUATION OF THE VIABILITY OF RECOVERED PHOSPHORUS AS A FERTILIZER

Tesis Doctoral
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Only if we understand, can we care.
Only if we care, we will help.
Only if we help, we shall be saved.

Jane Goodall

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SUMMARY/RESUMEN

Summary

Phosphorus (P) biogeochemical cycle is being dramatically and worldwide affected by human activities. Nowadays, we are facing two coupled problems: the global exhaustion of P reserves and the P enrichment of inland aquatic ecosystems. Actually, both aspects are strongly connected as P is translocated from terrestrial ecosystems (causing a depletion of P reserves) to aquatic ecosystems promoting eutrophication which represents the most important challenge for water resources managers. P has no substitute in food production and in a world of 9 billion people by 2050, securing sufficient P will be critical for future food security.

Previous studies have confirmed the suitability of using novel adsorbents such as magnetic adsorbents (MPs) for removing P from eutrophicated aquatic ecosystems as they can firstly trap dissolved P with a minimal alteration in water quality (e.g. de Vicente et al., 2010; Funes et al., 2016, 2018) and on the later, P adsorbed on MPs can be recovered. However, before using MPs in a whole-lake restoration project it is essential to get some insights about potential toxic effects on aquatic biota. Accordingly, the first concern of this PhD dissertation was to assess the toxicity of novel P adsorbents used for lake restoration on aquatic biota by using a multi-methodological approach such as standardized laboratory tests and microcosms experiments. Next, we also aim on reverting the problem of the exhaustion of P reserves and lake eutrophication. Considering that one of the most important P sources to eutrophicated aquatic ecosystems is wastewaters discharge, in this PhD we achieve the efficiency of MPs for trapping P in treated wastewaters. Then, we evaluate the viability of recovered P as a liquid fertilizer.

Firstly, our results from single-species acute and chronic toxicity tests using phytoplankton (Chlorella sp. and Raphidocelis subcapitata), zooplankton (Daphnia magna and Brachionus calyciflorus) and benthic organisms (Chironomus sp.) have shown that using MPs for reducing P concentration in lake water and lake sediment is a risk-less (no toxic effect) and efficient (high P adsorption capacity) tool for lake restoration. This statement is based on the higher values measured in this PhD for the concentration of MPs that caused an effect (EC50) or the death (LC50) in 50% of organims compared to the required MPs concentration. At this point, it is worth to note

that MPs concentration to be added in a real lake restoriation project, can be estimated considering the 53 mg MPs: mg P mass ratio as the adsorption efficiency ratio (de Vicente et al., 2010) and the typical concentration of lake water P concentration in eutrophicated ecosystems.

To get a comparison of the toxicity of MPs with other novel P adsorbents (magnetite, Phoslock® and CFH-12®), several toxicity tests were also run. In particular, the acute effects on both the green algae R. subcapitata (growth rate inhibition test) and on D. magna (immobilization) were assessed. To discriminate between chemical and physical effects of adsorbents on D. magna, the immobilization test was run both in direct and in indirect contact (by using a double beaker) with the different P adsorbents. Results evidenced that when organisms were exposed to a direct contact in the D. magna immobilization test, no statistically significant differences were found in the EC50 values among the four studied adsorbents. Additionally, the huge difference between direct and indirect contact experiments suggests that toxicity is mainly physically mediated. Finally, an uptake-depuration test was carried out for assessing the response of D. magna after being in direct contact with adsorbents for 24 h. Fe and La body burdens contents, monitored during 24 h-uptake and 24 h-depuration tests, showed that neither accumulation nor longer term effects of P adsorbents is expected. As a result, the risk for toxicity may, on a case-to-case basis, be acceptable.

In order to get a more realistic effect of MPs addition on the whole zooplankton community, microcosm experiments containing lake water and lake sediment from an hypertrophic ecosystem (Honda lake, Almería) were carried out. Results confirmed that MPs adition did not significantly affect zooplankton total abundance, species richness and species diversity. The absence of any effect of MPs on zooplankton was explained because MPs did not significantly alter any of its physico-chemical (e.g. temperature, pH, O2) or biological (e.g. food quantity and quality) drivers.

Secondly, we have proved the suitability of using MPs for removing P in treated wastewaters. Therefore, high values of both equilibrium adsorption capacity (q) and P removal efficiency of MPs have been measured when adding MPs to treated wastewaters with lastly discharge in the Fuente de Piedra Ramsar site. Accordingly, MPs addition can be proposed as a reliable countermeasure to reduce the impact of wastewater effluents in this Ramsar site. Even more, and considering both advantages (P removal efficiency, %) and disadvantages (economic price, € L-1) of using MPs, we

have identified an optimum ratio $\geq$ 0.16 g MPs mg-1 P when adding MPs to treated wastewaters.

In the last Chapter, and based on both nutrient concentrations used in previous studies and the cost-effectiveness of the method in a long-term, we select 0.1 M NH4OH (neutralized with H3PO4) as the best option for desorbing P from P loaded MPs. Actually, P concentration in the selected conditions was so high that it was necessary to dilute the supernatants, making profitable to use recovered P as a fertilizer. Fertirrigation experiments showed marked differences in the germination patterns among the three plants species. The first species in germinating was O. bassilicum but it later experienced a slower tendency and at the end of the experiment the highest percentage of germination was 80%, which corresponded to the control. The germination of the other two species, C. sativus and C. melo, started later (from day 4) and it generally reached values up to 100% at the end of the experiment. Even more, germination in the different treatments experienced notable differences among plants species. While in O. bassilicum, the germination in the Control was also higher than in the other treatments for any time of the experiment; for C. sativus and C. melo, the percentage of germination in all treatments was higher than in Control, evidencing the key role of adding nutrients for the germination of these species. In general, height and growth rate for the three plants species was significantly lower in control than in treatments. Additionally, it is worth to note that no significant differences were found when using commercial fertilizer or recovered P from the P loaded MPs. Similarly, shoot and root biomass as well as P concentration in shoot was significantly lower in control than in treatments while no significant differences when using a commercial fertilizer and recovered P were found. These results suggest that P desorbed from P loaded MPs can be used as a liquid fertilizer. As a result, our results are promising in order to counteract the widespread and coupled problems of the exhaustion of the P reserves and the eutrophication of aquatic ecosystems.

Resumen

El ciclo biogeoquímico del fósforo (P) se está viendo dramáticamente afectado por las actividades humanas. Hoy en día, nos enfrentamos a dos problemas íntrimamente acoplados: el agotamiento global de las reservas de P y el enriquecimiento en P de los ecosistemas acuáticos continentales. En realidad, ambos aspectos están fuertemente conectados, ya que el P se transloca desde los ecosistemas terrestres, provocando un agotamiento de las reservas de P, a los ecosistemas acuáticos promoviendo la eutrofización. Actualmente, el P no tiene sustituto para la producción de alimentos por lo que es esencial buscar alternativas para que permitan retirar el P de los ecosistemas acuáticos y utilizarlo posteriormente como fertilizante.

Estudios previos han confirmado la conveniencia de usar nuevos adsorbentes como son las partículas magnéticas (MPs) para eliminar el P de los ecosistemas acuáticos eutrofizados, ya que en primer lugar pueden atrapar el P disuelto con una alteración mínima en la calidad del agua (por ejemplo, de Vicente et al., 2010; Funes et al. al., 2016, 2018) y, posteriormente, se puede recuperar el P adsorbido sobre las MPs. Sin embargo, antes de utilizar a las MPs en un proyecto de restauración de todo el lago, es esencial evaluar los posibles efectos tóxicos sobre la biota acuática. En consecuencia, el primer objetivo de esta Tesis Doctoral ha sido evaluar la toxicidad de nuevos adsorbentes de P utilizados para la restauración de lagos sobre la biota acuática mediante una aproximación metodológica interdisciplinar, tanto con test de laboratorio como con experimentos de microcosmos. A continuación, y teniendo en cuenta que una de las fuentes de P más importantes a los ecosistemas acuáticos eutrofizados es la descarga de aguas residuales, en esta Tesis se ha evaluado la capacidad de las MPs para retirar P en aguas residuales tratadas. Finalmente, y en el contexto actual del agotamiento de las reservas de P, se ha evaluado la viabilidad de utilizar el P recuperado de ecosistemas acuáticos eutrofizados como fertilizante líquido.

En primer lugar, los resultados de los tests de toxicidad aguda y crónica con una única especie de fitoplancton (Chlorella sp. y Raphidocelis subcapitata), zooplancton (Daphnia magna y Brachionus calyciflorus) y organismos bénticos (Chironomus sp.) han demostrado que la utilización de MPs para la reducción de la concentración de P presenta una toxicidad reducida sobre los organismos acuáticos. Esta afirmación se basa

en que los valores de la concentración de MPs que causa un efecto (EC50) o la muerte (LC50) en el 50% de los organismos es superior a la concentración de MPs estimada para proyectos de restauración. En este punto, es importante tener presente que la concentración de MPs a adicionar se puede estimar considerando la relación 53 mg MP: mg P (de Vicente et al., 2010) así como la concentración típica en ecosistemas eutrofizados.

Para obtener una comparación de la toxicidad de las MPs con otros novedosos adsorbentes de P (magnetita, Phoslock® y CFH-12®), también se realizaron varias pruebas de toxicidad. En particular, se evaluaron los efectos agudos tanto sobre R. subcapitata (prueba de inhibición de la tasa de crecimiento) como en D. magna (inmovilización). Para discriminar entre los efectos químicos y físicos de los adsorbentes en D. magna, la prueba de inmovilización se realizó en contacto directo e indirecto (utilizando un doble vaso de precipitados) con los diferentes adsorbentes de P. Los resultados han mostrado claramente el predominio de un efecto físico de los adsorbentes sobre los organismos. Finalmente, se llevó a cabo una prueba de asimilación-depuración para evaluar la respuesta de D. magna después de estar en contacto directo con los adsorbentes durante 24 h. La concentración de Fe y de La en los organismos reflejó la ausencia de acumulación así como de efectos a largo plazo de los adsorbentes de P. Por tanto, podemos concluir que el riesgo de toxicidad puede, según el caso, ser aceptable.

Para obtener un efecto más realista de la adición de MPs en toda la comunidad de zooplancton, se llevaron a cabo experimentos de microcosmos que contenían agua y sedimento superficial de un lago hipereutrófico (laguna Honda, Almería). Los resultados confirmaron que la adición de MPs no causa efectos significativos ni en la abundancia total, ni en la riqueza de especies ni en la diversidad de especies del zooplancton. La ausencia de efectos de la adición de MPs sobre la comunidad zooplanctónica puede explicarse por la no alteración ni de los condicionantes físico-químicos (por ejemplo, temperatura, pH, O2) ni biológicos (por ejemplo, cantidad y calidad de alimento).

En relación al segundo objetivo de esta Tesis, las MPs han mostrado una elevada eficiencia para retirar P en aguas residuales tratadas. Esta afirmación se basa tanto en la elevada capacidad de adsorción de P en equilibrio (q) así como en la eficiencia de retirada de P por las MPs. Teniendo en cuenta tanto las ventajas (eficiencia de retirada de P) como las desventajas (coste económico) de usar MPs, hemos identificado que la dosis óptima a utilizar es ≥ 0.16 g MPs mg-1 P.

En el último Capítulo, y tras la comparación de la concentración de P medida en los extractos al utilizar tres diferentes soluciones básicas (NaOH; KOH y NH4OH), podemos concluir que la mejor opción para desorber P es la utilización de 0.1 M NH4OH (neutralizado con H3PO4). De hecho, la concentración de P en las condiciones seleccionadas fue tan alta que fue necesario diluir los sobrenadantes, lo que optimiza el uso del P recuperado como fertilizante.

Finalmente, en los experimentos de fertirrigación con O. bassilicum, C. sativus y C. melo, se observó que la altura y la tasa de crecimiento para las tres especies de plantas fue significativamente menor en el control (sin adición de nutrientes) que en los tratamientos. Más aún, es importante destacar la ausencia de diferencias significativas entre los tratamientos con fertilizante comercial y con P recuperado. De manera similar, la biomasa de las partes aéreas y de las raíces, así como la concentración de P en las partes aéreas fue significativamente más baja en los controles que en los tratamientos, mientras que no se encontraron diferencias significativas entre los tratamientos (fertilizante comercial vs P recuperado). Estos resultados sugieren que el P desorbido desde las MPs puede usarse como fertilizante líquido. Por tanto, las MPs podrían ser porpuestas para contrarrestar la alteración antrópica del ciclo biogeoquímico del P.

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1. Worldwide affection of phosphorus biogeochemical cycle

Although it was predicted a growing imprint of the humanity on the planet from Industrial Revolution, the huge magnitude of this imprint from 1950 has been totally unexpected (Steffen et al., 2015). Actually, human actions have become the main driver of global environmental change and a new era has arisen “Anthropocene”. Contrarily to Holocene where environmental change occurred naturally and Earth´s regulatory capacity maintained the conditions that allow human development, in the Anthropocene human activities are pushing the Earth system outside the stable environmental state with consequences that are detrimental or even catastrophic for large parts of the world (Steffen et al., 2015). In order to establish safety thresholds to avoid these negative effects, planetary boundaries (PB) were proposed as indicators of biophysics boundaries that must not be crossed since the consequences could be catastrophic (Steffen et al., 2015; Terradas, 2009; Butler, 2017).

Among the nine PB recognized by Steffen et al. (2015; Figure 1.1), one of these is related to the interference in the biogeochemical cycle of nitrogen (N) and phosphorus (P). For the case of P cycle, these calculations are mainly based in P loading to oceans due to human action (Terradas, 2009; Rockström et al., 2009). It is striking to note that three of the Earth-system processes- climate change, rate of biodiversity loss and interference with the N cycle- have already transgressed their boundaries while alteration of P cycle is close to be also exceeded.

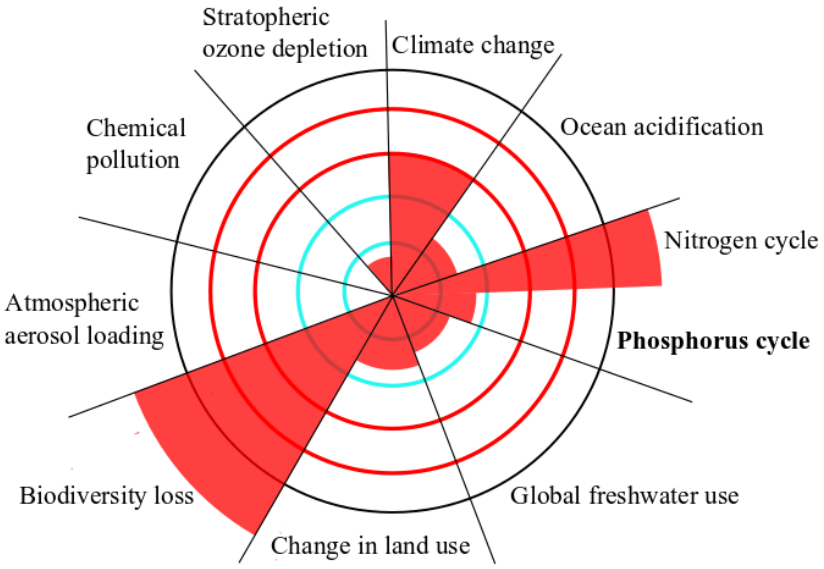


Figure 1.1. Current status of the nine planetary boundaries (modified from Rockstrom et al., 2009 and Steffen et al., 2015). The inner blue shading represents the safe operating space while red zones are an estimate of the current position for each variable.

Humans through industrial and agricultural activities have dramatically altered the biogeochemical P cycle. There exists a clear worldwide translocation of P from terrestrial to aquatic ecosystems (Figure 1.2). By one hand, modern human society depending on a large, continuous supply of mined P to sustain the global food supply (Schroder et al., 2011) is responsible for the global exhaustion of P reserves (Cordell et al., 2011; Gilbert, 2009). By the other hand, mobilized P is moved to the hydrosphere causing the eutrophication of aquatic ecosystems, which actually is the primary worldwide water quality issue (OECD, 1982; Sas, 1989; Cooke et al., 2005; Hupfer and Hilt, 2008).

In relation to the exhaustion of P reserves, Rockström et al. (2009) estimated that 20 million tonnes of P fossil mineral are extracted by year and between 8.5 and 9.5 million tonnes ends up in the oceans which is 8 times the natural influx. This may have as a consequence anoxic ocean events in 1000 years thresh (Rockström et al., 2009). The PB estimates for oceans are 11 million tonnes of P by year, but the critical thresholds have been exceeded for many freshwater systems (Rockström et al., 2009; Carpenter and

Bennet, 2011; Kahiluoto, 2015), while the relating to ocean anoxia and the exhaustion of P reserves are expected to be exceeded in coming years (Carpenter and Bennet, 2011). P has no substitute in food production and in a world of 9 billion people by 2050, securing sufficient P will be critical for future food security (Cordell et al., 2011). Actually, Cordell et al. (2009) estimated that peak P will occur by 2035, after which demand would outstrip supply (Figure 1.3).

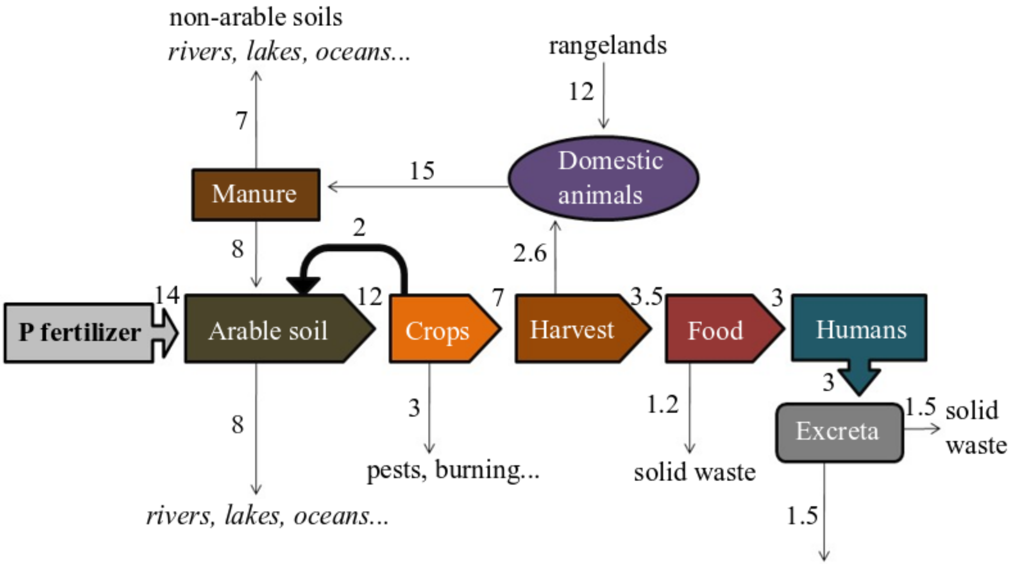


Figure 1.2. Flow diagram of the global P cycle (modified from Elser, 2012). The fluxes units are in MT year^{-1}.

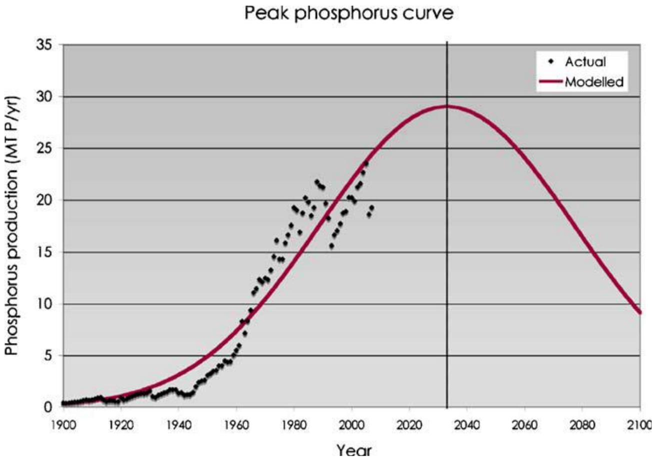


Figure 1.3. Peak phosphorus curve (Cordell et al., 2009).

Despite this bothered tendency, there are some reasons for being optimistic as the current food production and consumption system is highly inefficient with respect to P use and accordingly, some action for reverting this tendency can be still done. As Cordell et al. (2011) stated there is no single solution for achieving a P-secure future. Among others, it is necessary more investments and policies for increasing P use efficiency and for recovering and reusing P from all current waste streams throughout the food production and consumption system (Ragnarsdottir et al., 2011; Carpenter and Bennet, 2011; Elser, 2012; Whiteman et al., 2013). Additionally, some other possible strategies for not crossing this boundary have been proposed such as a lower irrigation and fertilizer use (Running, 2012); an effective population; redistribution of P (Steffen et al., 2015); the quantification of the effects of industrial and agricultural processes on P cycle (Whiteman et al., 2013); the reduction of P losses or the reduction of our dependency on P reserves (Conjin et al., 2018). It is clear that, overall, we need a comprehensive analysis considering the entire food system as suggested by Conijn et al. (2018). Although these authors found that the combination of all measures would be able to partly prevent transgression of the boundary, among others, for P flow into the ocean; global mineral P fertilizer inputs to water still exceeded its boundary in their study.

Apart from the worldwide depletion of P reserves, eutrophication is the other key challenge in the field of natural resources to be faced. It promotes negative effects both in the ecological and economical dimension (i.e. annual costs just in USA of approximately $2.2 billion; Dodds et al., 2009). As a result of eutrophication, the unbalanced ecosystem and changed chemical composition make the water body unsuitable for recreational and other uses, and the water becomes unacceptable for human consumption). Due to the complexity of the phenomena, the lack of the consistent data sets and the lack of a harmonised approach to assess eutrophication, the severity and extent of the problem had not been adequately characterized at an international level. In Europe, the Water Framework Directive (WFD – 2000/60/EC) provides a common framework to protect, manage and restore surface and groundwater. WFD prescribed as a legal requirement by 2015 that all water bodies must have achieved "good status" (Heiskanen et al., 2004). However, for the case of Spain, the first national overview of the ecological, chemical and overall status of surface water bodies (rivers, lakes, transitional and coastal), evidenced that almost 50% of all surface waters in Spain are in poor status and 43% of surface water bodies have not yet been evaluated (Willaarts et al., 2014). In order to achieve the legal requirements, implementation of appropriate tools and effective restoration methods are essential.

2. State of the art: strategies for restoring eutrophicated aquatic ecosystems

The essential and preliminary strategy for combating eutrophication by controlling algal biomass is the reduction of lake water P concentration, which is the main limiting nutrient for the primary production (e.g. Hupfer and Hilt 2008; Schindler et al., 2008). Nowadays, three different methods for reducing lake water P concentrations have been proposed (Hupfer and Hilt, 2008): (1) reduction of external P load, (2) increase of P retention capacity by sediment, and (3) increase of P export.

Among all of them, it is considered that the keystone of lake restoration is the reduction of external P load to annual average inlet concentration lower than 0.1 mg P L^{-1} (Reitzel et al., 2003; Cooke et al., 2005; Jeppesen et al., 2009). However, in most of the cases, the shift to a good ecological status requires a reduction of external P inputs to lower levels than found before the collapse (restoration hysteresis). This is a requirement that may be not feasible due to the dominance of non-point sources or the high cost implied (Deppe and Benndorf, 2002; Scheffer and Carpenter, 2003). The European Environmental Agency (EEA) recognized that the main source of N pollution is run-off from agricultural land, whereas most P pollution comes from households and industry. The rapid increase in industrial production and household consumption during the 20th century has resulted in greater volumes of nutrient-rich wastewater. Nutrient removal during wastewater treatment in sewage-treatment plants is vital to reduce the impact of N and P pollution on Europe’s water bodies. Before discharging wastewater into water bodies, P removal is usually obligatory, even though in many cases it is not performed (de-Bashan and Bashan, 2004). Up to date there exist many technologies for removing P from wastewater being the chemical precipitation (CP) and biological nutrient removal (BNR) the two most common methods (Oguz et al., 2003; Ping et al., 2008). Essentially, these methods are based on transferring P ions from the liquid phase to a solid fraction (sludge phase) which needs to be subsequently transported and disposed of elsewhere (Ping et al., 2008). Although recent studies are actually focused on assessing P removal from wastewaters by novel adsorbents such as zeolites (Ping et al., 2008) and struvite (de-Bashan and Bashan, 2004); we are still far for recognizing the best P adsorbent in wastewaters. Accordingly, it is necessary to continue developing new techniques in order to achieve an environmentally acceptable level with affordable costs.

A promising technology for treating wastewater is based on the effective role of wetlands as natural water purifiers. In this sense, constructed wetlands technology has been developed since 1980s (Kadlec and Knight, 1996; Kadlec, 2009). By definition, treatment wetlands are constructed ecosystems designed primarily to enhance physical, chemical and biochemical processes with the goal of reducing specific contaminants to acceptable levels (Kadlec, 2009). Despite the range of usage of wetland treatment technology is large and still expanding, the largest number and the longest history belong to the class that threats municipal and domestic wastewaters. A good example of the high efficiency of semi-natural ponds for treating secondary municipal effluents is exposed by de los Ríos-Mérida et al. (2017) in the Ramsar Fuente de Piedra wetland. This recent study has quantified a notable reduction in total N (23%) and total P (40%) concentrations in the water along it flows through the system containing three semi-natural ponds. However, it is essential to consider that despite the notable improvement in the quality of the treated wastewater, Fuente de Piedra is still characterized by extremely high values of total N and total P (Batanero et al., 2017) so much more efforts for reducing P inputs to the lake must be accomplished.

Apart from reducing external P load which is actually the first and necessary step for achieving the success of any restoration program of eutrophicated ecosystems (Jeppesen et al., 2009; Smith 2009), internal P loading frequently has a considerable impact on lake water P concentrations (Phillips et al., 1994). In fact, internal P loading is considered as an important factor explaining the delay in lake recovery after a reduction of external P load in lakes with a long eutrophication history (Søndergaard et al., 2003). The P exchange between sediment and water column is influenced by biological (bacterial activity, mineralization processes, and bioturbation), chemical (redox potential, pH, Fe:P ratio, nitrate availability), and physical factors (resuspension and sediment mixing; Søndergaard et al., 2001).

Next, we will focus on P sedimentary retention by P inactivation. P inactivation mechanism by salts addition consist on spreading alum (Al), iron (Fe) or calcium (Ca) salts into a lake with pipes, by boats and airplanes or aeration devices to increase the number of adsorption sites for P uptake (Hupfer and Hilt, 2008). Soluble P is precipitated as low soluble salts or adsorbed by colloidal aggregates whereas particulate P (algal biomass) is coagulated and settle to the lake bottom sediments. Once they reach the surface sediment, they may continue to act to reduce diffusion of dissolved P from the lake bed to the water column. A more novel concept related to P inactivation is refereed as "geoengineering" which is defined as "*the deliberate manipulation of lake processes using natural and engineered amendments to induce a desired chemical or ecological outcome" (Mackay et al., 2014)*. As these authors stated, the premise of geoengineering is to get a rapid reduction in P concentrations in the water column for lastly improve the ecological structure and function of the system. The range of products is growing and include engineered materials, commercially available salts, modified local soils and industrial by-products (Hickey and Gibbs, 2009). Among all these products, 50 lakes have been treated with Al-based compounds in the USA over the last 5 years, while in 2013, Phoslock©, a lanthanum (La)-modified bentonite clay product, was added to at least 30 lakes across the world (Mackay et al., 2014).

More in detail, Phoslock® (CSIRO, Finsterle, 2014) has a high capacity to adsorb P from water column and to increase P-sorption capacity of the sediment (Robb et al., 2003; Meis et al., 2012). Among its advantages, Phoslock® has shown to be efficient in a wide pH range (5-9) and even under anoxic conditions (Robb et al., 2003; Meis et al., 2012). Because of that, it is being increasingly used for lake restoration with promising results in P reduction in the water column (Douglas et al., 2000; Robb et al., 2003; Spears et al., 2016) and causing a decrease in cyanobacteria abundance (Lang et al., 2016). However, Phoslock® addition has also shown some weakness such as chemical interferences with humic substances or oxyanions (Lürling and Tolman, 2010; Lürling et al., 2014) which may cause a reduction in its effectiveness (Ross et al., 2008; van Oosterhout and Lürling, 2013) and it is not cost-effective (Spears et al., 2013).

More recently, an amorphous Fe oxide-based material (CFH-12®; Kemira) has been proposed to be used in eutrophicated lakes as it is characterized by a high P adsorption capacity (27.59 mg P g^{-1}; Lyngsie et al., 2014); its independency of redox conditions and it does not change medium pH (Fuchs et al., 2018). Additionally, previous studies have shown that the natural zeolites had negligible affinity for P due to the presence of structural negative charges on the zeolite framework (Hrenovic et al., 2008; Lin et al., 2011). However, modification of zeolites with components with high affinity for P may increase their adsorption capacity. Some novel modified zeolites are Aqual-P® (Blue Pacific Minerals, Matamata, NZ) which is an Al-modified zeolite (Gibbs et al., 2011); a zirconium-modified zeolite (ZrMZ; Yang et al., 2014) and La oxide-modified zeolite obtained from fly ash (Wang et al., 2016). All of them have shown a high P removal efficiency, although ZrMZ was only efficient in neutral or low alkaline conditions (Yang et al., 2014). Finally, another promising adsorbent is Bephos®, which is a natural bentonite with Cu, Fe and humic acids embedded in its interlayer (Zamparas et al., 2013). Although it has a high P adsorption capacity and P removal efficiency in a wide range of pH (5-9), it is still needed to investigate its efficiency in natural conditions as well as if it is safe for biota lake.

All in all, it is clear that the effectiveness to remove P of most P-sorbing materials is dependent on the pH, potential redox, and/or presence of other dissolved ions (Westholm 2006; de Vicente et al., 2008; Vohla et al., 2011) and accordingly, more research is required as no universally valid P-adsorbent exist. In her PhD, Funes (2016) summarizes some of the diverse drawbacks of P adsorbent application: (i) reduction of P adsorption efficiency with ageing (Al) and the increase of toxicity at high pH values found in eutrophic waters due to solubilization (Al) or competition with hydroxyl groups (Fe and Al; Marsden, 1989; de Vicente et al., 2008; Egemose et al., 2009; Reitzel et al., 2013), (ii) redox sensitivity of Fe (Kleeberg et al., 2013), (iii) reduction of the lake water pH due to formation of Al and Fe flocs (Hupfer and Hilt, 2008; Reitzel et al., 2013), (iv) reduction of sediment stability hindering macrophytes colonization by the presence of fresh Al floc (Egemose et al., 2009; Egemose et al., 2010), (v) complementary measures such as in-lake addition of buffer substances or frequent aeration and oxygenation are often needed (Hupfer and Hilt, 2008), (vi) short/medium-term effectiveness of adsorbents, being more long lasting Al (5-15 years) than Fe effects (Huser et al., 2015), and (vii) the potential mobilization of inactivated P under changing physico-chemical conditions such as pH, redox potential, temperature, biological activity or resuspension (Søndergaard et al., 1992; Perkins and Underwood, 2001).

3. Recent alternatives for worldwide depletion of phosphorus reserves

Apart from the need for restoring eutrophicated systems, it is essential to develop new strategies for combating the exhaustion of worldwide P reserves. The sustainable P use is straightforward linked to other challenges such as food security or freshwater use among others. Actually, one of the ways to reduce both P demand and greenhouse gas emissions is changing diets from high meat-based to more plant-based diets, which also may decrease the consumption of natural resources. This can be considered as one of the essential changes that could decrease our high P demands (Reijnders, 2014; Neset and Cordell, 2012).

Moreover, high concentrations of P are lost because of several deficiencies being essential to improve the efficiency along the whole production cycle from the mining stage and fertilizer production to the agricultural practices (Neset and Cordell, 2012; Reijnders, 2014). As an illustration, Gilbert (2009) quantified that between 40% and 60% of P is lost when its host rock is converted to fertilizer. To counteract the loss of P from agricultural soils occurred by runoff, some strategies have been proposed such as the addition of crops residues, cropping of perennials, using cover crops and contour cultivation (Reijnders, 2014). In relation to agricultural practices, it is also worth to note that while the USA and Europe, in the last two decades, have reduced the widespread over-application of fertilizers; in other countries such as China, farmers are applying nearly twice as much fertilizer as is needed in the production of wheat (Gilbert, 2009).

Other measures for preserving P resources could come through P reuse from manure, organic waste or human excreta (Neset and Cordell, 2012). Even more, the residues and solid wastes from harvest, food and feed processing plants may be used for the reutilization of P in agricultural soils (Reijnders, 2014). Wastewaters are specially P enriched being crucial to recover P from them. Several authors have described different ways to recover P from wastewaters such as nanofiltration (Blöcher et al., 2012) and sorption-based technologies (Hermann, 2009; Loganathan et al., 2014; Shimamura et al., 2013). However, it is needed to test the efficiency of the technology for desorbing and purifying P before its reutilization. Moreover, the ashes generated in the wastewater sludge incineration have been also considered as a source for P recovery, though this process eliminates essential plant nutrients (Zhang et al., 2001; Reijnders, 2014).

Finally, struvite has been proposed as a promising method for recovering nutrients (N and P) from wastewater. It is produced by spontaneous precipitation from wastewater, reducing high concentrations of ammonium and phosphate salts (Wang et al., 2015) and being able to be used later as a sustainable fertilizer (Sena and Hicks, 2018). However, although several authors have obtained promising results in fertilization experiments by using struvite as a fertilizer (Johnston and Richards, 2003; Barak and Stafford, 2006; Plaza et al., 2007; Kataki et al., 2016), it has been mentioned that struvite production is difficult and non-cost effective (Hao et al., 2013; Sena and Hicks, 2018). In any case, more research about its potential toxicity is required (Sena and Hicks, 2018).

4. Novel magnetic adsorbents for improving water quality

The main shortcoming of the mentioned P adsorbents, is that none of them can be recovered from the treated system and therefore, P is lost in the sediment. To counteract this limitation, new magnetic adsorbents have been recently proposed for recovering P from eutrophicated systems. In this context, magnetic particles (MPs) have been proposed for lake restoration as they adsorb P and later, P loaded MPs can be removed from medium by using a magnetic separation gradient (de Vicente et al., 2010a, 2011; Funes et al., 2016, 2017; Merino-Martos et al., 2011, 2015). Even more, de Vicente et al. (2010a) found that when washing P loaded MPs in basic solutions, MPs still adsorb P in a 2nd adsorption cycle. Reusing MPs would then notable reduce the initial investment and it would also allow the potential P recovery as a liquid fertilizer.

According to previous studies, MPs are characterized by the next advantages: (i) their effective adsorption of P under both batch and flow conditions (de Vicente et al., 2010a; Merino-Martos et al., 2011); (ii) their specific capacity to adsorb considerable amounts of P at pH > 7 despite being negatively charged (Daou et al., 2007; de Vicente et al., 2010a); (iii) the non-dependence on redox conditions of their P adsorption (Funes et al., 2016); (iv) the reduction in sedimentary mobile P concentrations caused by their addition (under both oxic and anoxic conditions), potentially contributing to a long-term reduction in P efflux; (v) their lesser cost, considering the maximum P adsorption capacity and price of each adsorbent, compared to other P adsorbents such as $AlCl_3 \cdot 6H_2O$ or Phoslock® if reused more than twice, (Funes et al., 2016); and (vi) the low toxic effects on plankton and benthic organisms (Álvarez- Manzaneda et al., 2017).

Although the chemical composition of natural and wastewaters drastically differ, our working hypothesis is that MPs addition increase P removal in treated wastewaters. Therefore, MPs become an excellent alternative for P removal in wastewaters as recovered P can be potentially desorbed and recovered for its later use as a fertilizer. At this point, it is important to consider that P removal from P enriched waters and its later reuse, would perfectly fit in the framework of the recent concept of bioeconomy. The European Commission defines the bioeconomy as "*the production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and bioenergy. Its sectors and industries have strong innovation potential due to their use of a wide range of sciences, enabling and industrial technologies, along with local and tacit knowledge*" (EC, 2012). More recently, Aguilar et al. (2018) considered that for the development of smart bioeconomies, excellent innovative and mission-oriented research needs to be supported long-term and stimulated by all stakeholders in science, industry and society.

5. The need for assessing toxicity of new adsorbents

Although the use of novel P adsorbents for restoring eutrophicated ecosystems has increased in the last years, it is essential to remark the lack of toxicological studies focused on assessing their effects on lake biota. Up to date, few studies about lethal and sublethal effects of Phoslock® on planktonic organisms there exist (Lürling and Tolman, 2010; van Oosterhout and Lürling, 2013; Yamada-Ferraz et al., 2015). Therefore, in this PhD, we consider that once the chemical properties of MPs have been throughly studied (among others, de Vicente et al., 2010a; 2011; Funes et al., 2016; 2018), it is worth to evaluate potential toxic effects before a whole-lake MPs application is considered.

A wide spectrum of methodological approaches for testing toxicity does exist. The procedures currently in use for conventional risk assessment have a first step that consists in the identification and characterization of hazards based, among others, in basic toxicity tests (Amiard-Triquet et al., 2015). However, although single-species toxicity tests performed under laboratory conditions are easier to carry out, they also exhibit marked limitations: (i) they are focussed on sensitive species and accordingly, they are not representative of the response of the natural community (Cairns and Pratt

1993; Rohr et al., 2016); (ii) the difficulty of recording population dynamics that occurs at longer times than the duration of laboratory tests and (iii) it is difficult to identify effects on structure and function of the ecosystems due to the simplicity of these tests (Van den Brink et al., 2005). Because of these reasons, it is essential to also assess the fate and effects of chemicals on different levels of organization by performing outdoor microcosm experiments that resemble natural ecosystems (Figure 1.4).

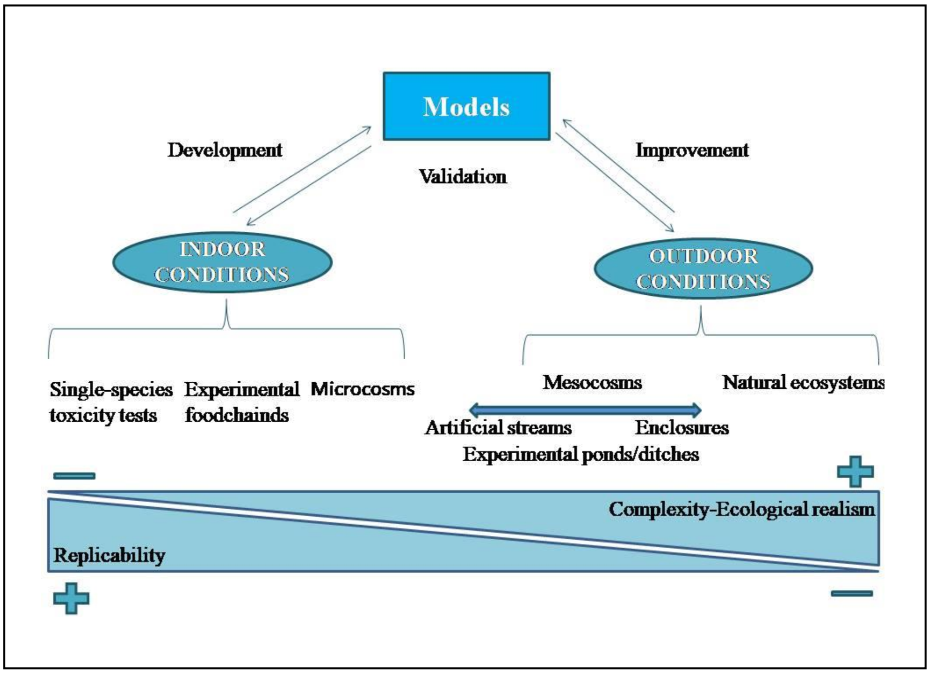


Figure 1.4. Experimental models performed in aquatic toxicology (modified from Caquet 2013)

In this context, the toxicity of novel P adsorbents (MPs; magnetite; Phoslock® and CFH-12®) on aquatic biota has been assessed by using a multi-methodological approach such as standardized laboratory tests and microcosms experiments.

Firstly, single-species toxicity tests were run by using different phytoplankton, zooplankton and benhic species. Algae were chosen due to the following consideration: (a) they belong to the first level of the trophic chain and so, any change in the composition and density of the phytoplankton could change the biological and chemical quality of an ecosystem (Lewis, 1995); (b) they seem to be more sensitive for some contaminants than animal species (Hoffman et al., 2003) and (c) they have a short life cycle, allowing the evaluation of toxic effects over several generations (Silva et al., 2009). At this point it is important to consider that the need for these studies is that MPs addition may alter environmental (physical) and nutritional factors (chemical) affecting the growth of microalgae and accordingly, the last effect t must be assessed before adding MPs in a whole-lake restoration plan. Among algae, *Chlorella* sp. (cell volume: 365 µm^3; diameter: 8.8 µm) and *Raphidocelis subcapitata* (40 to 60 µm^3) were selected as the test species because these unicellular green algae have a good sensibility to toxicants and they are easily cultured at laboratory (Rojickova and Marsalek, 1999; Ma et al., 2006; Silva et al., 2009).

Next, we evaluate the effects of MPs addition on one of the main components of the biological communities of aquatic systems, which is the zooplankton. In short, the function of zooplankton in aquatic food webs is crucial as: (i) it acts as a link between phytoplankton and secondary consumers (fish and some birds; Conde-Porcuna et al., 2004) as herbivorous zooplankton control phytoplankton community but it also represents great value resources for planktivorous fish and other organisms (Christoffersen et al., 1993; Jeppesen et al., 1999); and (ii) zooplankton actively participates in nutrients (N and P) recycling (Sterner 2009). Even more, zooplankton assemblages provide good elements for biogeographical studies (Xu et al., 2011) that may guide conservation polices needed to assess future shifts in biodiversity and community structure caused by anthropogenic impacts (Gilbert et al., 2014, 2015, 2017). As a result, zooplankton community has been proposed as an important criterion for wetlands conservation (Jeppesen et al., 2011). Despite of the key role of this community in aquatic ecosystems, there is a lack of studies focused on the assessment of the effect of P adsorbents used for lake restoration on zooplankton community. This is a relevant aspect as P adsorbents may direct (i.e. physical effects) or indirectly (i.e. by reducing phytoplankton biomass) affect to the zooplankton and the last effect of P adsorbents must be test by using more complex and realistic approaches than single-species toxicity tests. Among zooplankton, *D. magna* was selected as a test organism as it is widely used in toxicity tests due to it sensibility to contaminants (e.g. Khangarot and Ray, 1987; García et al., 2011) and because of its size, high fecundity, parthenogenetic reproduction, short life-cycle and its relatively facility for culturing (Núñez and Hurtado, 2005). In addition, and considering that MPs addition makes sense just in eutrophicated systems where the zooplankton community is dominated by rotifers instead of cladocerans (Gannon and Stemberger, 1978), *Brachionus calyciflorus* as a representative of rotifers, was considered as a test organism. Rotifers have been widely used in toxicological tests since they are good toxicity indicators (Alvarado-

Flores et al., 2012) such as: (i) acute toxicity tests; (ii) life-cycle tests and (iii) tests on suborganismic level (Dahms et al., 2011). Also, they have a key role in the ecosystems so that they are used as models in ecotoxicology (Snell and Janssen, 1995). Their short-life cycle, rapid reproduction (Fernández-Casalderrey et al., 1991) and the facility to conserve their cysts for long time, make them very suitable for toxicology.

In addition, benthic macroinvertebrates are a very suitable community to carry out ecotoxicological tests due to their easy collection, relatively slow mobility and their life expectancy, very useful for chronic toxicity tests (Iannacone and Alvariño, 2004). In particular, larvae of *Chironomus* sp. have a great ecological relevance for ecotoxicological researches (Iannacone and Alvariño, 2004).

Finally, it is important to keep in mind that the application of MPs for lake restoration may involve two kind of interaction with lake biota: i) direct and short-term effect caused by MPs and ii) indirect and long-term effect caused by the dissolved Fe (Tot-Fe_{dis}; after MPs removal). Therefore, it is essential to assess the potential effect of MPs and Tot-Fe_{dis}. Accordingly, in this PhD we combine both acute, which are mostly based on mortality as endpoint, and sublethal toxicity tests looking at growth and/or reproduction of the biota (Table 1.1). Even more, and although the majority of standardized ecotoxicity tests and biomonitoring in aquatic systems are based on the active component of invertebrate communities, dormant egg banks are crucial for the long-term survival and community dynamics of many aquatic organisms (Navis et al., 2013). In fact, the invertebrate dormant egg banks in the sediments of aquatic ecosystems constitute ecological and evolutionary reservoirs of species (De Stasio, 1989; Hairston and Munns, 1984; Hairston, 1996). Among invertebrate communities, rotifers are important components of such egg banks in freshwater systems and accordingly, the last effect of Tot-Fe_{dis} on hatching rate of *B. calyciflorus* has been also evaluated.

Table 1.1. Summary of the toxicity tests achieved during this PhD with different planktonic and benthic organisms. HQ: carbonyl Fe magnetic particles; Fe_3O_4: magnetite.

Test species	**End point**	**Adsorbent**	
Daphnia magna	Immobilization	HQ	*Chapter I*
Daphnia magna	Reproduction	HQ	*Chapter I*
Daphnia magna	Immobilization	HQ, Fe_3O_4, CFH-12®, Phoslock®	*Chapter III*
Daphnia magna	Immobilization (indirect contact)	HQ, Fe_3O_4, CFH-12®, Phoslock®	*Chapter III*
Daphnia magna	Uptake and depuration	HQ, Fe_3O_4, CFH-12®, Phoslock®	*Chapter III*
Brachionus calyciflorus	Mortality	HQ	*Chapter II*
Brachionus calyciflorus	Cysts hatching	HQ	*Chapter II*
***Chironomus* sp.**	Immobilization	HQ	*Chapter I*
***Chironomus* sp.**	Life cycle	HQ	*Chapter I*
***Chlorella* sp.**	Growth inhibition	HQ	*Chapter II*
Raphidocelis subcapitata	Growth rate inhibition	HQ	*Chapter III*
Zooplankton whole community	Total abundance, species richness, species diversity	HQ	*Chapter IV*

OBJECTIVES

2. OBJECTIVES

The main objectives of this PhD dissertation were:

(i) To assess the toxicity of novel P adsorbents used for lake restoration on aquatic biota by using a multi-methodological approach such as standardized laboratory tests and microcosms experiments *(Chapters I-IV)*

(ii) To achieve the efficiency of MPs for trapping P in secondary municipal effluents which lastly discharge in a Ramsar site (Fuente de Piedra, Málaga, Spain) *(Chapter V)*

(iii) To evaluate the viability of recovered P as a liquid fertilizer *(Chapter VI)*

The specific objectives were:

1. To evaluate both the acute (immobilization) of MPs and the chronic effects of dissolved Fe (Tot-Fe_{dis}) on planktonic (*D. magna)* and benthic species (*Chironomus* sp.) widely used in toxicity tests *(Chapter I).*

2. To assess the acute (algal growth) effects of MPs on the freshwater green algae *Chlorella* sp. and to identify potential drivers responsible for algal toxicity by monitoring physicochemical changes *(Chapter II).*

3. To evaluate both acute (mortality) of MPs and the chronic (hatching rates) effects of Tot-Fe_{dis} on the rotifer *B. calyciflorus (Chapter II).*

4. To assess, by using short-term laboratory tests, the acute effects of two magnetic (carbonyl iron and magnetite) and two non-magnetic P adsorbents (Phoslock® and CFH-12®) on *Raphidocelis subcapitata* (growth rate inhibition test) and on *D. magna* (immobilization; *Chapter III).*

5. To discriminate between chemical and physical effects and to quantify the uptake-depuration dynamic of *D. magna* in contact with four different P adsorbents *(Chapter III).*

6. To monitor temporal changes on physico-chemical conditions and on zooplankton composition and structure as a result of MPs addition *(Chapter IV).*

7. To assess the effect of adding MPs on the chemical composition of treated wastewaters and to select, based on, advantages (P removal efficiency, %) and disadvantages (economic price, € L^{-1}), the optimum ratio between MPs mass and initial dissolved inorganic P (DIP) concentration *(Chapter V).*

8. To identify the best working conditions for desorbing P from P loaded MPs previously used in a hypertrophic lake (*Chapter VI).*

9. To compare germination, height, growth rate, shoot and root biomass and P concentration in shoots and roots of three different plant species (*Ocimum basilicum, Cucumis sativus and Cucumis melo)* when using a commercial fertilizer and P recovered from a hypertrophic lake *(Chapter VI).*

GENERAL METHODOLOGY

3. GENERAL METHODOLOGY

1. General characterization of magnetic and non magnetic phosphorus adsorbents

In this PhD, we have used four different P adsorbents: two magnetic (HQ and Fe_3O_4) and two non-magnetic P adsorbents (Phoslock® and CFH-12®). All toxicity tests were run by using HQ (*Chapters I-IV*) but in *Chapter III*, the other adsorbents were also considered. The most important features of the four selected P adsorbents are shown in Table 3.1. All were commercially supplied. They greatly differ in their chemical composition, size and maximum P adsorption capacity. Briefly, HQ and Fe_3O_4 have spherical shape, relative polydispersion and a ferromagnetic behavior. CFH-12®, manufactured by Kemira (Oyj, Finland), is a dried amorphous solid consisting of poorly ordered Fe oxides (Fuchs et al., 2018; Lyngsie et al., 2014). Finally, Phoslock® is a La-based (5% La, 95% clay) engineered product in which the presence of Fe and Al apart from La, increases the number of P binding sites (Reitzel et al., 2013).

Table 3.1. Main physico-chemical features of the P adsorbents used for this study. Concentrations (total and atomic surface) have been taken from Funes et al. (2018).

Adsorbent	Total [1] and surface [2] concentration (%)	Size	Maximum P adsorption capacity (mg g^{-1})
HQ	Fe (97.5); C (0.9); O (0.5); N (0.9)[1]	805 ± 10 nm (de Vicente et al., 2010a)	18.83 (de Vicente et al., 2010a)
Fe_3O_4	C (59); Fe (10); O (31)[2]	50-100 nm (manufacturer)	5.85 (Funes et al., unpublished results)
CFH-12®	O (59); Fe (28); C(9); S (2); Ca and Mg (<1)[2]	0.85-2 mm (Fuchs et al., 2018)	15.1 (Funes et al., 2018)
Phoslock®	O (66); Si (19); C (6); Al (6); Mg, Na, Ca, Fe and La (<1)[2]	2-4 x 1-3 mm (Haghsereht, 2005)	13.6 (Funes et al., 2018)

2. Single-species toxicity tests for testing magnetic particles effects

2.1. On phytoplankton organisms

✓ Test organisms

Laboratory experiments were carried out with two different species of green algae: *Chlorella* sp. and *Raphidocelis subcapitata* (Korshikov). The need for using phytoplankton toxicological tests is based on its sensitivity to contaminants (Hoffman et al., 2003) and its key role in the aquatic ecosystems (Lewis, 1995). *Chlorella* sp. (cell volume: 365 mm^3; diameter: 8.8 mm) was selected as the test species because this unicellular green alga has a good sensibility to toxicants and it is easily cultured at laboratory (Silva et al., 2009). By the other hand, *R. subcapitata* is an unicellular green algae (Chlorophyta) with crescent-shaped (40-60 µm^3), which can be found in eutrophic or oligotrophic epicontinental aquatic systems (Granados et al., 2004). Further details about culture conditions can be found in *Chapters II* (*Chlorella* sp.) and *III* (*R. subcapitata).*

✓ Toxicity Tests

The acute effects of MPs on *Chlorella* sp. and on *R. subcapitata* were assessed by using algal growth inhibition tests. Considering that MPs are efficient P adsorbents and they may therefore affect nutrient availability for phytoplankton, during the *Chlorella* sp. experiments a through monitoring of physicochemical changes in the aqueous solutions was accomplished. In particular, at the end of the experiment, physico-chemical (conductivity, pH and dissolved oxygen concentration) and chemical (dissolved inorganic nitrogen, dissolved inorganic P and Tot-Fe_{dis}) variables were monitored. In the *R. subcapitata*, acute effects of the other three P adsorbents (Fe_3O_4; Phoslock® and CFH-12®) were also tested.

2.2. On zooplankton organisms

- ✓ **Test organisms**

Two different zooplankton species were considered in this PhD: *Daphnia magna* which is a cladoceran widely used in toxicity tests and *Brachionus calyciflorus,* a rotifer very frequent in eutrophic ecosystems. *D. magna* was selected based on its sensibility to contaminants (e.g. Khangarot and Ray, 1987; García et al., 2011) and because of its size, high fecundity, parthenogenetic reproduction, short life-cycle and its relatively facility for culturing (Núñez and Hurtado, 2005). By the other hand, *B. calyciflorus,* as rotifer, is characterized by a short-life cycle and a rapid reproduction (Fernández-Casalderrey et al., 1991) and their cysts can be store dried for long time. Further details about culture conditions can be found in *Chapters I, III and IV).*

- ✓ **Toxicity tests**

Regarding *D. magna*, in *Chapter I*, we analyzed the acute and chronic effects of MPs and of Tot-$Fe_{dis,}$ respectively. First, direct effects of MPs on daphnids immobilization (when animals are not able to swim within 15 s, after gentle agitation of the test vessel) were recorded after 24 and 48 h of contact time. Second, suspensions containing different MPs concentrations were prepared and after 24 h, MPs were removed by using magnetic techniques. Next, and with the remaining solutions (containing Tot-Fe_{dis}) the reproduction test (a slight modification of 211 OECD, (OECD, 2012)) was run. Later on, in *Chapter III,* we aim on discriminating between chemical and physical effects of four different P adsorbents (HQ; Fe_3O_4; Phoslock® and CFH-12®) on *D. magna*. For that purpose, the immobilization test was run both in direct and in indirect contact (by using a double beaker, Skjolding et al., 2016) with the different P adsorbents. Finally, in *Chapter III*, an uptake-depuration test was also carried out for assessing the response of *D. magna* after being in direct contact with adsorbents for 24 h. In particular, temporal changes in Fe and La body burdens contents were monitored during 24 h-uptake and 24 h-depuration tests.

For the case of *B. calyciflorus,* acute toxicity and hatching tests were run (*Chapter II*). Mortality test was carried out during 24 hours with ten individuals (<2 h) of B. calyciflorus in contact with different MPs concentration and following American Society for Testing and Materials International (ASTM) standardized protocol (Allen, 1998). Then, and considering that although the majority of standardized ecotoxicity tests and biomonitoring in aquatic systems are based on the active component of invertebrate communities, dormant egg banks are crucial for the long term survival and community dynamics of many aquatic organisms (Navis et al., 2013), hatching test were carried out using cysts (from MicroBioTests, Gent, Belgium). The rationale behind this test is the fact that the invertebrate dormant egg banks in the sediments of aquatic ecosystems constitute ecological and evolutionary reservoirs of species (De Stasio, 1989; Hairston and Munns, 1984; Hairston, 1996) and accordingly, potential toxic effects must also be tested.

2.3. On benthic macroinvertebrates

✓ Test organisms

Benthic macroinvertebrates are a very suitable community to carry out ecotoxicological tests due to their easy collection, relatively slow mobility and their life expectancy (Iannacone and Alvariño, 2004). In particular, larvae of *Chironomus* sp. have a great ecological relevance for ecotoxicological researches (Iannacone and Alvariño, 2004). In *Chapter I*, up to 100 individuals of *Chironomus* sp. were collected from river Beiro (Granada, Southern Spain) using a kick net with 250 µm of mesh. More details about culture conditions can be found in *Chapter I*.

✓ Toxicity tests

Both acute and chronic toxicity tests were run following OECD standardized methods. Briefly, while in the acute test, *Chironomus* sp. was directly exposed to MPs for 48 h, in the chronic test organisms were not in contact with MPs but with the Tot-F_{edis}. In this case, the test last 30 days, and every day the number of dead larvae, pupae and the adults’ emergence were recorded.

3. A community approach for testing magnetic particles effects on zooplankton

*3.1. **Study site***

Apart from single-species toxicity tests, it is essential to get an insight about the response of the whole zooplankton community to MPs addition. To achieve this goal, microcosm experiments (15 PVC black containers; Ø = 38 cm; h = 58 cm) were developed by using lake water (40 L) and lake sediment (6600 cm^3) from a hypertrophic lake: Honda lake (Almería, Spain; *Chapter IV*). Honda lake is a well study shallow (surface area = 9 ha, Z_{mean} = 1.3 m; Z_{max} = 3.2 m) and hypertrophic wetland (Carrillo et al., 1987; Cruz-Pizarro et al., 2003; de Vicente and Cruz-Pizarro, 2003; Funes et al., 2016). It is included in Albufera de Adra Natural Reserve, a Ramsar site that represents one of most important wetlands in south Spain. Its high trophic state is the result of both, the great extension devoted to intensive agricultural practices in its catchment area, which is responsible for the high external P load; and the relevance of P release from the lake sediment (internal P loading; de Vicente et al., 2006, 2010b).

3.2. Sampling and microcosms monitoring

Briefly, on July 2015, 100 dm^3 of surface sediment were collected with an Ekman dredge at the deepest site of the lake. Surface water was also collected near the shore with a peristaltic pump and kept at 14 °C until use. Once in the laboratory, microcosms were randomly distributed in an outdoor roofed area to minimize bias. After a one-week stabilization period, baseline physicochemical data were gathered from the microcosms (day 0), which were then allocated in a randomized manner to one of three treatments (5 replicates per treatment): (1) MP addition on the surface of the water (T-W); (2) MP addition on the surface of the sediment (T-S); or (3) no MP addition (control). MPs concentration was based on a MP:P_{Mobile} molar ratio of 85:1, which was three-fold higher than the ratio selected by de Vicente et al. (2010a) to test the P adsorption effectiveness of MPs in batch experiments. This ratio was increased in order to counteract possible chemical interferences (de Vicente et al. 2011). The experiment lasted for 70 days and physico-chemical variables (temperature, pH, dissolved oxygen, conductivity, total dissolved solids) and zooplankton data were recorded on days 0, 2, 21, 35 and 70.

Finally, this study was framed in a broader project focused on determining the consequences of MPs application on: water quality and sediment P pools (Funes et al., 2017); phytoplankton composition and structure (del Arco et al., *in preparation*) and on zooplankton composition and structure (*Chapter IV*).

4. Using magnetic particles for reducing phosphorus inputs from treated wastewaters to a Mediterranean Ramsar wetland

4.1. Study site

The Fuente de Piedra lake (37° 6′ N, 4° 44′ W) is a hypersaline wetland of great extension (13.5 km^2; NE-axis: 6.8 km; SE-axis: 2.5 km). It became the third Spanish wetland to be included in the Ramsar convention (in 1983) and it was declared as regional Nature reserve in 1984. The site is an ideal habitat for migrating and non-migrating birds supporting one of the most important breeding colonies of the greater flamingo (e.g., *Phoenicopterus roseus*; Rendón et al., 2014). Despite its protection, one of the major problems of this ecosystem is the discharge of treated wastewater from the nearby Fuente de Piedra town, even though it fulfills the standards for the treatment of urban wastewater (RD 509; de-los-Ríos-Mérida et al., 2017). Since 1999, secondary municipal effluents go through several small semi-natural ponds before treated wastewater is discharged in the Ramsar site. Although a recent study has quantified a notable reduction in nutrient (N and P) concentrations in the water along it flows through the semi-natural ponds system (de-los-Ríos-Mérida et al., 2017), nutrient concentrations are still high and they may cause a water quality impairment of the lake.

4.2. Sampling and experimental set-up

On May 2018, water samples at the inlet (station A) and at the outlet (station B) of the semi-natural Laguneto pond (27801 m^2 and a maximum depth of 1.42 m; *Chapter V*) were collected. Once in the laboratory, an initial characterization of water samples was carried out. Later on, a set of batch experiments were run for assessing the effect of

adding MPs on the chemical composition (major cations, major anions and metal concentration) of treated wastewaters. Additionally, we aim on identifying the number of adsorption cycles (by reusing MPs) which are able to still trapping a high percentage of P (>50%). Finally, the optimum ratio between MPs mass and initial dissolved inorganic P (DIP) concentration was selected based on advantages (P removal efficiency, %) and disadvantages (economic price, € L^{-1}) of using MPs.

5. Phosphorus recovery and reuse

5.1. Optimizing phosphorus desorption

Firstly, P loaded MPs were obtained from a previous experiment (Funes et al., 2017) were MPs were added to microcosms comprising surface sediment and lake water from the hypertrophic Honda (Albufera de Adra, Almería). MPs were added to two from three treatments, to get a final concentration of 1.4 g MPs l^{-1}. After a contact time of 24 h (day 1), P loaded MPs were recovered by fully immersing a magnetic rake in the microcosms down to the sediment surface.

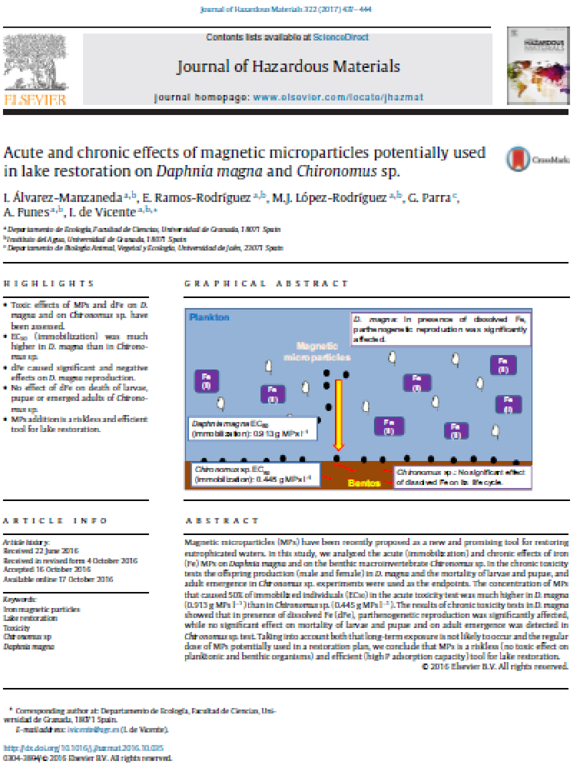
Once P loaded MPs were recovered from microcosms, they were firstly washed and sonicated (1 min) three times with distilled water. Finally, MPs were dried for 24 h at 60ºC. Then, dried P loaded MPs were washed by using three different basic solutions (NaOH, KOH and NH_4OH) at four different concentrations (0.1; 0.5; 1 and 3 M). The supernatants were neutralized with two different acids: HCl and H_3PO_4 and the extraction procedure was repeat up to four times, with each concentration of every basic solution, for assuring the complete P desorption. Finally, the chemical composition of all supernatants was determined by using ICP-MS (Al, B, Ca, Cu, Co, Fe, K, Mg, Mn, Mo, Na, S and Zn), ion chromatography (F^-, Cl^-, Br^-, NO_3^-, PO_4^{3-} and SO_4^{2-}) and an elemental analyzer (C, N and H).

5.2. Preparation of the plant nutritive solutions and experimental set-up

A total of four plant nutritive solutions (Treatments) were considered. One of them was prepared by reproducing the chemical composition of a typical commercial fertilizer (T1) while the others (T2; T3 and T4) were prepared by using P desorbed from P loaded MPs (extracted by using 0.1 M NH_4OH and by neutralizing with H_3PO_4). Considering previous studies (Stradiot, 2002), in T2, T3 and T4 supernatants were diluted up 68 times with irrigation water (tap water). While T2 only contains P desorbed from P loaded MPs; T3 was enriched in macroelements and T4 was amended with both micro and macroelements.

Next, we run a fertirrigation experiment using three different species (*Ocimum basilicum, Cucumis sativus* and *Cucumis melo)* for testing if plant growth and biomass differ among different P sources (recovered P or commercial P). Basically, a total of 330 horticultural nurseries, 110 for each species, were used containing a nutrient poor composite substrate of black peat (15%), yellow peat (70%) and pearlite (15%). A control with no addition of any nutrient was also considered and all treatments were run in 22 replicates. The experiment was run in a canopy area located in the Andalusian Institute of Agricultural Research and Training (IFAPA). During the experiment, which lasted for 31 days, plant germination was daily recorded and plant height was measured three days a week. Finally, at the end of the experiment, seedlings were collected for measuring root and leaf biomass and for quantifying P content.

Chapter I: Acute and chronic effects of magnetic microparticles potentially used in lake restoration on *Daphnia magna* and *Chironomus* sp.



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Acute and chronic effects of magnetic microparticles potentially used in lake restoration on *Daphnia magna* and *Chironomus* sp.

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HIGHLIGHTS

- Toxic effects of MPs and dFe on *D. magna* and on *Chironomus* sp. have been assessed.
- EC_{50} (immobilization) was much higher in *D. magna* than in *Chironomus* sp.
- dFe caused significant and negative effects on *D. magna* reproduction.
- No effect of dFe on death of larvae, pupae or emerged adults of *Chironomus* sp.
- MPs addition is a riskless and efficient tool for lake restoration.

GRAPHICAL ABSTRACT

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ABSTRACT

Magnetic microparticles (MPs) have been recently proposed as a new and promising tool for restoring eutrophicated waters. In this study, we analyzed the acute (immobilization) and chronic effects of iron (Fe) MPs on *Daphnia magna* and on the benthic macroinvertebrate *Chironomus* sp. In the chronic toxicity tests the offspring production (male and female) in *D. magna* and the mortality of larvae and pupae, and adult emergence in *Chironomus* sp. experiments were used as the endpoints. The concentration of MPs that caused 50% of immobilized individuals (EC_{50}) in the acute toxicity test was much higher in *D. magna* (0.913 g MPs l^{-1}) than in *Chironomus* sp. (0.445 g MPs l^{-1}). The results of chronic toxicity tests in *D. magna* showed that in presence of dissolved Fe (dFe), parthenogenetic reproduction was significantly affected, while no significant effect on mortality of larvae and pupae and on adult emergence was detected in *Chironomus* sp. test. Taking into account both that long-term exposure is not likely to occur and the regular dose of MPs potentially used in a restoration plan, we conclude that MPs is a riskless (no toxic effect on planktonic and benthic organisms) and efficient (high P adsorption capacity) tool for lake restoration.

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Abstract

Magnetic microparticles (MPs) have been recently proposed as a new and promising tool for restoring eutrophicated waters. In this study, we analyzed the acute (immobilization) and chronic effects of iron (Fe) MPs on *Daphnia magna* and on the benthic macroinvertebrate *Chironomus* sp. In the chronic toxicity tests the offspring production (male and female) in *D. magna* and the mortality of larvae and pupae, and adult emergence in *Chironomus* sp. experiments were used as the endpoints. The concentration of MPs that caused 50% of immobilized individuals (EC_{50}) in the acute toxicity test was much higher in *D. magna* (0.913 g MPs l^{-1}) than in *Chironomus* sp. (0.445 g MPs l^{-1}). The results of chronic toxicity tests in *D. magna* showed that in presence of dissolved Fe (dFe), parthenogenetic reproduction was significantly affected, while no significant effect on mortality of larvae and pupae and on adult emergence was detected in *Chironomus* sp. test. Taking into account both that long-term exposure is not likely to occur and the regular dose of MPs potentially used in a restoration plan, we conclude that MPs is a riskless (no toxic effect onplanktonic and benthic organisms) and efficient (high P adsorption capacity) tool for lake restoration.

1. Introduction

Biogeochemical cycles are being dramatically and worldwide affected by human activities. For the case of phosphorus (P), human intervention has mobilized nearly half a billion tons of this element from phosphate rock into the hydrosphere over the past half century (Cordell et al., 2011). As a result, nowadays, we are facing two problems: the exhaustion of P reserves, essential for making fertilizers, and the P enrichment of inland aquatic ecosystems, which is the responsible for eutrophication. On the one hand, experts disagree on how much P is left and how quickly it will be exhausted but many argue that a shortage is coming and that it will leave the world's future food supply hanging in the balance (Gilbert et al., 2009). On the other hand, eutrophication is currently considered as a worldwide problem which affects 30% of the inland aquatic ecosystems (OECD, 1982; Sas, 1990; Cooke et al., 2005). As the main limiting nutrient of the primary production in aquatic ecosystems is P, it is essential to consider as a preliminary strategy the reduction in P concentration in the water column. To achieve this goal, three different but complementary approaches have been proposed (Hupfer and Hilt, 2008): (i) a reduction in P external loading, (ii) an increase in P retention by the sediment and (iii) an increase in P export from the system. Controlling the external load is an essential step to manage and restore the eutrophicated systems, in fact, it has been observed that an insufficient reduction in P external loading results in a long-term failure in lake restoration (8–10 yr; Smith 2009).

Up to date, there is no a management tool as panacea for eutrophicated inland waters. Although chemical adsorbents such as Fe, aluminum (Al) and calcium (Ca) salts seem to be the most convenient, it is relevant to consider that, although inactivated, P remains in sediments and may be released to water column under changing physico-chemical and biological conditions such as temperature, pH, redox potential, biological activity or resuspension (Jensen et al., 1992; Søndergaard et al., 1992; Rydin and Welch, 1998; Egemose et al., 2009; Funes et al., 2016). In order to by pass these difficulties, great attention has recently been paid for developing new and efficient adsorbents that are able to reduce P levels in water bodies. One of the most promising methods is the addition of magnetic microparticles (MPs) for P removal to aquatic ecosystems as we get the P out of the system, so this method conducts to an increase in P export (P is removed from both lake water and lake sediment; Funes et al., 2016). Therefore, MPs are used to adsorb contaminants from aqueous effluents and after the adsorption is carried out, the adsorbent can be separated from the medium by a simple high gradient magnetic separation process (de Vicente et al., 2010). Once P is trapped, it can be later desorbed and recovered and simultaneously, MPs can be reused for adsorbing more P because they still maintain a high P adsorption capacity (de Vicente et al., 2010). All in all, several outstanding advantages of using these particles for lake restoration can be highlighted: (i) the high P: MPs molar ratio under both batch and flow conditions (de Vicente et al., 2010; Merino-Martos et al., 2011); (ii) the fast P adsorption process (in just 2 hunder batch conditions; (Funes et al., 2016); (iii) the ability for adsorbing P even in anoxic conditions (Funes et al., 2016); (iv) the recovery of MPs from the solution, reducing both economic costs and toxic effects on the biota and (v) the potential reusability of the recovered P as a fertilizer.

Despite the excellent advantages of using MPs, before using them in a “whole-lake application”, it is essential to assess their toxicological effects on both planktonic and benthic organisms. Up to date, there only exist studies focused on the toxicity of nano and no magnetic particles (Baun et al., 2008; Navarro et al., 2008; García et al., 2011; Keller et al., 2012; Shinde et al., 2012; Yah et al., 2012; Baumann et al., 2014) but no similar studies have been developed for magnetic Fe MPs. The procedures currently in use for conventional risk assessment have a first step that consists in the identification and characterization of hazards based, among others, in basic toxicity tests (Amiard-Triquet et al., 2015). Present study is the first step in the MPs ecotoxicological assessment but experimental designs mimicking a natural environment (microcosms and mesoscosms) and in situ assays will be necessary to be conducted in the near future. Additionally, it is important to consider that in a whole-lake application, MPs would be removed after 24 h but dissolved iron (dFe) could be mobilized to the water column and stay longer time in contact with aquatic biota, with the subsequent potential toxic effects. In this context, the general aim of this paper was to assess, by laboratory tests and following standardized Organization for Economic Cooperation and Development (OECD) protocols, the short- and long-term effects of magnetic MPs on both benthic and planktonic organisms. In particular, the specific aims were to evaluate both the acute effects (immobilization) of MPs on *D. magna* and *Chironomus*sp. and the chronic effects of dFe on *D. magna* and *Chironomus* sp.

2. Material and methods

2.1. Sampling and culturing of test organisms

Experiments were carried out with *D. magna* and *Chironomus* sp. *D. magna* is a cladoceran which has been widely used intoxicity tests due to it sensibility to contaminants (e.g. (Khangarot et al., 1987; Garcia et al., 2011)) and because of its size, high fecundity, parthenogenetic reproduction, short life-cycle and its relatively facility for culturing (Núñez et al., 2005). For this study, *D. magna* was isolated from Lake Grande (Jaén, Southern Spain). In the laboratory, a single clone from a parthenogenetic female was obtained. Daphnia cultures were maintained with densities ranging from 20 to 30 ind l^{-1} (EPA, 2002) in 1 l glassbeakers containing hard (209 mg $l^{-1}$of total hardness) commercial mineral water (<4 µg P l^{-1}). Daphnids were fed ad libidum (5 × 10^4 cells ml^{-1}, 0.0027 mg C) three times a week with a pure culture of the chlorophycean algae *Chlorella* sp. *Chlorella* sp. (365 m^3, diameter: 8.8 µm), which was originated from a culture collection of the University of Granada, was maintained in an 800 ml volume with Bold's Basal Medium (BBM; Bold, 1949). Photoperiod was set to 16 h light: 8 h dark cycle and temperature at 22 ± 0.5 ºC. To avoid the sedimentation of algae's cells, the culture was shaken at 100 rpm. Algal cell concentration was estimated using Neubauer's counting chamber.

On the other side, benthic macroinvertebrates are a very suitable community to carry out ecotoxicological tests due to their easy collection, relatively slow mobility and their life expectancy (Iannacone et al., 2004). In particular, larvae of *Chironomus* sp. have a great ecological relevance for ecotoxicological researches (Iannacone et al., 2004). In the present study, up to 100 individuals of *Chironomus* sp. were collected from river Beiro (Granada, Southern Spain) using a kick net with 250 µm of mesh. Once in the laboratory, chironomid larvae were placed in a 50 × 26 × 36 cm aquarium, containing silica sand and three aerators to prevent anoxia. Hard (209 mg l^{-1} of total hardness) commercial mineral water (<4 μg P l^{-1}) was used to fill the aquarium. Chironomids feeding was carried out three times a week by using fish flakes food (EPA, 1993).

2.2. General characterization of magnetic microparticles

Micronsized iron (Fe) particles were kindly supplied by BASF (Germany) and used without further treatment to make the suspensions. According to the manufacturer, the composition of this powder is 97.5% Fe, 0.9% C, 0.5% O and 0.9% N. Previous studies have characterized in detail their magnetization properties, electrophoretic mobility, particle size distribution and P adsorption properties (de Vicente et al., 2010; Merino-Martos et al., 2011; de Vicente et al., 2011). In brief, MPs used in this work are spherical in shape, relatively polydisperse and with a mean diameter of 805 ± 10 nm. As expected, a ferromagnetic behavior was found for MPs with a negligible remnant magnetization. MPs present a thin oxide surface layer and hence behave as amphoteric solids with surface charges controlled by the pH in the aqueous medium, with anisoelectric point around pH 6.5. Although MPs experienced a slight decrease in P removal efficiency with increasing pH, P removal efficiency was larger than 85% at pH 7. Finally, reused MPs have asimilar P maximum adsorption capacity (18.83 mg P g^{-1} Fe) as bare MPs (15.80 mg P g^{-1} Fe).

2.3. Toxicological tests with **Daphnia magna**

Tests were made according to different OECD standardized protocols (2004, 2012) and using as reference solution of 1 g MPs l^{-1} concentration, which is the concentration with a high P removal efficiency (de Vicente et al., 2010). Following OECD protocols, no adjustment of pH was carried out as the pH remained in the range 6–9. In particular, pH was quite stable after MPs addition with a mean value of 7.96 ± 0.06, for all MPs concentrations.

2.3.1. Acute immobilization test with magnetic particles

To run the immobilization test, 202 OECD Part I standardized protocol was followed (OECD, 2004). We used, <24-h-old, F2-generation females of our clone of *D. magna*. Thirty five *D. magna* females were isolated and fed with 0.0035 mg C (37,000 cells) of *Chlorella* sp. ml^{-1}. Individually, female neonates were randomly distributed in groups of five individuals in 50 ml glass beakers containing the following MPs concentrations:

0.01; 0.05; 0.1; 0.5; 0.7; 1 and 2 g MPs l^{-1} (concentration selection was carried out based on the results of a preliminary test as recommended by OECD protocol). All control and treatments were run in five replicates. All glass beakers were randomly placed in a culture chamber at 23ºC and a 14:10 light:dark cycle. After 24 and 48 h, mortality, immobilization (when animals are not able to swim within 15 s, after gentle agitation of the test vessel) and abnormal behaviors were recorded. As it is stated in the standardized OECD protocol, organisms were not fed during the experiment.

2.3.2. Reproduction test with dissolved iron

Following a slight modification of 211 OECD (OECD, 2012) standardized test, a reproduction test was run to assess sublethal effects of dFe on *D. magna* after 21 days. To carry out this chronic test, suspensions containing the following MPs concentrations were prepared: 0.01; 0.05; 0.1; 0.5; 1 and 2 g MPs l^{-1}. Similarly to a real lake-application (de Vicente et al., 2010; Merino-Martos et al., 2011; Funes et al., 2016), after 24 h, MPs were removed by using magnetic techniques, and with the remaining solutions (containing dFe) the reproduction test was run. dFe concentration was measured in the filtrate (Whatman GFC filters) by using the spectrophotometric ferrozine method proposed by Gibbs (1979), and all dFe concentrations were lower than the detection limit for all treatments. The reproduction test consisted of placing individually, <24-h-old, F3-generation females of our clone of *D. magna* into 100 ml glass tubes containing 50 ml of the above mentioned solutions enriched in dFe. Daphnids were fed with 0.1 mg C $Daphnia^{-1}$ of algal concentration (1.8×10^6 cells ml^{-1}) in an isolated room at 22 ± 0.5ºC and a light:darkness cycle of 16: 8 h. Each treatment was run in ten replicates, and the medium was renovated three times a week. Every day, the number of female and male offspring and the survivorship of *D. magna* individuals were recorded. Every day, the offspring were removed. The survivorship of *D. magna* was always 100% in control and treatments.

2.4. Toxicological tests with **Chironomus** *sp.*

2.4.1. Immobilization test with magnetic particles

Immobilization test was performed according to the 235 OECD standard method (OECD, 2011). The experimental design consisted of adding five larvae of the same cohort to each 50 ml glass beaker. Four replicates per treatment, including the control, were considered. For this test, concentrations were the same as those used for Daphnia immobilization test (section 2.3.1): 0.01; 0.1; 0.5; 0.7; 1 and 2 g MPs l^{-1}. Each beaker was randomly placed in the laboratory at 23ºC and under a natural light cycle. After the 24 and 48 h-exposure, observations were carried out to each individual for 15 s. In this period of time the immobilization, as well as any signal of affectation, was recorded.

2.4.2. Chronic exposure test with dissolved iron

Solutions enriched in dFe were prepared similarly to those used for the reproduction test with Daphnia (section 2.3.2). Nominal MPs concentrations were: 0.01; 0.05; 0.1; 1 and 2 g MPs l^{-1}. dFe concentration was measured following the method above described for reproduction test for *Daphnia magna*. As suggested by OECD for *Chironomus* sp., acute immobilization test, long-term test (30 days) was run with four replicates (control and treatments). An additional replicate for each concentration was used for measuring physico-chemical variables (temperature, conductivity and dissolved oxygen concentration with a multiparameter probe Eutech PCD650). The methodological approach consisted of placing, in each 50 ml glass beakers, five chironomids from the same cohort. They were fed three times a week with 2 ml of food flake fish diluted in 100 ml of mineral water. Beakers were placed randomly in the laboratory at 23ºC and under a natural light cycle. Every day, any signal of stress, adult emergency and physico-chemical variables were recorded.

2.5. Statistical analysis

To estimate the MPs concentration that causes the immobilization of 50% of the individuals during the exposure period (EC_{50}–48 h), as well as its 95% confidence limits, a Probit analysis with the statistical program SPSS was carried out (de Vicente et al., 2011). This analysis is a kind of regression model to analyze a binominal response variable. To analyze the results of the chronic exposure tests in Daphnia and Chironomus, the R program was used considering the recommendations of Sokal and Rohlf (1995).

Normality and homogeneity of variances were checked by the Kolmogorov–Smirnov test and Levene's test, respectively (Gibbs, 1979). Our data did not satisfied normality and homocedasticity assumptions (Shapiro-Wilk and Levene tests, respectively with $p < 0.05$), and transformations did not achieve data to follow a normal distribution. In consequence, a non-parametric one way analysis of variance (Kruskal-Wallis ANOVA) (Quinn and Keough, 2002) was performed to test the effects of dFe on the number of Daphnia offspring (males and females), the number of dead larvae and pupae and the number of emerged adults of Chironomus. Mann–Whitney U tests, corrected for multiple testing with the sequential Bonferroni test (Rice, 1989) were used for examining differences in all these response variables between pairs of treatments.

3. Results

3.1. Toxicological tests with **Daphnia magna**

3.1.1. Immobilizatión test with magnetic particles

In the final immobilization test with *D. magna* no immobilization effects were registered in the control, as expected, while the percentage of immobilized organisms increased when increasing MPs concentration (Fig. 1). In addition, when organisms were exposed to 0.01 g Fe l^{-1} no immobilization effect was recorded on the population, while in the highest concentration (2 g Fe l^{-1}) all animals were affected. The EC_{50} (always referred to 48 h) was 0.913 g l^{-1} (our data were adjusted to a normal distribution; Pearson's adjustment; $p>0.05$).

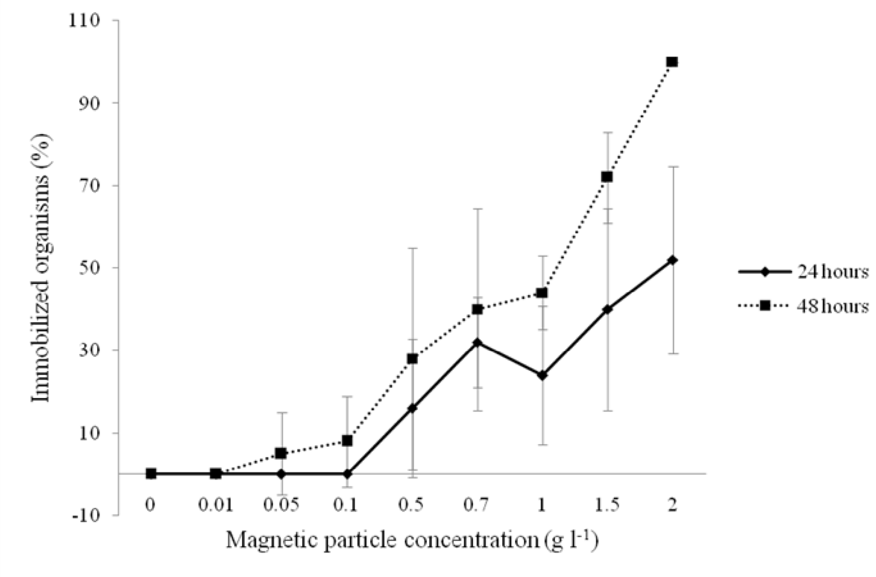

Figure 1. Individuals of *D. magna* immobilized (%) after their contact with MPs for 24 h and 48 h. Vertical error bars show standard deviation (SD). n=5.

3.1.2. Reproduction test with dissolved iron

Significant effects of dFe on the production of females offspring in *D. magna* have been found (Kruskal-Wallis ANOVA, χ^2= 16.14, $p<0.05$). Daphnia raised in any concentration of dFe had significantly lower total number of female neonates than control did (Fig. 2a). In the presence of dFe, median values of female neonates ranged from 0 (2 g l^{-1}) to 4.5 (0.01 g l^{-1}). However, no differences were found between dFe concentrations, exclusive of control, for this trait (Fig. 2a). The post-hoc analysis, after applying the Bonferroni's correction, showed significant differences between the control and any treatment ($p<0.05$ in all the cases).

For the case of male offspring, dFe did not stimulated their production in *D. magna* (Kruskal-Wallis ANOVA; χ^2= 10.26; $p>0.05$; Fig. 2b). Median values was 0 in all control and treatments and the number of male neonates ranged from 0 to 8 (0.1 and 1 g l^{-1}).

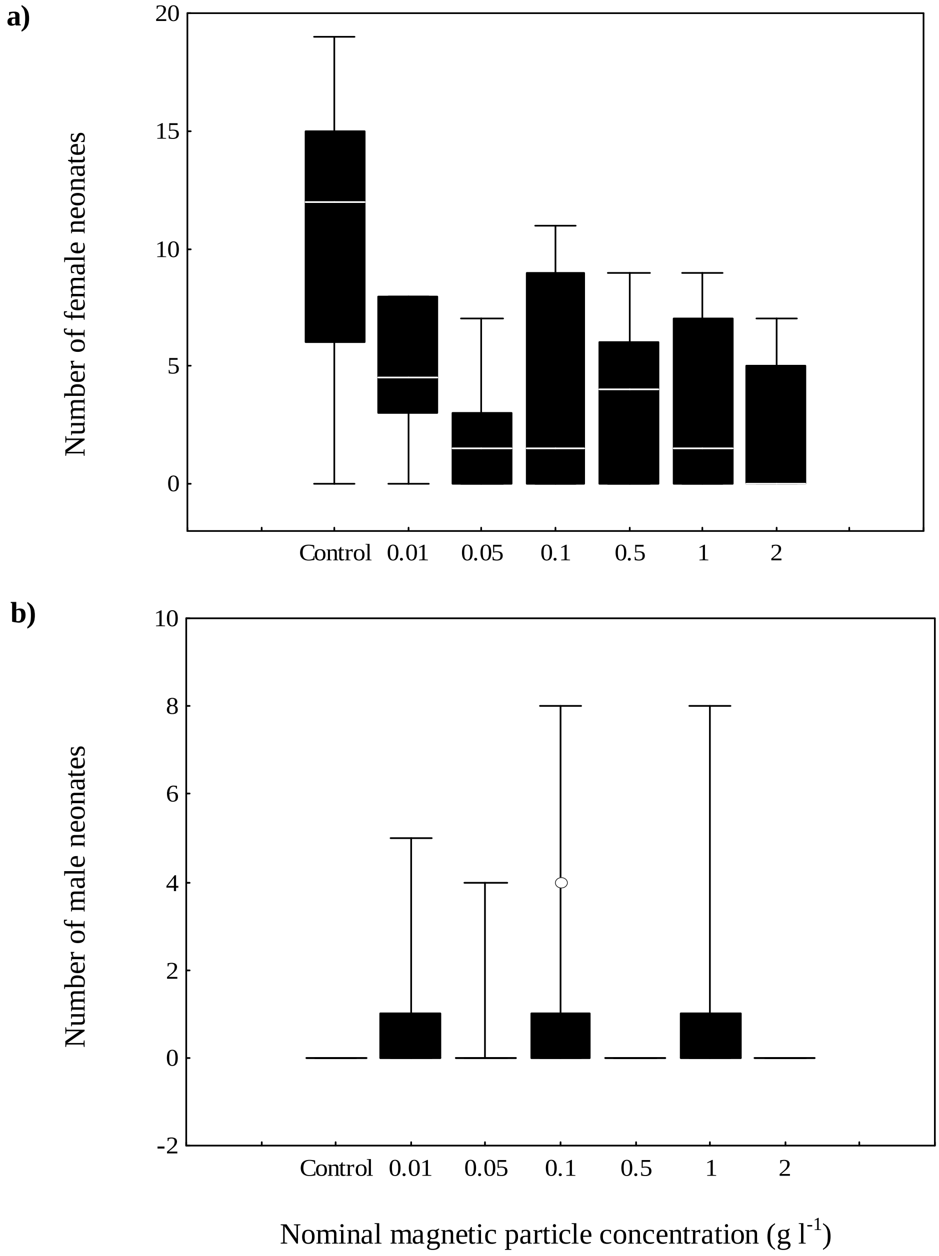

Figure 2. Number of female offspring (a) and male offspring (b) of *D. magna* produced during 21 days in contact with dFe. *Line* median. *Boxes* 25%-75%. *Whiskers* min–max. n=10. *White circle* represent the outlier.

3.2. Toxicological tests with **Chironomus** *sp.*

3.2.2. Immobilization test with magnetic particles

As Fig. 3 shows, immobilization increased with MPs concentrations and the total immobilization in *Chironomus* sp. population was recorded at 2 g Fe l^{-1}. Data fit to a normal distribution ($p>0.05$; Pearson adjustment) and 0.445 g Fe l^{-1} was identified as the concentration that caused the immobilization in half of the population (EC_{50}).

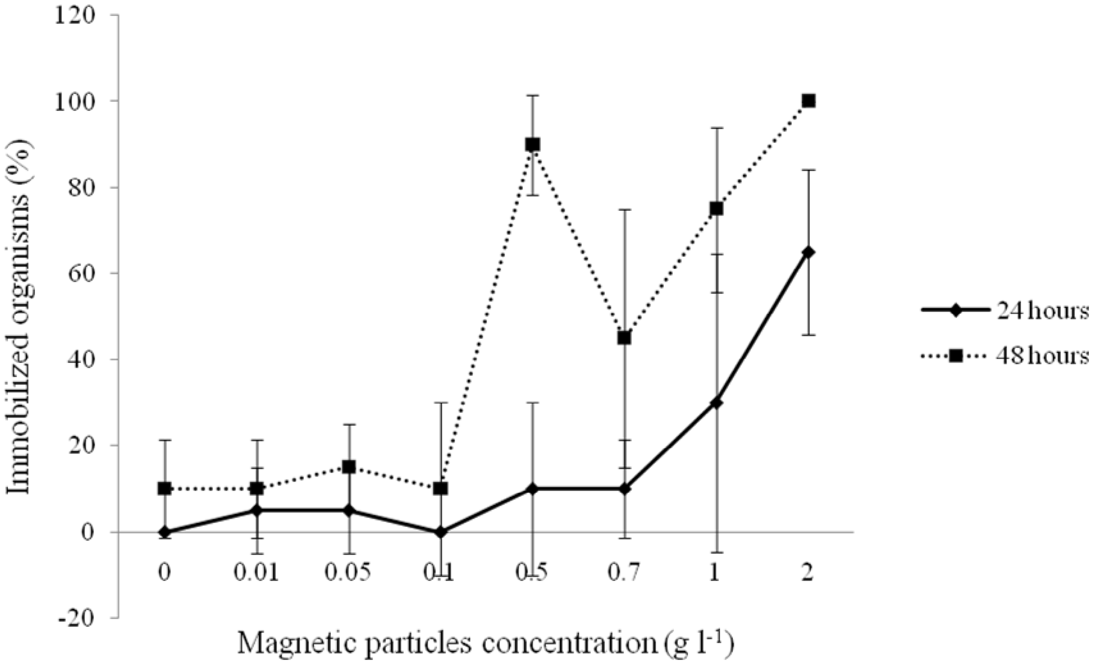

Figure 3. Individuals of *Chironomus* sp. immobilized (%) after their contact with MPs for 24 h and 48 h. Vertical error bars show standard deviation of data (SD). n=4.

3.2.3. Chronic exposure test with dissolved iron

Table 1 summarizes physico-chemical variables recorded along the chronic experiment with *Chironomus* sp. In brief, pH was slightly basic and average values of electric conductivity, dissolved oxygen concentration and temperature ranged from 1.56 to 1.82 mS cm^{-1}, from 4.00 to 5.00 mg l^{-1} and from 19.4 to 19.9ºC, respectively.

Table 1. Physico-chemical parameters recorded during the long-term experiment with *Chironomus* sp. Data are mean ± SD (min-max).

MPs (g l^{-1})	**pH**	**Conductivity (mS cm^{-1})**	**O_2 (mg l^{-1})**	**T (ºC)**
Control	8.30 ± 3.83 (7.04 - 8.81)	1.77 ± 0.55 (1.01 - 2.73)	4.30 ± 1.36 (1.39 - 7.43)	19.9 ± 0.3 (18.8 - 20.8)
0.01	8.48 ± 3.98 (7.8 - 9.05)	1.82 ± 0.68 (1.02 - 3.40)	4.93 ± 0.75 (3.33 - 7.48)	19.6 ± 0.3 (18.6 - 20.8)
0.05	8.48 ± 3.97 (7.8 - 9.07)	1.66 ± 0.48 (1.00 - 2.63)	4.72 ± 0.74 (2.94 - 7.55)	19.5 ± 0.4 (19.2 - 20.2)
0.1	8.57 ± 4.01 (7.79 - 9.03)	1.67 ± 0.46 (0.99 - 2.63)	5.00 ± 0.60 (3.75 - 7.54)	19.6 ± 0.4 (19.2 - 20.5)
0.5	8.58 ± 3.98 (7.76 - 9.02)	1.67 ± 0.48 (0.99 - 2.70)	4.66 ± 0.53 (3.61 - 7.29)	19.4 ± 0.4 (18.6 - 20.3)
1	8.54 ± 3.98 (7.66 - 8.99)	1.62 ± 0.45 (0.99 - 2.72)	4.23 ± 0.72 (1.57 - 7.18)	19.5 ± 0.4 (18.8 - 20.3)
2	8.54 ± 4.01 (7.66 - 9.37)	1.56 ± 0.41 (0.99 -2.57)	4.00 ± 0.56 (1.33 - 5.71)	19.5 ± 0.5 (19.1 - 20.6)

The long-term experiment results have evidenced the absence of any significant effect of dFe concentration on the number of dead larvae, dead pupae and emerged adults (Fig. 4 a, b and c respectively; Kruskal-Wallis ANOVA: for the number of dead larvae: X^2= 5.0327, p>0.05; for the number of dead pupae: X^2=6.602, p>0.05; and for the number of emerged adults: X^2= 4.251, p>0.0).

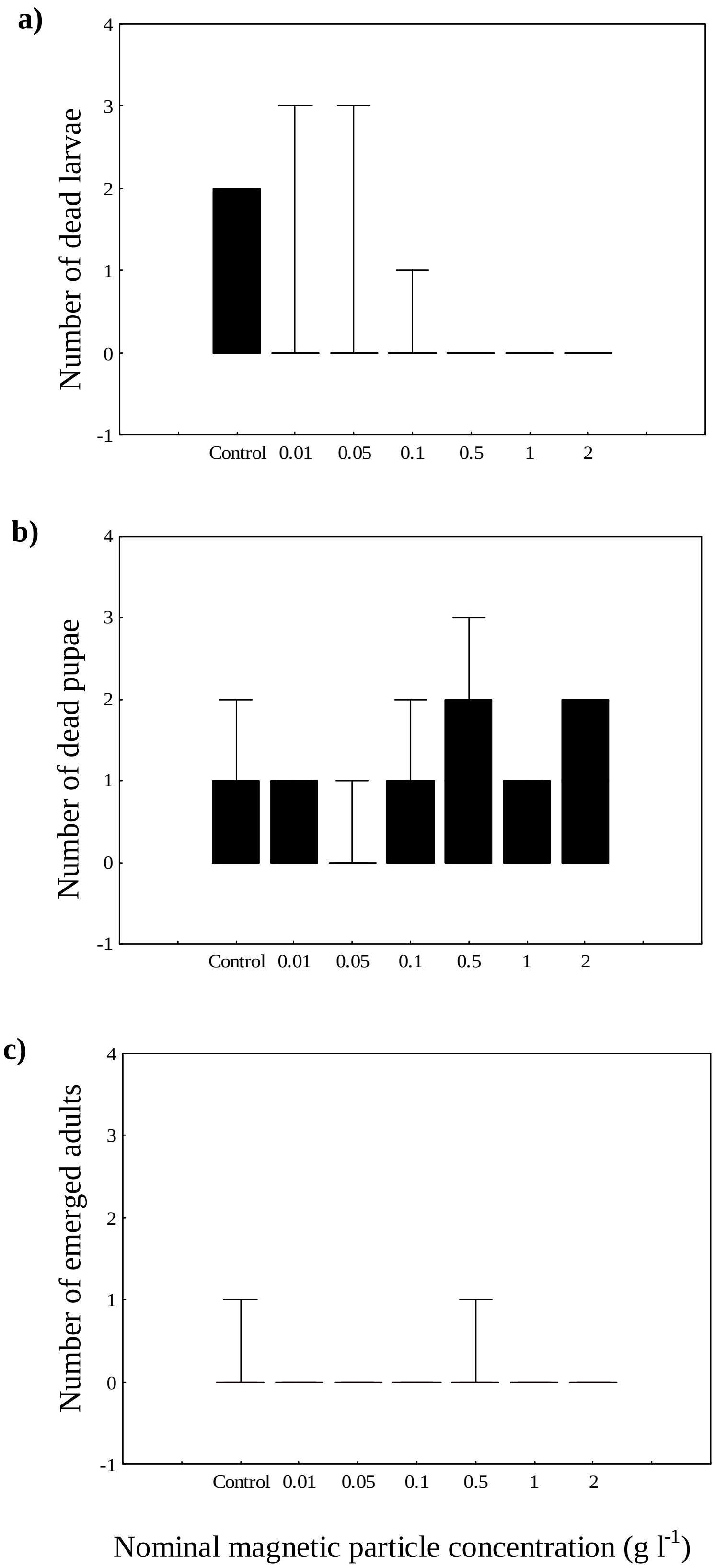

Figure 4. Number of dead larvae (a), dead pupae (b) and emerged adults (c) of *Chironomus* sp. in the long-term test with dFe. *Line* median. *Boxes* 25%-75%. *Whiskers* min–max. n=5.

4. Discussion

4.1. Effects of MPs on the organism immobilization

EC_{50} referred to immobilization was notably lower in *Chironomus* sp. (0.445 g Fe l^{-1}) than in *D. magna* (0.913 g Fe l^{-1}), showing that the benthic organism was more sensitive than the planktonic one. This is likely to be the result of drastic differences in the lifestyle of these organisms. In fact, chironomids are benthic animals and hence they will be in contact with precipitated Fe throughout the experiment, while *D. magna*, a planktonic organism, is much less time in contact with MPs as these particles rapidly settle down in the water column. At this point, it is relevant that considering the 53 mg MPs: mg P mass ratio as the adsorption efficiency ratio, reported in previous studies (de Vicente et al., 2010; Merino-Martos et al., 2011; Table 2); the addition of 0.4 g MPs l^{-1} (EC_{50} for *Chironomus* sp.) and 0.91 g MPs l^{-1} (EC_{50} for *D. magna*) would correspond to a treatment scenario of 8.4 and 19.7 mg P l^{-1}, respectively, which are extremely high values for typical inland waters. In fact, considering the annual mean TP concentration typical for eutrophic (30-100 µg l^{-1}) and hypereutrophic (>100 µg l^{-1}) systems following (Nürnberg, 1996), it would be necessary to add just 1.4 and 4 mg MPs l^{-1}, respectively. Even more, MPs concentrations lower than 50 mg MPs l^{-1} would be necessary to be added if we consider representative P concentration in porewater of eutrophic lakes (≈1000 µg l^{-1}). Therefore, only slight effects on immobilization of test organisms (*Chironomus* sp. and *D. magna*) are expected when adding MPs in relation to realistic P concentration in a restoration strategy.

Table 2. Comparative values of EC_{50} for *Daphnia magna* and *Chironomus* sp. for the most frequently used P adsorbent in lake restoration. P removal efficiency is also shown. [1]de Vicente et al. (2010); [2]Lürling and Tolman (2010) and [3]de Vicente et al. (2008). Mortality* reported in acute tests and Life cycle** in chronic tests.

Adsorbent	**Test species**	**End point**	**EC_{50} (mg l^{-1})**	**P removal efficiency (g product g^{-1} P)**	**References**
MPs	*Daphnia magna*	Immobilization	913	53[1]	This study
	Chironomus sp.	Immobilization	445		This study
Phoslock	*Daphnia magna*	Growth (weight based rate)	871	100[2]	Lürling and Tolman (2010)
		Growth (length based rate)	1557		
Aluminum sulphate	*Daphnia magna*	Mortality*	38.2	66[3]	Kimball in Gostomski (1990)
	Daphnia magna	Life cycle**	0.742		Kimball in Gostomski (1990)
Aluminum chloride	*Daphnia magna*	Mortality*	25.3		Brooke et al. (1985) in Gostomski (1990)

Moreover, it is important to note that standardized OECD immobilization protocols with *D. magna* and *Chironomus* sp. are referred to an exposure of 24 h and 48 h. However, when applying this technique in a whole-lake experiment, MPs would be added to the lake water and after 24 h they would be removed as previous studies have found that maximum P adsorption occurred during this contact time (Funes et al., 2014).

For this reason, toxic effects which may result from the application of MPs are likely to be even less than those detected in these laboratory tests.

In order to compare toxicity of MPs with other P adsorbents (Phoslock, alum, Zeolites, calcite) used for lake restoration, a wide literature review has been done. We have focused our attention on *D. magna* and *Chironomus* sp. An evident scarcity in this type of toxicity and well standardized tests render a tricky comparison (Table 2). If we compare MPs and Phoslock, EC_{50}, although referred to different endpoints, was in the same order of magnitude for both adsorbents. However, it is crucial to take in consideration that P removal efficiency was half for Phoslock compared to MPs; thus, it is expected major toxic effects of Phoslock on *D. magna* than of MPs in a whole-lake restoration project. In relation to the EC_{50} for MPs and alum, it is clear from Table 2 that much higher values have been found for MPs reflecting the lower toxicity of this adsorbent compared to alum. In fact, Gostomski (1990) remarked that *D. magna* is one of the most sensitive invertebrate species to alum. (2012) evaluated, by means of a laboratory microcosm experiment, the effect of adding alum, calcite and both alum + calcite on the survival of different planktonic and benthic species but no EC_{50} values were reported. They found that in general, the restoration techniques had neither acute nor chronic toxic effects on survival of *D. magna*. They also found that the alum + calcite technique impaired the survival of *Chironomus riparius*, and that the midge emergence was much higher compared to alum only and control. A recent and interesting study was carried out by (2014) but no planktonic organisms were considered, just native benthic-dwelling macroinvertebrates and fish. These authors compared, by laboratory mesocosms, the lethal and sublethal effects of alum or Aqual-P (aluminum amended zeolite) and they found no significant effect of both adsorbents on survival or growth of the studied animals.

Currently, there is a complete lack of research on the effect of magnetic Fe microparticles on aquatic organisms, but studies have focused on nanoparticles. Nanoparticles, with lower size than MPs used in our tests, restrict the access of food in some organisms, staying in their filtering systems (Traunspurger and Drews, 1996). Toxicological studies on nanoparticles have shown that particles size and their aggregation have an important role in the determination of toxicity (Baun et al., 2008) García et al. (2011) reported some data about the lethal concentration for half of the population (LC_{50} = 0.23 mg l^{-1}) of *D. magna* of magnetite nanoparticles (Fe_3O_8). Although the end point of the test is different, immobilization *vs* mortality, we can infer that magnetite nanoparticles are much more toxic for daphnids than our MPs. More recently, Baumann et al. (2014) observed that coating Fe oxide nanoparticles drastically affected to daphnids mobilization, reporting EC_{50} values which ranged from 27.9 (dextran coated nanoparticles) to >100 mg l^{-1} (polymer coated nanoparticles). These values are again far below EC_{50} values obtained in our study, which reflect the lower toxicity of our MPs for aquatic organisms.

4.2. Long-term effects of dissolved iron on test organisms

A typical example of response to a stress factor at organism level is the decrease in reproduction, resulting in a decrease in the size of the organism's population (Tannebaum, 2010). In some cases, a sublethal effect which result in an unable individual to produce viable offspring could be considered like a lethal effect because of the biological efficiency of the individual could be equal to a death individual (Newman and Unger, 2003).

Our results suggested that dFe had a negative effect on reproductive output in *D. magna* as it significantly reduced the number of female offspring but no effect on the number of male offspring was observed. However, it is essential to consider that is difficult to compare dFe concentrations that negatively affect *D. magna* reproduction with other metals reported in the literature (Enserik et al., 1991; Wollenberger et al., 2000) as it is well known that toxicity vary many orders of magnitude across metals since it depends on metal speciation and intrinsic toxicity. Even more, it is important to consider that the addition of MPs makes sense just in eutrophicated ecosystems, where the zooplankton community is dominated by rotifers instead of cladocerans (Conde-Porcuna et al., 2004; Haberman and Haldna, 2014), so much more research about long-term effects of MPs on rotifers is required before applying MPs in a whole-lake restoration strategy.

For the case of the long-term experiment with *Chironomus* sp., no effect of dFe on the number of dead larvae, dead pupae or emerged adult have been observed. Despite the above mentioned restrictions when comparing toxicity of different metals, next we present some evidences from the literature. Previous studies with *Chironomus riparius* and Fe^{+2} observed, in a 48 h test, a significant mortality in larvae for concentrations up to 400 mg l^{-1} (Rousch et al., 1997). It has been reported that indirect effects of dissolved colloids of Fe are more harmful than direct toxic impact of Fe^{+2} (Linton et al., 2007). These authors found that the number of invertebrates decreased with increasing Fe concentration, detecting physiological stress (which conducts to a decrease in reproduction and growth), and being the most tolerant families Tipulidae and Baetidae.

On the other hand, Rasmussen and Lindegaard (1988) observed that a lot of invertebrates which can live in eutrophic environments can tolerate high concentration of Fe.

4.3. Implications for lake restoration

If we consider a whole-lake application of MPs for removing P from both lake water and lake sediment, it is necessary to note the constrains for inferring MPs toxicity found in this study, under very controlled and simple conditions, to natural conditions. All the following features will evidence the overestimation of MPs toxicity in laboratory experiments compared to that expected under natural conditions: (i) in a real restoration project, MPs would be in contact with the plankton organisms for a very short time as MPs are characterized by a high settling velocity (considering MPs and water densities and following Stokes law, estimated value for MPs settling rate is 3.7 µm s^{-1}); (ii) in relation to MPs toxicity on benthic organism, it is also expected a lower affection as MPs will be in contact with them for just 24 h instead of the 48 h used in the standardized OECD toxicity tests; (iii) if we consider that the maximum P adsorption capacity by MPs (under batch conditions) was 18.83 mg P g^{-1} MPs (de Vicente et al., 2010), and that 100% of immobilization in *D. magna* and *Chironomus* sp. have been reported in this study for 2 g MPs l^{-1} (which correspond to 37.66 mg P l^{-1}), we can conclude that it is very unlikely to cause toxic effects on aquatic organisms under natural conditions as lower MPs concentration are likely to be necessary to apply and (iv) the complexity of the inland waters matrix may promote the occurrence of chemical reactions such as metal complexation which lastly may cause a reduction in dFe toxicity. In this sense, Sorvari and Sillanpää, (1996) found that after complexation of some metals such as Fe^{+3} with free EDTA and DTPA the metal toxicity on *D. magna* was drastically reduced.

5. Conclusions

According to the results obtained in the immobilization test with *D. magna*, MPs concentration responsible for the immobilization in half of the population of daphnids was 0.913 g Fe l^{-1} (EC_{50}). The presence of dFe (at any concentration) significant and negatively affected to the number of female neonates and, as a result, it affected to the reproduction of *D. magna*. In addition, in the reproduction test with *D. magna*, no effect of dFe concentration on the number of male neonates was reported. The outcomes of this study is that MPs and dFe effects on immobilization and on reproduction, respectively, are lower than other reported in the literature for nanoparticles. Even more, considering that the addition of MPs makes sense just in eutrophicated ecosystems where the zooplankton community is dominated by rotifers instead of cladocerans, much more research about long-term effects of MPs on rotifers is required before applying MPs in a whole-lake restoration strategy. In relation to the toxicity assays with *Chironomus* sp., EC_{50} for MPs was notably lower (0.445 g Fe l^{-1}) than that measured for *D. magna* (0.913 g Fe l^{-1}), which is likely to be the result of their different behavior (benthic *vs*. pelagic). Anyway, these MPs concentration are far above the MPs concentration required in a whole-lake restoration project if we consider the 53 mg MPs: mg P mass ratio reported in previous studies (de Vicente et al., 2010; Merino-Martos et al., 2011). The long-term exposition test on *Chironomus* sp. with dFe evidenced the absence of significant effect on larvae and pupae mortality and on the emergency of adults. Therefore, we can conclude that using MPs for reducing P concentration in lake water and lake sediment is a riskless (no toxic effect) and efficient (high P adsorption capacity) tool for lake restoration although more research on toxicological effects on other plankton and benthic organisms is required.

Acknowledgments

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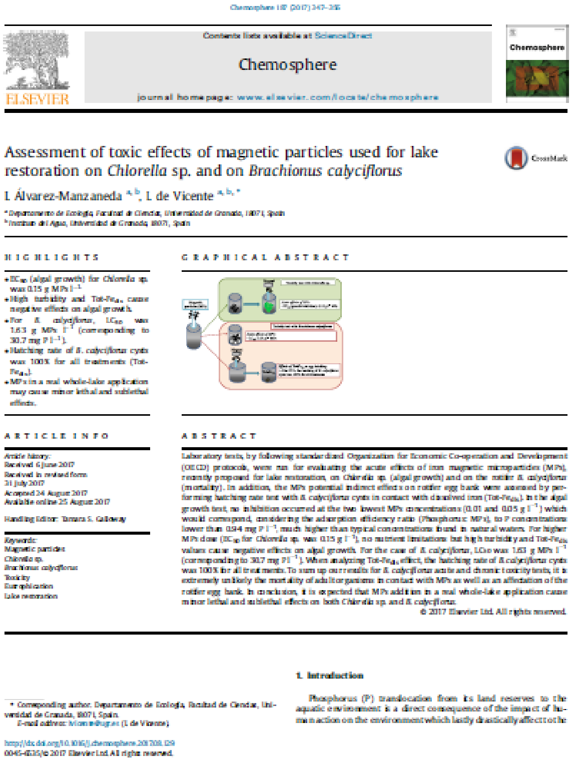
Chapter II: Assessment of toxic effects of magnetic particles used for lake restoration on *Chlorella* sp. and on *Brachionus calyciflorus*



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Assessment of toxic effects of magnetic particles used for lake restoration on *Chlorella* sp. and on *Brachionus calyciflorus*

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HIGHLIGHTS

- EC_{50} (algal growth) for *Chlorella* sp. was 0.15 g MPs l^{-1}.
- High turbidity and Tot-Fe_{dis} cause negative effects on algal growth.
- For *B. calyciflorus*, LC_{50} was 1.63 g MPs l^{-1} (corresponding to 30.7 mg P l^{-1}).
- Hatching rate of *B. calyciflorus* cysts was 100% for all treatments (Tot-Fe_{dis}).
- MPs in a real whole-lake application may cause minor lethal and sublethal effects.

GRAPHICAL ABSTRACT

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ABSTRACT

Laboratory tests, by following standardized Organization for Economic Co-operation and Development (OECD) protocols, were run for evaluating the acute effects of iron magnetic microparticles (MPs), recently proposed for lake restoration, on *Chlorella* sp. (algal growth) and on the rotifer *B. calyciflorus* (mortality). In addition, the MPs potential indirect effects on rotifer egg bank were assessed by performing hatching rate test with *B. calyciflorus* cysts in contact with dissolved iron (Tot-Fe_{dis}). In the algal growth test, no inhibition occurred at the two lowest MPs concentrations (0.01 and 0.05 g l^{-1}) which would correspond, considering the adsorption efficiency ratio (Phosphorus: MPs), to P concentrations lower than 0.94 mg P l^{-1}, much higher than typical concentrations found in natural waters. For higher MPs dose (EC_{50} for *Chlorella* sp. was 0.15 g l^{-1}), no nutrient limitations but high turbidity and Tot-Fe_{dis} values cause negative effects on algal growth. For the case of *B. calyciflorus*, LC_{50} was 1.63 g MPs l^{-1} (corresponding to 30.7 mg P l^{-1}). When analyzing Tot-Fe_{dis} effect, the hatching rate of *B. calyciflorus* cysts was 100% for all treatments. To sum up our results for *B. calyciflorus* acute and chronic toxicity tests, it is extremely unlikely the mortality of adult organisms in contact with MPs as well as an affectation of the rotifer egg bank. In conclusion, it is expected that MPs addition in a real whole-lake application cause minor lethal and sublethal effects on both *Chlorella* sp. and *B. calyciflorus*.

1. Introduction

Phosphorus (P) translocation from its land reserves to the aquatic environment is a direct consequence of the impact of human action on the environment which lastly drastically affects to the

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Abstract

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1. Introduction

Phosphorus (P) translocation from its land reserves to the aquatic environment is a direct consequence of the impact of human action on the environment which lastly drastically affect to the biogeochemical P cycle (Cordell et al., 2011). Hence, we are facing two coupled and worldwide increasing problems: (i) the global food resources depletion link to the reduction of P reserves essential for making fertilizers P (Gilbert, 2009) and (ii) the eutrophication, nutrient enrichment, of aquatic ecosystems (OECD, 1982; Sas, 1989; Cooke et al., 2005).

Eutrophication is the leading cause of water pollution for many freshwater and coastal marine ecosystems and is a rapidly growing problem in the developing world (Harper, 1992; Schindler, 2006; Smith and Schindler, 2009). Because of the main limiting nutrient for the aquatic primary production is P, it is essential to reduce P concentration when restoring eutrophicated systems. It is well accepted that P availability in lake water can be reduced by three ways (Hupfer and Hilt, 2009): (i) Reducing P external load, (ii) Increasing P retention by the sediment and (iii) Increasing P export from the system. Among these techniques, the control of P external load is an essential and preliminary step before implementing any other management strategy (Smith, 2009; Jeppesen et al. 2009). In fact, controlling catchment-derived nutrient loading is a prerequisite for lasting lake restoration efforts, otherwise internal stocks of nutrients will be replenished (Cooke et al., 2005).

As a result of the two above mentioned problems (the depletion of P reserves and the eutrophication of aquatic ecosystems), new and innovative methods are required. In this context, iron (Fe) magnetic particles (MPs) have been recently proposed as convenient P adsorbent (de Vicente et al., 2010; de Vicente et al., 2011; Merino-Martos et al., 2011). Briefly, MPs are used to adsorb P from aqueous solutions and after the adsorption is carried out, P loaded MPs can be separated by using a high gradient magnetic separation process. Later, P can be desorbed and potentially used as a fertilizer while the bare MPs can be reused. Next, we summarize the most relevant advantages for using MPs as P adsorbent for lake restoration (de Vicente et al. 2010; Merino-Martos et al. 2011; Funes et al. 2016, 2017; Álvarez- Manzaneda et al. 2017): (i) high P adsorption capacity under both batch and flow conditions; (ii) the insignificant dependence on physico-chemical conditions (redox and pH) of their P adsorption; (iii) the reduction in sedimentary P_{Mobile} concentrations caused by their addition (under both oxic and anoxic conditions), potentially contributing to a long-term reduction in P efflux; (iv) their lesser cost in comparison to other P adsorbents (e.g. $AlCl_3 \cdot 6H_2O$ and Phoslock®); and (v) the low toxic effects on plankton and benthic organisms. Accordingly, the use of MPs would help to counteract both the depletion of P reserves and the eutrophication of aquatic systems by removing P from eutrophicated systems and by using the recovered P as a fertilizer.

However, it is clear that before adding MPs in a whole-lake application strategy it is essential to gain more knowledge about MPs potential toxic effects on lake biota. The procedures currently in use for conventional risk assessment have a first step that consists in the identification and characterization of hazards based, among others, in basic toxicity tests (Amiard-Triquet et al., 2015). Accordingly, acute and chronic effects of MPs on *Daphnia magna* and on *Chironomus* sp. have been already evaluated (Álvarez- Manzaneda et al. 2017). However, and considering that MPs addition makes sense just in eutrophicated systems where the zooplankton community is dominated by rotifers instead of cladocerans (Gannon and Stemberger, 1978), it is essential to test MPs effects on rotifers. Apart from rotifers, algae were also chosen as test organisms in this study due to the following consideration: (a) they belong to the first level of the trophic chain and so, any change in the composition and density of the phytoplankton could change the biological and chemical quality of an ecosystem (Lewis, 1995); (b) they seem to be more sensitive for some contaminants than animal species (Hoffman et al., 2003) and (c) they have a short life cycle, allowing the evaluation of toxic effects over several generations (Silva et al., 2009). In addition to basic toxicity tests, experimental designs mimicking a natural environment (microcosms) are also recommended (Caquet, 2013). Therefore, by using microcosms from an hypertrophic coastal lake, potential changes on species composition and abundance of phytoplankton (del Arco et al., unpublished) and on zooplankton community (Álvarez-Manzaneda et al., unpublished) after MPs addition have been assessed.

Although the majority of standardized ecotoxicity tests and biomonitoring in aquatic systems are based on the active component of invertebrate communities, dormant egg banks are crucial for the long term survival and community dynamics of many aquatic organisms (Navis et al., 2013). In fact, the invertebrate dormant egg banks in the sediments of aquatic ecosystems constitute ecological and evolutionary reservoirs of species (De Stasio, 1989; Hairston and Munns, 1984; Hairston, 1996). Among invertebrate communities, rotifers are important components of such egg banks in freshwater systems. Most planktonic rotifers reproduce via cyclical parthenogenesis (Snell and Janssen, 1995), incorporating both asexual (amictic) and sexual (mimic) reproduction into their life cycle (Preston and Snell, 2001). The application of MPs for lake restoration may involve two kind of interaction with lake biota: i) direct and short-term effect caused by MPs and ii) indirect and long-term effect caused by the dissolved Fe (Tot-Fe_{dis}; after MPs removal). Therefore, and for the case of rotifers, it is essential to assess the potential effect of MPs and Tot-Fe_{dis} on both adult organisms and cysts.

In this context, our working hypothesis is that MPs addition for lake restoration cause lethal and sublethal effects on algae and on rotifers. Accordingly, in this paper we combine both acute, which are mostly based on mortality as endpoint, and sublethal toxicity tests looking at growth and/or reproduction of the biota. In particular, the general aim of this paper was to assess, by laboratory tests and following standardized Organization for Economic Co-operation and Development (OECD) protocols, the acute effects of MPs on *Chlorella* sp. (algal growth) and on the rotifer *Brachionus calyciflorus* (mortality). In addition, hatching test with *B. calyciflorus* cysts were performed for assessment the MPs indirect effects due to the Tot-Fe_{dis} increase. As MPs are efficient P adsorbent and they may therefore affect nutrient availability for phytoplankton, during the *Chlorella* sp. experiments a through monitoring of physico-chemical changes in the aqueous solutions was accomplished.

2. Material and methods

2.1. Test organisms

Laboratory experiments were carried out with two species belonging to two different trophic levels, the freshwater green algae *Chlorella* sp. and the rotifer *B. calyciflorus*

Chlorella sp. (cell volume: 365 µm^3; diameter: 8.8 µm) was selected as the test species because this unicellular green alga has a good sensibility to toxicants and it is easily cultured at laboratory (Silva et al., 2009).

The stock culture of *Chlorella* sp., provided by the Department of Ecology of the University of Jaén, was cultivated in an 800 ml volume with Bold’s Basal Medium (BBM; Bold, 1949). This freshwater algae medium was chosen based on previous studies who found that it is better than natural medium for toxicity tests with *Chlorella* sp. (Polonini et al., 2015). The culture was maintained in an isolated room at a temperature of 22 ± 0.5 ºC and a cycle of light: darkness of 16: 8 h. In order to avoid the sedimentation of algae cells, the culture was shaken at 100 rpm and the cell density was estimated by using a Neubauer counting chamber.

Rotifers are organisms frequently used in toxicity tests such as: i) acute toxicity tests with mortality endpoints; ii) life-cycle tests and short-term toxicity tests and iii) tests on suborganismic level (Dahms et al., 2011). Because of their sensitivity to toxic compounds they are good toxicity indicators (Alvarado-Flores et al., 2012). In addition, they often play a key role in the ecosystems dynamics, being useful as models in ecotoxicology (Snell and Janssen, 1995). Rotifers are characterized by a short-life cycle and a rapid reproduction (Fernández-Casalderrey et al., 1991) and their cysts can be store dried for long time. All of these reasons make them very suitable for toxicology.

B. calyciflorus were used for toxicological tests by following the American Society for Testing and Materials International (ASTM) standardized test (Allen, 1998). Cysts were purchased dried (from MicroBioTests, Gent, Belgium) and they were kept, at darkness, at a temperature of 5 ± 2ºC. Hatching was carried out about 16 h before the beginning of the test, at 25ºC and under continuous illumination of 3000-4000 lux, in rotifer medium (96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4 \cdot 7H_2O$ and 4 mg KCl per liter of distilled water; Allen, 1998). Then, *Chlorella* sp., originated from a culture collection of the University of Jaén, served as food for the stock rotifers with a density of $3x10^6$ cells ml^{-1}, required concentration for assuring a high population growth (Sarma et al., 2001; Lucía-Pavón et al., 2001) and a temperature between 20 and 25ºC. In addition, algal cell concentration was estimated using Neubauer counting chamber, replacing and counting the solution used as food for rotifers every week.

2.2. General characterization of magnetic microparticles

The composition of the micronsized Fe particles used is 97.5% Fe, 0.9% C, 0.5% O and 0.9% N, according to the manufacturer (BASF, Germany). Its magnetization properties, electrophoretic mobility, particle size distribution and P adsorption properties have been previously characterized (de Vicente et al., 2010; de Vicente et al., 2011; Merino-Martos et al., 2011). Briefly, MPs have spherical shape, are relatively polydisperse and with a mean diameter of 805±10 nm. They present a ferromagnetic behavior with a negligible remnant magnetization as well as a thin oxide surface layer which determines that surface charges are controlled by the pH in the aqueous medium (isoelectric point is around pH 6.5).

2.3. Toxicological test with **Chorella** *sp.*

2.3.1. Growth inhibition test

Growth inhibition test was made according to an OECD’s modified protocol (1984) and by using a sonicated (5 min) 50 g MPs l^{-1} stock solution.

First, each glass flask was inoculated with 25000 cells ml^{-1} from *Chlorella* sp. stock. Next, different volumes of the MPs stock solution were added to each treatment for getting a final concentration of: 0.01; 0.05; 0.1; 0.5; 0.7; 1; 1.5 and 2 g MPs l^{-1} in a final volume of 100 ml. All treatments and controls (no MPs addition) were run in four replicates. Test flasks were randomly placed on a horizontal shaker at 125 rpm for avoiding the algae precipitation.
After 24 h of contact time, MPs were removed by applying a magnetic field gradient exerted by a permanent magnet (volume ¼ 25.6 cm^3; NB032, Aiman GZ, Spain). Removal process of MPs was carried out by immersing the magnet twice for 3 s in the vessels.

The experiment, which lasted for three days, was performed following the OECD standardized protocol (1984) with a temperature between 24 and 25.5ºC and under continuous illumination of 6660 lux. Algal cells concentration was measured five times: before MPs addition; right after MPs removal and 24, 48 and 72 h after MPs removal. A Nageotte's counting chamber was used due to the low cell density (Lund et al., 1958; Sabiri et al., 2011; Gubelit et al., 2015).

Following the OECD protocol, the mean value of the cell concentration was plotted against time to obtain growth curves. Next, the area below the growth curves was calculated according to this formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \qquad (1)$$

where:

A: area
N_0: number of cells ml^{-1} at time t_0
N_1: number of cells ml^{-1} at t_1
N_n: number of cells ml^{-1} at t_n.
t_1: time of first measurement after beginning of the test.
t_n: time of the nth measurement after beginning of the test.

Finally, the percentage inhibition of the cell growth (I_A) was calculated as the difference between the area under the control growth curve (A_c) and the area under the growth curve at each concentration (A_t) according to the following equation:

$$I_A = \frac{A_C - A_T}{A_C} x100 \qquad (2)$$

For the estimation of the EC_{50}, I_A values were plotted on semilogarithmic paper against the corresponding concentrations. The intercept of the regression line with the parallel drawn to the abscissa at I_A was the EC_{50} (OECD, 1984).

2.3.2. *Physico-chemical variables*

At the end of the experiment, physico-chemical variables were monitored. Conductivity, pH and dissolved oxygen concentrations (DO) were measured by using a multiparameter probe (Hanna Instrument, HI 9829) while turbidity was measured with a turbidimeter ISO 7027 (LW-TN3024). Ammonium (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-), Dissolved Inorganic P (DIP) and Tot-Fe_{dis} were also measured at the end of the test after filtration (Whatman GF/F). The analytical methods were as follows: NH_4^+ was determined following the phenate method (Rodier, 1989); NO_2^- were analyzed following the sulfanilamide method (Rodier, 1989); NO_3^- were quantified by using the ultraviolet spectrophotometric screening method (APHA, 1995); DIP was analyzed by the molybdenum blue method (Murphy and Riley, 1962) and Tot-Fe_{dis} was measured by using spectroscopy emission by plasma of inductive coupling (ICP-OES PERKIN-ELMER OPTIMA 8300).

2.4. *Toxicological test with* Brachionus calyciflorus

2.4.1. *Mortality test with magnetic particles*

This toxicological test, which lasted for 24 h and with no feeding during the test, was carried out following the ASTM's standardized protocol (Allen, 1998). First, from a stock solution containing 5 g of MPs l^{-1}, we prepared the next test concentrations: 0.1; 0.5; 0.7; 1; 1.5; 2; 2.5; 3; 3.5; 4 and 5 g l^{-1} of MPs, which were sonicated for 5 min. All treatments and controls (no MPs addition) were run with four replicates.

When placed in the recommended medium (Allen, 1998), rotifer cysts hatch in about 16 h at 25°C. Later, ten individuals (< 2 h) of *B. calyciflorus* were placed in each well of a multiple-well plate (48 well plates. Total volume per well: 1.4 ml) by using a stereomicroscope and a micropipette and, immediately, 1 ml of the different solutions was added. The plate, covered with parafilm to avoid evaporation, was placed in a culture chamber at darkness and at temperature of 25 ± 1ºC.

After 24 h of contact time with the MPs, the organisms were observed by using a stereomicroscope and the number of living and dead organisms was recorded. If the animal did not move the mastax or foot for 5 seconds it was recorded as a dead individual. Rotifers mortality in control was always lower than 10%.

For estimating the LC_{50} (concentration of MPs which cause the 50% of mortality of the total organisms) in the *B. calyciflorus* test, Probit analysis was carried out with the statistical program SPSS. This is a parametric statistical method very used in toxicology to analyze doses-response tests transforming sigmoidal dose-response curves to a straight line that can be analyzed (Vincent, 2014).

2.4.2. *Cysts hatching in the presence of dissolved iron*

With this experiment, we aim on assessing the effect of the Tot-Fe_{dis} (dissolved from MPs) on the cysts hatching in *B. calyciflorus.* Firstly, suspensions containing different MPs concentrations (0; 0.1; 0.5; 0.7; 1; 1.5; 2; 2.5; 3; 3.5; 4 and 5 g l^{-1}) were prepared from an stock solution of 5 g of MPs l^{-1}. They were sonicated for 5 min and after 24 h, MPs were removed by applying a magnetic field gradient exerted by a permanent magnet (the same as described in section 2.3.1). For running the toxicological experiment, one cyst was placed in each well of the multiple-well plate (96 well plates. Total volume per well: 0.37 ml) containing 0.35 ml of each MPs concentration and the plate was covered with parafilm to avoid evaporation. The multiple-well plate was placed in a culture chamber at 3000-4000 lux of continuous illumination and at temperature of 25±1ºC. All treatments were run with eight replicates. After 16 h, cysts were observed every half hour until the last cyst hatched and the number of hatched cysts were recorded. Finally, time of the first hatching (TFH; Gutierrez et al., 2017) and synchronized time (Ts; Ortega-Salas, 2013) were calculated. In particular, Ts was estimated as the difference between T_{90} (time when 90% of cyst hatched) and T_0 (time when the first neonate hatched).

2.4.3. Physico-chemical variables

Temperature, hardness and pH were measured at the beginning and at the end of the test while DO was measured just at the beginning. Tot-Fe_{dis} concentrations were also measured at the end of the experiment by using the method already described in the section 2.3.2.

2.5. Statistical data analysis

In the algal inhibition test, As both non transformed and logarithmically transformed data did not fit a normal distribution, non parametrical tests (Kruskal-Wallis ANOVA and U of Mann-Whitney; software SPSS) were carried out for identifying if there exists significant differences between control and treatments.

3. Results and discussion

3.1. Effect of magnetic particles on **Chlorella** *sp.*

In the algal growth inhibition test, no inhibition occurred at the two lowest MPs concentrations (0.01 and 0.05 g l^{-1}) while the highest MPs concentration (2 g l^{-1}) caused an average inhibition of 83%. The EC_{50} was very low (0.15 g l^{-1}; Fig. 1).

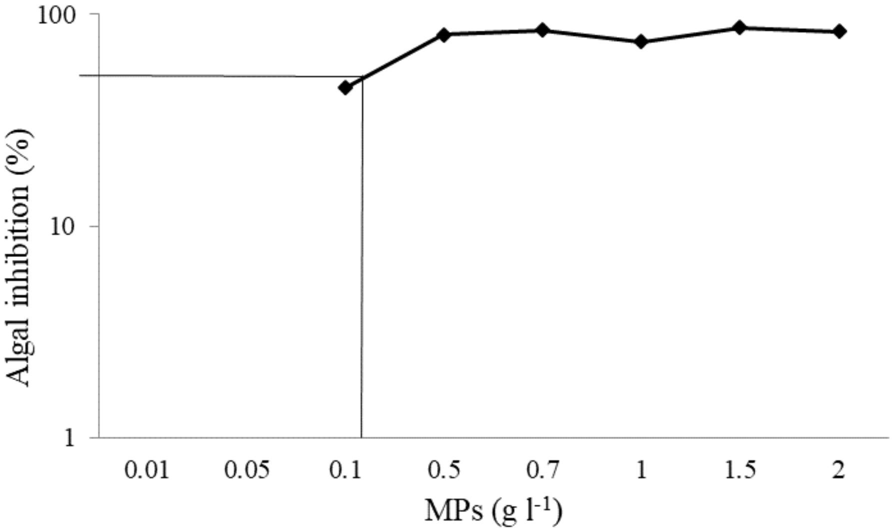

Figure 1. *Chlorella* sp. growth inhibition (%) as a function of MPs concentration. Please note that EC_{50} is shown.

When comparing these results with those found for the same MPs and *Selenastrum capricornutum* (EC_{50} of 1.5 g l^{-1}; Álvarez-Manzaneda et al., unpublished) we noticed great differences which could be determined by the different chemical composition of the solution (BBM was used in the present study while ISO medium was used by Álvarez-Manzaneda et al. (unpublished). Similarly, Millington et al. (1988) noted that the toxic values from algae toxicity tests are affected by the medium composition. Although EC_{50} for *Chlorella* sp. was very low, this concentration was much higher than others found for metals or nanoparticles (NPs) of titanium, zinc, aluminum or silica for *Chlorella* sp. (Mehta and Gaur, 1999; Ji et al., 2011; Iswarya et al., 2015). In similar studies performed with others green algae and NPs, EC_{50} was also lower than that found in the present study (Christensen and Nyholm, 1984; Griffitt et al., 2008; Becaro et al., 2014; Bhuvaneshwari et al., 2015; Adam et al., 2015). However, it is important to bear in mind the limitations for these comparisons as eventually, toxic effects will dramatically depend on the type of particle and on the alga specie. In this sense, Menard et al. (2011) highlighted the great heterogeneity in the results obtained by using the same NPs but different alga species. Even more, it is well known the close inverse relationship between specific surface area of the particles and its toxic effect on the organisms (Fujiwara, 2008; Van Hoecke et al., 2008; Navarro et al., 2008; Ji et al., 2011; Clément et al., 2013) and so, it is risky to compare toxic effects of microparticles and NPs.

Similarly to the estimations made by Álvarez-Manzaneda et al. (2017), and considering the 53 mg MPs: mg P mass ratio as the adsorption efficiency ratio (de Vicente et al., 2010; Merino-Martos et al., 2011), the addition of 0.15 g MPs l^{-1} (EC_{50} for *Chorella* sp.) would correspond to a scenario of 2.8 mg P l^{-1}, which is an extremely high value for typical inland waters (hypereutrophic category correspond to annual mean TP concentration > 100 µg l^{-1}; Nürnberg, 1996). This highlights that the use of MPs in effective concentrations for P removal is likely to have no effect or minor effects in phytoplankton community.

For comparing the toxicity of MPs with other P adsorbents (Phoslock, alum, Zeolites, calcite) used for lake restoration, a wide literature review has been done. Similarly, to a recent study performed with *D. magna* and *Chironomus* sp. (Álvarez-Manzaneda et al., 2017), an evident scarcity of well standardized tests makes difficult to establish a thorough comparison. In fact, no previous studies have been done with *Chlorella* sp. and other P adsorbents. For *Scenedesmus obliquus*, van Oosterhout and Lürling (2013) found that the threshold Phoslock® concentration for causing a depletion in algal biovolume was 0.5 g l^{-1}. Considering the 100:1 Phoslock®: P dose ratio, recommended by the manufacturer (Reitzel et al., 2013), 0.5 g Phoslock l^{-1} would match to 5 mg P l^{-1} which is a rather atypical P concentration in natural waters.

Factors affecting the growth of microalgae may be included in two categories: environmental factors (physical) and nutritional factors (chemical). Physical factors include pH, temperature, light intensity and the aeration of the system; while nutritional factors comprise the composition and amount of the chemical species in culture medium (C, N, P, among others; Daliry et al., 2017). Accordingly, during this experiment a thorough chemical monitoring of the solutions was achieved for identifying the main factor driving algal growth inhibition. No significant changes were observed between control and treatment for DO (Tables 1 and 3). pH significantly increased when increasing MPs concentration, except for the lowest MPs concentration; while Conductivity was only significantly higher in the treatments with some of the highest MPs concentration (1 and 2 g MPs l^{-1}) compared to control.

Table 1. Physico-chemical parameters at the end of the *Chlorella* sp. growth inhibition test (mean ± SD). Italics bold numbers show significant differences compared to control. [1]Kruskal-Wallis ANOVA and [2]U of Mann-Whitney test.

MPs ($g\ l^{-1}$)	**[2]pH**	**[2]Conductivity ($\mu S\ cm^{-1}$)**	**[1]O_2 ($mg\ l^{-1}$)**	**[2]Turbidity (NTU)**
Control	5.78 ± 0.48	508 ± 7	7.8 ± 0.1	17.90 ± 9.77
0.01	6.35 ± 0.06	509 ± 18	7.9 ± 0.1	***53.15 ± 18.50***
0.05	***7.08 ± 0.30***	541.25 ± 22	7.7 ± 0.3	***160.75 ± 118.11***
0.1	***6.85 ± 0.30***	557 ± 35	7.7 ± 0.1	***216.47 ± 189.87***
0.5	***7.39 ± 0.28***	577.5 ± 47	7.8 ± 0.1	***517.00 ± 412.48***
0.7	***7.52 ± 0.19***	540 ± 17	7.7 ± 0.1	***536.50 ± 437.00***
1	***7.44 ± 0.35***	***606 ± 9***	7.7 ± 0.0	***548.50 ± 431.38***
1.5	***7.67 ± 0.20***	597.5 ± 69	7.5 ± 0.4	***677.50 ± 287.20***
2	***7.89 ± 0.30***	***583 ± 161***	7.7 ± 0.1	***641.75 ± 345.59***

As expected, turbidity was significantly higher in treatments than in control (Table 2). In fact, turbidity increased with MPs following a logarithmic law (r= 0.88; $p<0.05$; Fig. 2a). Therefore, algal growth inhibition could be due to the MPs "shading effect". Previous studies have also shown inhibitory effects of NPs which trapped the cells (Ji et al., 2011; Gong et al., 2011; Huang et al., 2016; Cupi and Baun, 2016). This "shading effect" may mask or limit the chemical toxicity because algal cells with slow growth, due to low light intensity, are less sensitive to toxics than those with faster growth (Hjorth et al., 2016). Van Oosterhout and Lürling (2013) also observed a notably increased in turbidity (up to 211 NTU) when adding Phoslock®. This shading effect caused that algae growth rather than be affected by toxicity is affected by physical inhibition (Sørensen et al., 2016). Our results evidence that algal growth inhibition was higher than 80% when adding 0.5 g MPs l^{-1} which lastly caused extreme turbidity values (>500 NTU). However, in a real whole-lake application, these negative effects are unlikely to occur as 0.5 g MPs l^{-1} would correspond to extremely high P concentration (9.4 mg l^{-1}) and secondly, because MPs are characterized by a fast sedimentation rate (Funes et al. 2017) and so, turbidity quickly decreased with time. In fact, del Arco et al. (unpublished) found that after just one hour, turbidity decreased around 40%. Unexpectedly, turbidity values obtained in the present study are extremely higher than those found by del Arco et al. (unpublished) when using the same MPs in the same concentration range. The only difference which could explain so huge variation in NTU values is the solution (medium) where MPs were dissolved: BBM in the present study and commercial mineral water in del Arco et al. (unpublished). Therefore, it is expected that in a real whole-lake application turbidity values will be much lower than those found in the present study.

Table 2. Chemical parameters measured at the end of the *Chlorella* sp. growth inhibition test (mean ± SD). Italics bold numbers show significant differences in compared to control. ^{1}Kruskal-Wallis ANOVA and ^{2}U of Mann-Whitney test.

MPs (g l^{-1})	**^{1}N-NH_4^+ (mg l^{-1})**	**^{2}N-NO_3^- (mg l^{-1})**	**^{2}N-NO_2^- (mg l^{-1})**	**^{2}P-PO_4^{3-} (mg l^{-1})**	**^{2}Tot-Fe_{dis} (mg l^{-1})**	**^{2}Growth inhibition/ Control (%)**
Control	0.26 ± 0.51	105.46 ± 35.10	0.02 ± 0.02	75132.40 ± 24000.75	1.11 ± 0.11	
0.01	0.00 ± 0.00	114.34 ± 31.25	***0.00 ± 0.00***	74311.39 ± 16447.17	1.59 ± 0.08	0
0.05	0.00 ± 0.00	107.09 ± 21.17	0.64 ± 0.71	64551.76 ± 5297.70	1.85 ± 0.10	0
0.1	0.02 ± 0.11	132.19 ± 17.38	***0.4 ± 0.16***	73500.80 ± 12020.38	2.44 ± 0.47	44.9 ± 51.4
0.5	0.03 ± 0.10	145.10 ± 7.78	***1.27 ± 1.35***	44729.53 ± 20953.80	11.11 ± 6.29	***80.3 ± 15.3***
0.7	0.02 ± 0.09	***150.84 ± 12.68***	***1.39 ± 1.22***	34220.57 ± 21573.58	12.65 ± 3.24	***84.1 ± 8.7***
1	0.18 ± 0.26	147.30 ± 11.49	***1.59 ± 1.3***	43539.06 ± ***12934.86***	16.94 ± 7.77	***74.2 ± 29.2***
1.5	0.12 ± 0.22	***154.32 ± 10.8***	***1.78 ± 0.95***	***35657.35 ± 15225.11***	***30.24 ± 6.64***	***86.6 ± 29.2***
2	0.09 ± 0.16	***144.49 ± 7.44***	***1.67 ± 1.08***	***31552.28 ± 11920.22***	***22.62± 2.57***	***82.8 ± 19.2***

Nutrient concentration in the experiment was checked along the experiment. In relation to DIN fractions, NH_4^+ did not show significant differences between control and treatments; while marginal significant differences were found for NO_3^- and NO_2^- concentrations (Table 2). As it was expected, because an algal growth culture medium very rich in inorganic nutrients (BBM) was used for this experiment, DIN concentration was much higher than that consider by Reynolds (1992, 1999) as limiting for primary producers (80 µg N l^{-1}). In the case of DIP only significant differences between control and the two highest concentrations of MPs were found (Table 2). It is important to note that MPs caused a significant reduction in P concentration (Fig. 2b) but still P-SRP concentrations were extremely higher than P threshold concentration (3 µg l^{-1}) proposed by Reynolds (1992, 1999) for identifying P as a limiting nutrient of the primary production. Even more, when estimating DIN:SRP molar ratio, an extremely low value (< 0.02) was found for all cases, reflecting that *Chlorella* sp. was N limited. Similarly, Wu et al. (2014) found that *Chlorella vulgaris* can grow with lower N concentrations than P.

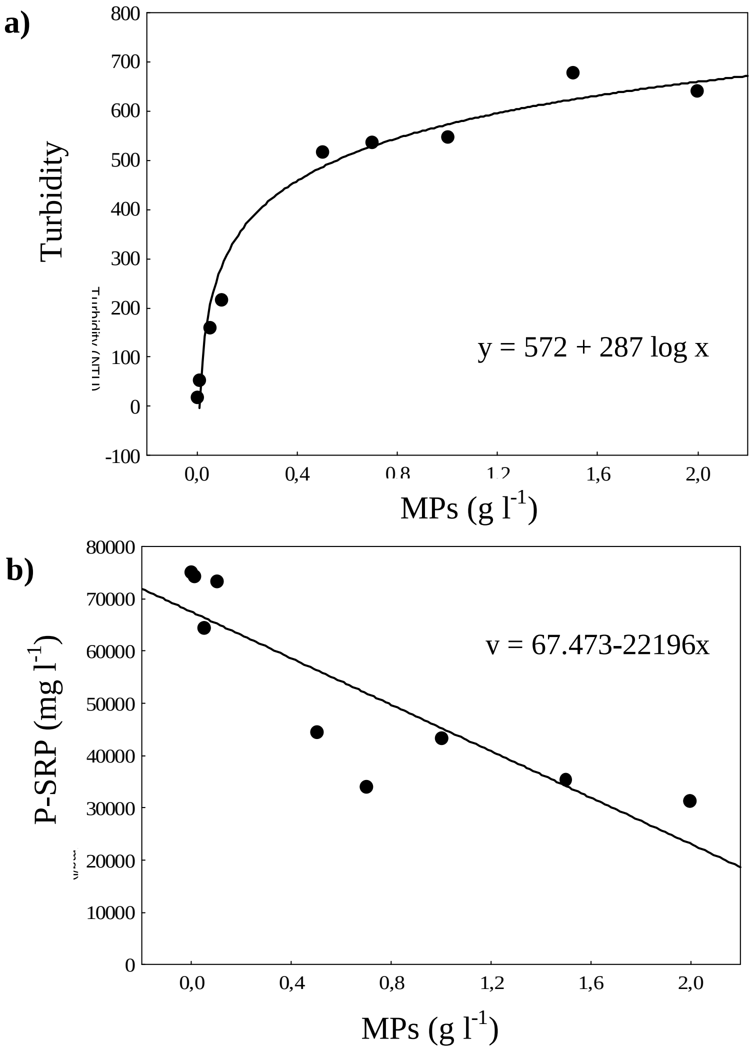

Figure 2. MPs effect on water turbidity (a) and on P-SRP concentration (b).

At last, Tot-Fe$_{dis}$ concentration was significantly higher in 1.5 and 2 g MPs l^{-1} treatments than in control (Table 2). Average Tot-Fe$_{dis}$ concentration was 1.11 and 12.43 mg l^{-1} in control and treatments, respectively. These values are much higher than other reported by Funes et al. (2016; 2017) when adding MPs, under anoxic and oxic conditions, to natural waters. Therefore, our results reflect that MPs are much easily dissolved in BBM (algal growth medium) than in natural waters. However, Tot-Fe$_{dis}$ concentrations are in the range of those used by Estevez et al. (2001) for studying iron-dependent oxidative stress in *Chlorella vulgaris.* These authors found that, algal growth was estimulated when Fe availability (EDTA:Fe, 2:1) was lower than 100 µM (5.6 mg Fe l^{-1}); while Fe concentrations higher than 200 µM (11.2 mg Fe l^{-1}) led to a drastic decrease in the growth of the cultures. Similarly, we have found inhibition growth in *Chlorella* sp. higher than 80% for Tot-Fe$_{dis}$ > 10 mg l^{-1} (Table 2).

3.2. Ecotoxicological tests with **Brachionus calyciflorus**

3.2.1. Mortality test with magnetic particles

LC$_{50}$ in *B. calyciflorus* was 1.63 g MPs l^{-1} (Figure 3) which is in the range of EC$_{50}$ (immobilization; at 24 h) values reported for other planktonic (1.99 g MPs l^{-1} for *D. magna*, Álvarez-Manzaneda et al., 2017) and benthic organisms (1.57 g MPs l^{-1} for *Chironomus* sp.; Álvarez-Manzaneda et al., 2017). Although LC$_{50}$ and EC$_{50}$ are obviously different endpoints, Jones et al. (1991) noted that sublethal toxicant concentration may change organism behaviour (in this case their mobility) conducting to long-term consequences on the survival rates. Therefore, and especially for planktonic organisms, there exists an explicit relationship between both endpoints. In this sense, experiments with *D. magna* in contact with MPs have recently shown a physical immobilization of the organisms (Álvarez-Manzaneda et al., unpublished) caused by the attachment of the particles in their bodies decreasing the movement capacity of the organism as others authors have found for other type of particles (Skjolding, 2016).

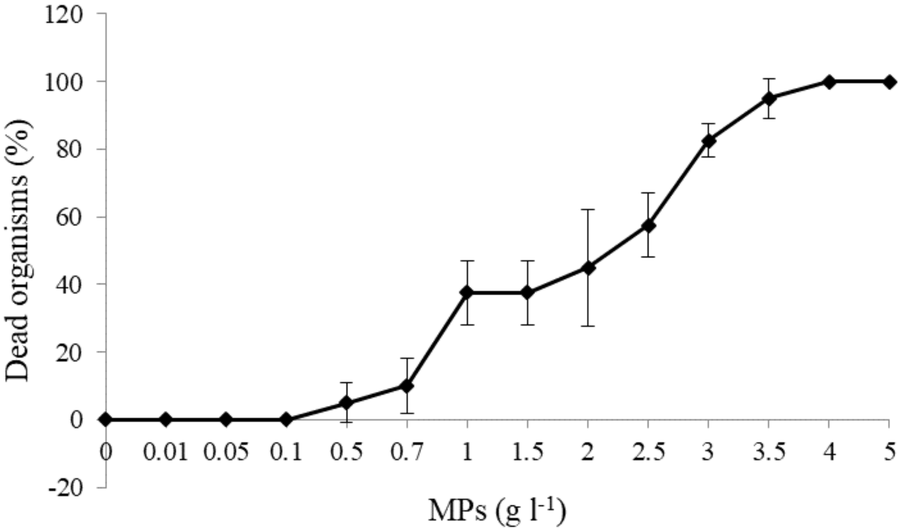

Figure 3. Dead organisms (%) of *B. calyciflorus* in contact, for 24 h, with MPs. Vertical error bars show standard deviation of data (SD). n=9.

There exists a clear inconsistency between our results from acute standardized tests and those obtained from outdoor microcosm experiments (Álvarez-Manzaneda et al., unpublished). In fact, our results have shown that the presence of 1.5 g MP l^{-1} caused the death of 40% of the population of *B. calyciflorus*; while when the whole plankton community (microcosm experiments) was exposed to 1.4 g MP l^{-1} no significant effect was observed neither in the zooplankton biomass or diversity. More specifically, in the microcosm experiments no significant changes in *B. calyciflorus* abundance were observed after 1.4 g MP l^{-1} addition. Similarly, Pascoe et al. (2000) in a comparative study about toxicological effects of pollutants (three reference chemicals) in laboratory and fields experiments found that No Observed Effect Concentrations (NOEC) was lower in laboratory than in field, reflecting a higher organisms sensibility under laboratory conditions.

At this point it is important to consider that there exists a continuum of experimental contexts used in aquatic toxicology which ranges from single-species toxicity tests to natural ecosystems (Caquet, 2013). Standardized laboratory tests, which are used for determining environmentally safe concentrations of pollutants (Sih et al., 2004), can have high throughput, their results can easily be interpreted and compared among laboratories, and they often correctly predict lethal or sublethal toxic effects on natural communities (Chalcraft et al., 2005; Versteeg et al., 1999). However, this approach usually does not consider additive or synergistic effects of multiple biotic and abiotic stress factors (Sih et al., 2004; Mikó et al., 2015). In fact, microcosm studies reflect more environmentally realistic exposure conditions, including natural abiotic conditions (OECD, 2006). Accordingly, it is evident that *B. calyciflorus* is much less vulnerable to MPs under more natural conditions (microcoms) than in single-species toxicity tests and therefore, in a future context of a whole lake application of MPs and considering a real MPs dose, it is expected that MPs toxic effects on *B. calyciflorus* are negligible.

Experiences with other P adsorbents such as Phoslock® are likely to reveal a major toxicity than MPs. In particular, van Oosterhout and Lürling (2013) found that *B. calyciflorus* population growth notably decreased when Phoslock® concentrations were higher than 0.2 g l^{-1}, which would correspond, following the same calculations explained above in section 3.1., to 2 mg P l^{-1} (LC_{50} for MPs is 1.63 g l^{-1} matching to 30.75 mg P l^{-1}).

Despite of the limitations for comparing the toxicity of pollutants which ultimately depend on many factors (e. g. valence of the element, redox conditions), our results are likely to indicate that MPs have a lower toxicity for *B. calyciflorus* than other compounds reported in the literature which cause abnormal behaviour on this species a few hours after the contact (Charoy and Janssen, 1999). Similarly, Zweerus et al. (2017) found a decrease in the population growth of *B. calyciflorus* when they were exposed to copper concentrations (50 to 100 µg l^{-1}) much lower than MPs concentrations used in the present study.() The LC_{50} obtained in our study is much higher than that found for *B. calyciflorus* in contact with other compounds such as copper (26 µg l^{-1}; De Schamphelaere et al., 2006), endosulfan (5.15 mg l^{-1}; Fernández-Casalderry et al., 1992), lindane (8.5 mg l^{-1}; Ferrando et al., 1993) or lead (125 µg l^{-1}; Grosell et al., 2006). When comparing our results for MPs with other metallic NPs, we found out that LC_{50} is higher for MPs than for NPs (Manusadžianas et al., 2012) as a result of the previously described strong dependency of toxicity on particle size. In fact, NPs have a much smaller size than MPs (≈800 nm; de Vicente et al., 2010) penetrating in the wall gut and tissues (Rothhaupt, 1990; Vadstein et al., 1993; Snell and Hicks, 2009).

Finally, it is important to consider that during the acute test, organisms were exposed to the best conditions, except that they were unfed during the 24 h experiment (as it is established in the standardized protocol). In this sense, some authors have suggested that in presence of food these results could have changed (Luna-Andrade et al., 2002; Perez and Sarma, 2008), although food privation begins to cause mortality about 32 h at 25ºC (Allen, 1998).

3.2.2. *Cysts hatching in the presence of dissolved iron*

After 22 h, the hatching of *B. calyciflorus* cysts was 100% for all treatments (Figure 4) and so, Tot-Fe_{dis} released after MPs addition did not affect rotifer hatching rate.

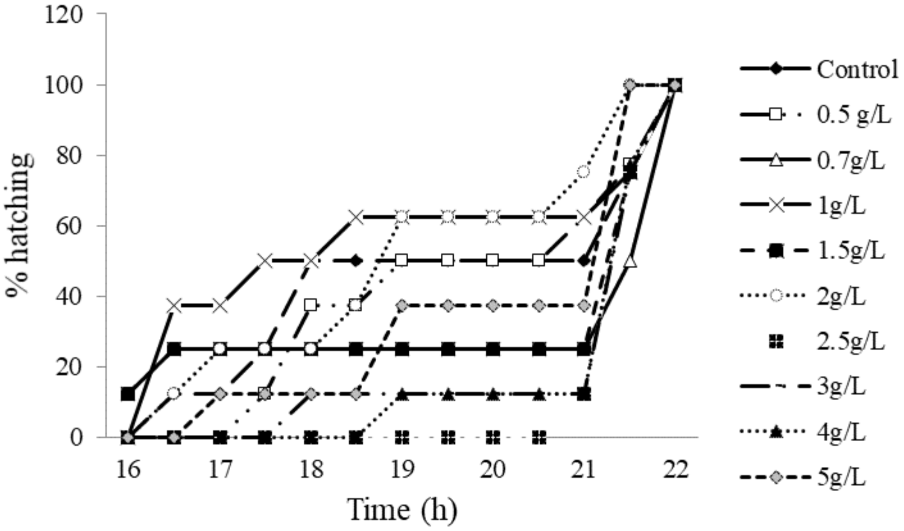

Figure 4. Temporal evolution of hatched cyts of *B. calyciflorus* (%) in the different treatments. Nominal MPs concentrations are represented in different lines.

Similarly, Ts and TFH were not significantly different between control and treatments (Table 3). In particular, Ts ranged from 4.2 (treatments) to 5.2 h (control). Therefore, this situation corresponds to optimal conditions for the rotifers hatching (Snell and Persoone, 1989; Ortega-Salas, 2013). Some authors have noticed that the suitable conditions for cysts rotifers hatching are just a minimum of light and oxygen concentrations (Gilbert, 2017); while others authors have found the need to stimulate the cysts more than once until their hatching (Martínez- Ruíz and García-Roger, 2015).

Table 3. Physico-chemical parameters at the beginning (T_0) and at the end (T_f); synchronized time (Ts) and Time of the First Hatching (TFH) of the experiment with *B. calyciflorus* (mean ± SD).

	T (°C)	**pH**	**Hardness**	**O_2 (%)**	**Ts (h)**	**TFH (h)**
Control						
T_0	21.07	10.45	2.33	83.56	5.2	16.5
T_f	22.9	10.36	2.33			
Treatments						
T_0	21.11 ± 0.09	10.37 ± 0.04	2.35 ± 0.02	83.61 ± 0.14	4.2± 1.6	17.5 ±1.6
T_f	22.82 ± 0.07	10.37 ± 0.03	2.30 ± 0.02			

The high hatching rate is in accordance with the extremely low Tot-Fe_{dis} concentrations, ranging from 0 to 0.061 mg l^{-1} (Figure 5). These values were much lower than those recorded as negative for the survival of *B. calyciflorus* (Couillard et al., 1989; Santos-Medrano and Rico-Martínez, 2013). Tot-Fe_{dis} concentrations were much lower than those found in the *Chlorella* sp. experiment (section 3.1) when the same MPs but different medium were used, thus confirming the relevance of the medium where MPs are dissolved.

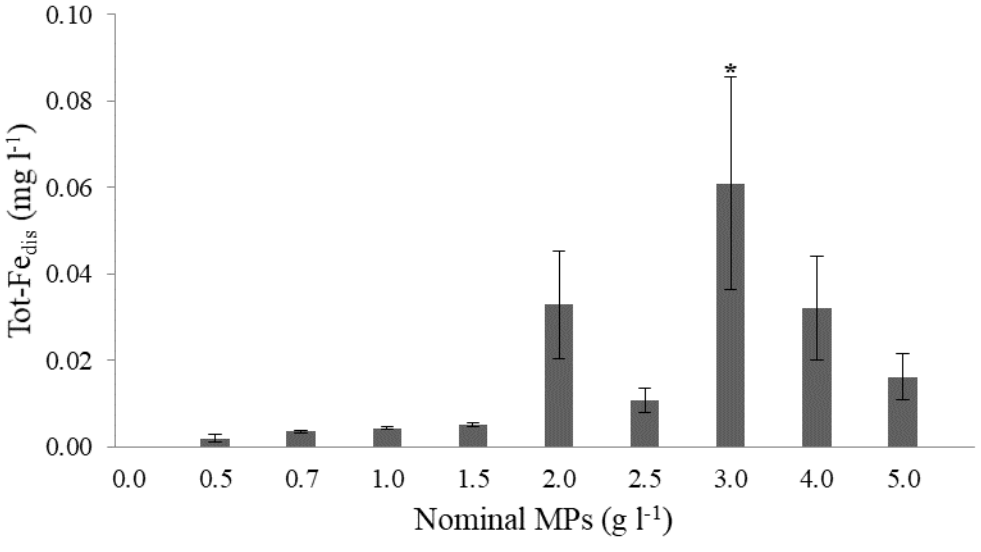

Figure 5. Dissolved iron concentration (Tot-Fe_{dis}) as a function of nominal MPs concentration during the *B. calyciflorus* hatching experiment. Vertical error bars show standard deviation of data (SD). Asterisk show significant differences (Kruskal Wallis and U of Mann-Whitney test).

In relation to the physico-chemical parameters, no significant changes were observed at beginning and at the end of the test (Table 3).

Contrarily to our results, previous studies have evidenced the sensibility of *Brachionus patulus* and *B. calyciflorus* to metals such as cadmium, copper or chromium, which drastically affect the production of offspring (Sarma et al., 2006; Gama-Flores et al., 2007). Similarly, insecticides significantly affect the survival and reproduction of rotifers (Ferrando et al., 1996).

All in all, although there exist a clear lack of studies focused on assessing the effects of other substances discharged to the natural environment on cysts hatching of *B. calyciflorus*, we may conclude that MPs effects are likely to be negligible. In fact, the null effect on hatching rate of rotifer cysts indicate that MPs addition for lake restoration would not endanger the long-term presence of *B. calyciflorus.*

4. Conclusions

In the algal growth test, no inhibition occurred at the two lowest MPs concentrations (0.01 and 0.05 g l^{-1}). Considering the 53 mg MPs: mg P mass ratio as the adsorption efficiency ratio (de Vicente et al., 2010; Merino-Martos et al., 2011), these concentrations would match to P concentrations lower than 0.94 mg P l^{-1}, which are much higher than typical concentrations found in natural waters (hypereutrophic category correspond to annual mean TP concentration > 100 µg l^{-1}; Nürnberg, 1996). Therefore, it is unlikely that MPs addition in a whole-lake application may cause negative effect on algal growth. For higher MPs dose (EC_{50} for *Chlorella* sp. was 0.15 g l^{-1}), no nutrient limitations but high turbidity ("shading effect") and Tot-Fe_{dis} values cause negative effects on algal growth. In fact, algal growth inhibition was higher than 80% when adding 0.5 g MPs l^{-1} which lastly caused extreme turbidity values (>500 NTU). Additionally, Tot-Fe_{dis} concentration was significantly higher in 1.5 and 2 g MPs l^{-1} treatments than in control. For the case of *B. calyciflorus*, LC_{50} was 1.63 g MPs l^{-1} which is in the range of EC_{50} (immobilization; at 24 h) values reported for other planktonic (1.99 g MPs l^{-1} for *D. magna,* Álvarez-Manzaneda et al., 2017) and benthic organisms (1.57 g MPs l^{-1} for *Chironomus* sp.; Álvarez-Manzaneda et al., 2017). When analyzing Tot-Fe_{dis} effect on hatching rate, no significant effects were found (after 22 h, the hatching of *B. calyciflorus* cysts was 100% for all treatments). Therefore, it is unlike that the increase on Tot-Fe_{dis} by MPs addition in a whole-lake application may cause any negative effect on rotifer community. The high hatching rate is in accordance with the extremely low Tot-Fe_{dis} concentrations, ranging from 0 to 0.061 mg l^{-1}, which are much lower than those recorded as negative for the survival of *B. calyciflorus*. To sum up our results for *B. calyciflorus* lethal and sublethal toxicity tests, it is extremely unlikely the mortality of adult organisms in contact with MPs (LC_{50} was 1.63 g MPs l^{-1} which correspond to 30.7 mg P l^{-1}) as well as an affectation of the rotifer egg bank. In conclusion, it is expected that MPs addition in a real whole-lake application cause minor lethal and sublethal effects on both *Chlorella* sp. and *B. calyciflorus*. However, further research for assessing MPs effects on lake biota is required. An outstanding aspect is to study the effect of double exposition which may considerably reduce LC_{50} values as well as MPs addition effects on ecological processes such as primary and secondary production.

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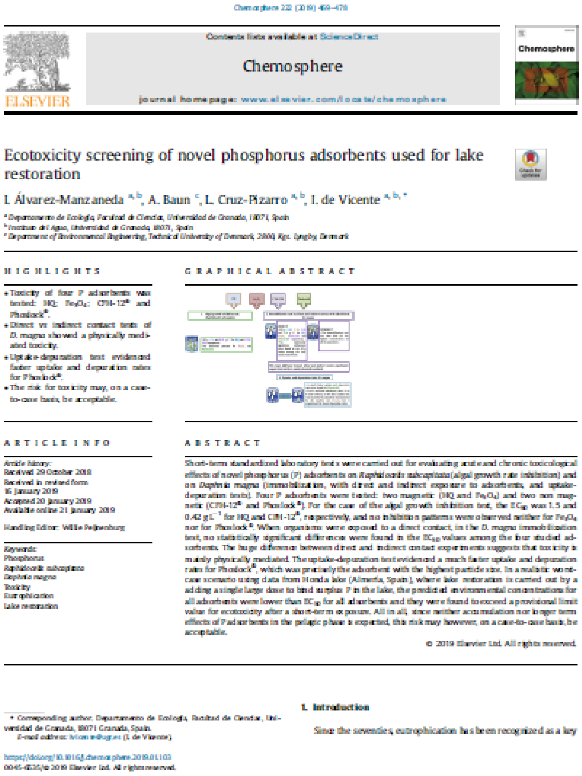
Chapter III. Ecotoxicity screening of novel phosphorus adsorbents used for lake restoration



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Ecotoxicity screening of novel phosphorus adsorbents used for lake restoration

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HIGHLIGHTS

- Toxicity of four P adsorbents was tested: HQ; Fe_3O_4; CFH-12® and Phoslock®.
- Direct vs indirect contact tests of *D. magna* showed a physically mediated toxicity.
- Uptake-depuration test evidenced faster uptake and depuration rates for Phoslock®.
- The risk for toxicity may, on a case-to-case basis, be acceptable.

GRAPHICAL ABSTRACT

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ABSTRACT

Short-term standardized laboratory tests were carried out for evaluating acute and chronic toxicological effects of novel phosphorus (P) adsorbents on *Raphidocelis subcapitata* (algal growth rate inhibition) and on *Daphnia magna* (immobilization, with direct and indirect exposure to adsorbents, and uptake-depuration tests). Four P adsorbents were tested: two magnetic (HQ and Fe_3O_4) and two non magnetic (CFH-12® and Phoslock®). For the case of the algal growth inhibition test, the EC_{50} was 1.5 and 0.42 g L^{-1} for HQ and CFH-12®, respectively, and no inhibition patterns were observed neither for Fe_3O_4 nor for Phoslock®. When organisms were exposed to a direct contact, in the *D. magna* immobilization test, no statistically significant differences were found in the EC_{50} values among the four studied adsorbents. The huge difference between direct and indirect contact experiments suggests that toxicity is mainly physically mediated. The uptake-depuration test evidenced a much faster uptake and depuration rates for Phoslock®, which was precisely the adsorbent with the highest particle size. In a realistic worst-case scenario using data from Honda lake (Almería, Spain), where lake restoration is carried out by adding a single large dose to bind surplus P in the lake, the predicted environmental concentrations for all adsorbents were lower than EC_{50} for all adsorbents and they were found to exceed a provisional limit value for ecotoxicity after a short-term exposure. All in all, since neither accumulation nor longer term effects of P adsorbents in the pelagic phase is expected, this risk may however, on a case-to-case basis, be acceptable.

1. Introduction

Since the seventies, eutrophication has been recognized as a key

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Abstract

Short-term standardized laboratory tests were carried out for evaluating acute and chronic toxicological effects of novel phosphorus (P) adsorbents on *Raphidocelis subcapitata* (algal growth rate inhibition) and on *Daphnia magna* (immobilization, with direct and indirect exposure to adsorbents, and uptake-depuration tests). Four P adsorbents were tested: two magnetic (HQ and Fe_3O_4) and two non magnetic (CFH-12® and Phoslock®). For the case of the algal growth inhibition test, the EC_{50} was 1.5 and 0.42 g l^{-1} for HQ and CFH-12®, respectively, and no inhibition patterns were observed neither for Fe_3O_4 nor for Phoslock®. When organisms were exposed to a direct contact, in the *D. magna* immobilization test, no statistically significant differences were found in the EC_{50} values among the four studied adsorbents. The huge difference between direct and indirect contact experiments suggests that toxicity is mainly physically mediated. The uptake-depuration test evidenced a much faster uptake and depuration rates for Phoslock®, which was precisely the adsorbent with the highest particle size. In a realistic worst-case scenario using data from Honda lake (Almería, Spain), where lake restoration is carried out by a adding a single large dose to bind surplus P in the lake, the predicted environmental concentrations for all adsorbents were lower than EC_{50} for all adsorbents and they were found to exceed a provisional limit value for ecotoxicity after a short-term exposure. All in all, since neither accumulation nor longer term effects of P adsorbents in the pelagic phase is expected, this risk may however, on a case-to-case basis, be acceptable.

1. Introduction

Since the seventies, eutrophication has been recognized as a key problem impairing the quality of worldwide water resources (Cooke et al., 2005; OECD, 1982; Sas, 1989). As phosphorus (P) is the main limiting nutrient for primary production in aquatic freshwater ecosystems, the first attempt for restoring eutrophicated systems is based on reducing P concentration in the aquatic ecosystems. This reduction can be achieved by decreasing external and internal (from the sediment) P loads and by increasing P export from the systems (Hupfer and Hilt, 2008).

Actually, one of the most promising methods consists on adding P-sorbing materials (e.g. modified clays, industrial by-products, flocculants and physical barriers) for stripping P from the water column and making it non-bioavailable for primary producers (Spears et al., 2016). Iron (Fe) and Aluminum (Al) oxides have been traditionally used for lake restoration (Cooke et al., 2005). Although Fe oxides are characterized by a high P adsorption capacity, the permanent P fixation requires in most cases continuous complete mixing or aeration (Jeppesen et al., 2009) due to the fact that Fe oxides are highly redox sensitive. Furthermore, Al salts as alum ($Al_2(SO_4)_3$) or polyaluminum chloride form solid $Al(OH)_3$ with high affinity for P in lake water. Despite Al salts have been used in lakes around the world for nearly half a century (Kennedy et al., 1987; Landner, 1970), they present the next disadvantages: (i) the drop in lake water pH during the formation of $Al(OH)_3$ floc; (ii) toxicity for the lake biota when pH is out of the range 6-8 (Lin et al., 2017; Nogaro et al., 2016; Pacioglu et al., 2016) and (iii) the strong reduction of P adsorption capacity with time; as it has been found that $Al(OH)_3$ lost 75% of the maximum P adsorption capacity in 90 days (de Vicente et al., 2008).

To counteract the mentioned limitations of both Fe and Al salts, novel P adsorbents have been recently proposed for restoring eutrophicated aquatic ecosystems. Lanthanum (La) modified bentonite (Phoslock®) is being increasingly used in lakes for P control (Douglas et al., 2000; Robb et al., 2003; Spears et al., 2016). It was firstly developed by the Common Wealth Scientific and Industrial Research Organization (CSIRO, Australia; Finsterle, 2014) for adsorbing P from the water column and for increasing P sorption capacity of the sediment (Meis et al., 2012; Robb et al., 2003). Among the most important advantages of this compound we remark that it can be used in a wide pH (5-9) range and even under anoxic conditions (Meis et al., 2012; Robb et al., 2003). However, Phoslock® application also shows some drawbacks such as chemical interferences with humic substances or oxyanions (Lürling and Tolman, 2010; Lürling et al., 2014) and its high-economic cost (Spears et al., 2013). More recently, a dried amorphous Fe oxide, CFH-12® (Kemira), has been proposed as a novel agent for lake restoration as (i) it does not change water pH; (ii) it causes a notable P efflux reduction when it is added in a 7.6-8.1 Fe: P molar ratio and (iii) it is not dependent on redox conditions (Fuchs et al., 2018).

All the above mentioned (Phoslock® and CFH-12®) innovative adsorbents lack of the possibility of being recovered from the lake water. In this scenario, magnetic particles (MPs) have been recently proposed for lake restoration as MPs adsorb P and later P loaded MPs can be efficiently removed from solution by applying a magnetic separation gradient (de Vicente et al., 2010, 2011; Funes et al., 2016, 2017a; Merino-Martos et al., 2011, 2015). Therefore, the use of MPs may contribute to mitigate the two coupled and worldwide increasing problems affecting biogeochemical P cycle: (i) the eutrophication, nutrient enrichment, of aquatic ecosystems (de Jonge et al., 2002; Glibert, 2017; OECD, 1984; Withers et al., 2014) and (ii) the global reduction of P reserves (Cordell et al., 2011; Gilbert, 2009). In more detail, the underlying mechanism is that by one hand, MPs trap dissolved P with a minimal alteration in water quality (e.g. Funes et al., 2016, 2018) and on the other hand, P adsorbed on MPs can be desorbed, in basic solutions, and therefore recovered P can be eventually used as a fertilizer (Álvarez-Manzaneda et al., unpublished). Additional advantages of using MPs include: (i) high (18.83 mg P g^{-1}) and fast (less than 1 h) maximum P adsorption capacity; (ii) their magnetic properties allows for recovery of MPs from solution by applying a magnetic separation gradient; (iii) the non-dependence on redox conditions of their P adsorption properties; (iv) their lower economic cost compared to other P adsorbents (e.g. $AlCl_3{\cdot}6H_2O$ or Phoslock®) and (v) they can be easily supplied by companies (i.e. BASF) or by synthesis under laboratory conditions and hence, size and specific properties can be thoroughly selected (de Vicente et al., 2010; Funes et al. 2016, 2017b).

Despite of the increasing use of novel adsorbents for inactivating P in lake sediment, it is especially striking that few studies are focused on assessing their toxic effects on lake biota (Álvarez-Manzaneda and de Vicente, 2017; Álvarez-Manzaneda et al., 2017; Lürling and Tolman, 2010; van Oosterhout and Lürling, 2013; Yamada-Ferraz et al., 2015). In general, those studies are focussed on assessing lethal and sublethal effects of Phoslock® and MPs on planktonic and benthic organisms but no research on discrimination between physical and chemical effects nor on uptake and depuration availability were carried out. In this context, our working hypotheses are firstly, that P adsorbents used for lake restoration cause both chemical and physical effects on algae and on cladocerans and secondly, that cladocerans show fast uptake and depuration mechanisms when they are exposed to low concentration of P adsorbents. Accordingly, in this study we combine toxicity tests (inhibition growth and immobilization) and uptake-depuration tests. In particular, the general aim of this paper was to assess, by short-term laboratory tests and following ISO (International Standardization Organization) and OECD (Organization for Economic Cooperation and Development) protocols, the acute effects of two magnetic (carbonyl iron, HQ and magnetite, Fe_3O_4) and two non-magnetic P adsorbents (Phoslock® and CFH-12®) on both the green algae *Raphidocelis subcapitata* (growth rate inhibition test; ISO, 2012) and on *D. magna* (immobilization; OECD, 2004). To discriminate between chemical and physical effects of adsorbents on *D. magna*, the immobilization test was run both in direct and in indirect contact (by using a double beaker, Skjolding et al., 2016) with the different P adsorbents. Finally, an uptake-depuration test was carried out for assessing the response of *D. magna* after being in direct contact with adsorbents for 24 h. In particular, temporal changes in Fe and La body burdens contents were monitored during 24 h-uptake and 24 h-depuration tests.

2. Material and methods

2.1. Test organisms

Laboratory experiments were carried out with two different species belonging to different trophic levels, the green algae *R. subcapitata* (Korshikov) and *D. magna*. The need for using phytoplankton toxicological tests is based on its sensitivity to contaminants (Hoffman et al., 2003) and its key role in the aquatic ecosystems (Lewis, 1995). *R. subcapitata* is an unicellular green algae (Chlorophyta) with crescent-shaped (40-60 μm^3), which can be found in eutrophic or oligotrophic epicontinental aquatic systems (Granados et al., 2004). The stock culture of *R. subcapitata*, provided by Department of Environmental Engineering of the Technical University of Denmark, was cultivated in the ISO 8692 medium (ISO, 2012).

Daphnia magna was selected among cladocerans as it is widely considered as an organism test in toxicity assays (García et al., 2011; Khangarot and Ray, 1987) and it is easily cultured in laboratory conditions (Núñez and Hurtado, 2005). *D. magna* has been cultured at the Department of Environmental Engineering of the Technical University of Denmark since 1978 from the original culture from Birkedammen, Denmark. The culture was maintained with 15 adult organisms kept in 800 mL of Elendt M7 medium (OECD, 2004) at 20 ± 1ºC and a 16:8 h light-dark cycle. The daphnids were fed daily with *R. subcapitata*. Algal cell concentration was estimated using a Coulter Counter (Multisizer Z2, Beckman Coulter) attached to a computer with Coulter AccuComp version 3.01 software (Beckman Coulter Corporation 2000). The medium of the daphnids culture was renewed twice a week.

2.2. General characterization of magnetic and non magnetic phosphorus adsorbents

The most important features of the four selected P adsorbents are shown in Table 1. All were commercially supplied. They greatly differ in their chemical composition, size and maximum P adsorption capacity. Briefly, HQ and Fe_3O_4 have spherical shape, relative polydispersion and a ferromagnetic behavior. CFH-12®, manufactured by Kemira (Oyj, Finland), is a dried amorphous solid consisting of poorly ordered Fe oxides (Fuchs et al., 2018; Lyngsie et al., 2014). Finally, Phoslock® is a La-based (5% La, 95% clay) engineered product in which the presence of Fe and Al apart from La, increases the number of P binding sites (Reitzel et al., 2013).

Table 1. Main physic-chemical features of the P adsorbents used for this study. Concentrations (total and atomic surface) have been taken from Funes et al. (2018).

Adsorbent	**Total [1] and surface [2] concentration (%)**	**Size**	**Maximum P adsorption capacity (mg g^{-1})**
HQ	Fe (97.5); C (0.9); O (0.5); N (0.9)[1]	805 ± 10 nm (de Vicente et al., 2010)	18.83 (de Vicente et al., 2010)
Fe_3O_4	C (59); Fe (10); O (31)[2]	50-100 nm (manufacturer)	5.85 (Funes et al., unpublished results)
CFH-12®	O (59); Fe (28); C(9); S (2); Ca and Mg (<1) [2]	0.85-2 mm (Fuchs et al., 2018)	15.1 (Funes et al., 2018)
Phoslock®	O (66); Si (19); C (6); Al (6); Mg, Na, Ca, Fe and La (<1) [2]	2-4 x 1-3 mm (Haghsereht, 2006)	13.6 (Funes et al., 2018)

2.3. Algal growth inhibition test

The algal growth inhibition test was performed following the ISO Standard “Water quality – Fresh water algal growth inhibition test with unicellular green algae” (ISO, 2012). Chemical composition of algal growth medium is shown in Table 2.

Table 2. Composition of the test solution used in the algal growth inhibition test (modified of ISO, 2012).

Major nutrients	**mg L^{-1}**
NH_4Cl	15
$MgCl_2 \cdot 6H_2O$	12
$CaCl_2 \cdot 2H_2O$	18
$MgSO_4 \cdot 7H_2O$	15
KH_2PO_4	1.6
$NaHCO_3$	50
Trace elements	**µg L^{-1}**
$FeCl_3 \cdot 2H_2O$	64
$Na_2EDTA \cdot 2H_2O$	100
H_3BO_3	185
$MnCl_2 \cdot 4H_2O$	415
$ZnCl_2$	3
$CoCl_2 \cdot 6H_2O$	1.5
$CuCl_2 \cdot 2H_2O$	0.01
$Na_2MoO_4 \cdot 2H_2O$	7

The concentrations of each adsorbent were 0.05, 0.1, 0.5, 1 and 1.5 g l^{-1} for HQ and Fe_3O_4; 0.02, 0.04, 0.1, 0.24 and 0.6 g l^{-1} for CFH-12® and 0.1, 0.5, 1, 1.5 and 2 g l^{-1} for Phoslock®. These concentrations were selected in view of the results obtained in preliminary tests as ISO (2012) recommended. Each test comprised three replicates of each concentration and six untreated controls. The pH values and the EC_{50} obtained for the reference toxicity test were within the validity criteria specified by the guideline (ISO, 2012).

For the case of the two magnetic adsorbents (HQ and Fe_3O_4), after 24 h of contact time, adsorbents were removed by applying a magnetic gradient exerted by a cylindrical teflon coated magnet (50x8 mm). Once magnetic adsorbents were removed, algal cells (50.000 cells ml^{-1}) were inoculated. Test vessels (20 mL) were located for 48 h in an isolated room, on a shaker (200 rpm) at 20 ± 2ºC and continuously illuminated at 100 ± 15 µE m^{-2} s^{-1}. In order to check possible changes in pH linked to adsorbents addition, pH was measured before and after MPs removal.

It is important to consider that a higher algal cell density than that recommended by ISO (2012) was used for a better discrimination between the background noise owing to particles and/or colored solutions and algal pigment fluorescence (Hartmann et al., 2013). The initial algal biomass of the stock culture was quantified before the inoculation, by using a Coulter Counter (Multisizer Z2, Beckman Coulter). Particle number was recorded as particle number per mL using the software Coulter AccuComp version 3.01 (Beckman Coulter Corporation 2000). Algal growth rates were estimated as a function of the fluorescence of algal pigments in acetone extracts (Mayer et al., 1997) by using a fluorescence spectrophotometer (Hitachi F-2000) at 430 and 670 nm excitation and emission wavelengths, respectively. In particular, samples (0.4 mL) were collected at 0, 24 and 48 h in each vial. EC values (95% confidence limits) and concentration-response curves were estimated by using a nonlinear-regression program (Christensen et al., 2009).

2.4. Toxicity and uptake/depuration tests with Daphnia magna

Tests were carried out following a standardized protocol (OECD, 2004) using HQ, Fe_3O_4, CFH-12® and Phoslock® as test substances. Medium pH was adjusted to 8.0 according to the protocol. The test suspensions of each adsorbent were prepared by adding the required volume of a 10 g l^{-1} strongly shaked stock solution to 100 ml volumetric flasks containing Elendt M7 medium.

2.4.1. Immobilization test by direct contact with adsorbents

This test was carried out with *D. magna* neonates (<24 h old) after testing the toxicity of the reference compound (potassium dichromate) as it is specified in the guideline (OECD, 2004). Preliminary tests were run for determining the appropriate concentrations of the four P adsorbents. Lastly, suspensions containing the following adsorbents concentrations were prepared: 0.0385; 0.096; 0.24; 0.6; 1.5; 2.25; 3 and 3.75 g l^{-1}. Neonates were distributed in groups of five individuals and they were placed into 100 mL glass beakers containing 25 mL of the different concentrations. All glass beakers, comprising four replicates of all control and treatments, were randomly located at 20 ± 1ºC in the dark. Organisms were not fed during the experiment and the number of immobile animals, those who are not able to swim within 15 s after gentle agitation of the test vessel, was counted after 24 and 48 h.

2.4.2. *Immobilization test by indirect contact with adsorbents*

For assessing indirect effects by the studied adsorbents, the same standard 48 h acute immobilization test (OECD,2004) as described in section 2.4.1, was carried out but using a double-beaker test setup. It consisted in using a net (0.33x0.33 mm) which separate the neonates (<24 h old) from direct contact with any aggregated particles. The same adsorbent concentrations, number of exposed organisms and replicates were used as it is described in section 2.4.1. Finally, after 24 and 48 h at 20 ± 1ºC in the dark, the number of immobile animals was recorded. The organisms were stored in 75% nitric acid (HNO_3) and Fe and La content were determined by using spectrophotometry technique (ICP-OES).

2.4.3. Uptake and depuration experiments

An uptake-depuration test was carried out for each P adsorbent following the methodology described by Skjolding et al. (2014). Briefly, a 24 h uptake phase followed by a 24 h depuration period was performed by using a selected concentration of each adsorbent. These concentrations were lower than EC_{10} obtained in the immobilization test with *D. magna* in direct contact with the particles. Adsorbent concentrations were: 0.2; 0.5; 1.4 and 0.05 g l^{-1} for HQ, Fe_3O_4, CFH-12® and Phoslock®, respectively. The method consisted of placing five neonates (<24 h) in 25 mL of the selected adsorbent concentrations. All beakers were incubated at 20ºC in the dark and organisms were sampled after 1, 2, 3, 4, 6 and 24 h of contact time. At each sampling time, mobile neonates of three beakers were collected for measuring their Fe and La content, similarly as it is described in the previous experiment (section 2.4.2). Three beakers were additionally included as control treatments. For the depuration test, after 24 h of exposition, the mobile organisms were transferred to pure medium. Again, the beakers were located in the dark at 20ºC and mobile organisms from three beakers were collected at 1, 2, 3, 4, 6 and 24 h. Finally, Fe and La content in the daphnids was measured similarly to it has been described above.

The natural logarithm of the data was fitted to a linear regression to determine the uptake and depuration rate constants (K_u and K_d). Moreover, the elimination half-life

($t_{1/2}$), which can be defined as the time that the particles stay inside the organisms once the exposure has stopped, was also calculated as Djomo et al. (1996) indicated:

$$t_{\frac{1}{2}} = \frac{Ln2}{K_d}$$

2.5. Statistical analysis

Concentration-response curves and mean effective concentration (EC_{50}) of the growth inhibition test with *R. subcapitata* were described by a log-logistic equation, fitted by least squares, and estimated by using a nonlinear-regression program (Christensen et al., 2009). EC_{50} and corresponding 95% confidence limits for the *D. magna* immobilization (under direct and indirect contact) tests were estimated by using Probit analysis with the program SPSS. In addition, statistical differences when comparing EC_{50} values were reported when non-overlapping confidence limits occurred (Abot et al., 1995; Jeske et al., 2009; Liu et al., 2003).

Finally, in order to deliver data for a preliminary risk assessment of the four studied adsorbents, predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC) were calculated. PNEC was estimated as the lowest EC_{50} found in algal or daphnia tests divided by an assessment factor of 1000 in accordance with the Technical Guidance Document for setting environmental quality standards in the Water Framework Directive (TGD-EQS, 2011). The PNEC forms the basis for determining the relevant environmental quality standard. Since adsorbents are added once in lake restorations, continuous exposure to adsorbents is not expected to occur in receiving water bodies. The environmental quality standard of relevance is hence the so-called “MAC-QSfw,eco”, i.e. the maximum allowable concentration for the freshwater ecosystem (TGD-EQS, 2011). For this an assessment factor of 100 was chosen, in accordance with the TGD-EQS (2011), and thus the MAC-QSfw,eco for all compounds was 10 times higher than the PNEC (see Table 4). Additionally, a realistic worst-case PEC was calculated for a real case application in a hypertrophic ecosystem: Honda lake (Almería, Spain). Estimations were based on: (i) the mass of mobile P in the upper sediment layer (0-10 cm; Funes et al., 2017a); (ii) lake morphometry and (iii) maximum P adsorption capacity of each adsorbent.

3. Results and discussion

3.1. Algal growth rate inhibition tests

Results from *R. subcapitata* growth rate inhibition tests differed markedly among the four P adsorbents. While no pattern of algal growth rate inhibition was observed for Fe_3O_4 and Phoslock®, EC_{50} for HQ and CFH-12® was 1.50[1.46; 1.54]$_{95\%}$ and 0.42 g l^{-1}[0.35; 0.51]$_{95\%}$, respectively. It should be noted that the EC_{50} for HQ was much higher than the 0.085g l^{-1} found in a similar experiment with *Chlorella* sp. (Álvarez-Manzaneda and de Vicente, 2017), in which the inhibition of the cell growth was recorded. These differences can be due to interspecies differences in sensitivity (Menard et al., 2011) and they may also be related to methodological aspects such as contact time (48 h in this experiment and 72 h in Álvarez-Manzaneda and de Vicente, 2017) and different medium composition as described by e.g. Chen and Lin (1997) and Millington et al. (1988).

As it was expected, inhibition of algal growth rates increased with CFH-12® particles concentration (Figure 1). It should be mentioned that CFH-12® suspension was red-colored and a shading effect cannot be excluded as Sørensen et al. (2016) found for platinum nanoparticles.

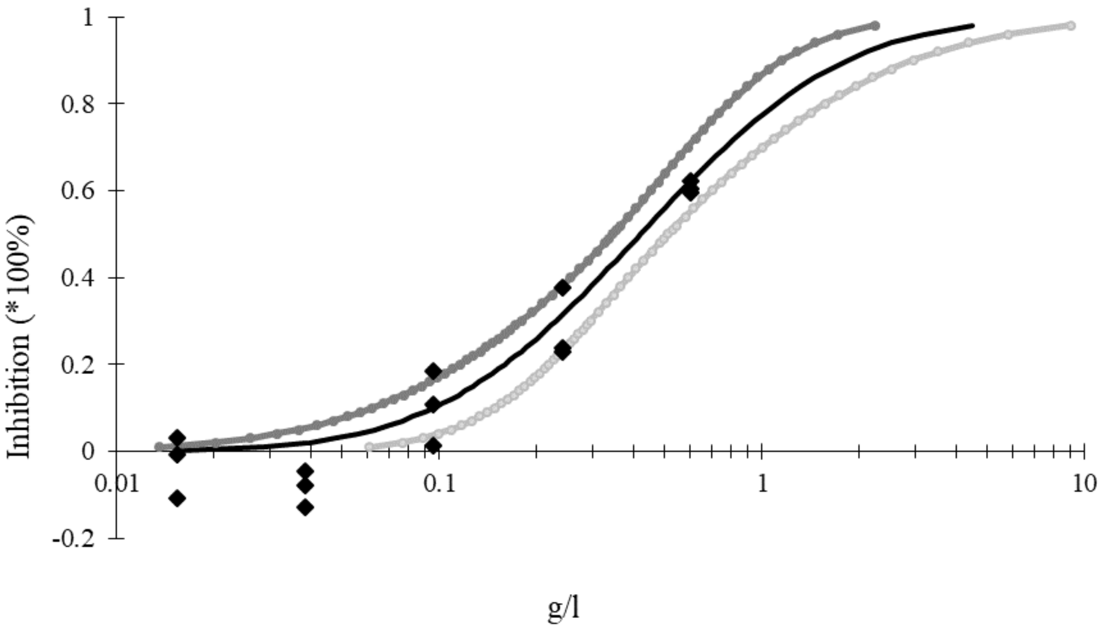

Figure 1. Concentration-response data and fitted curves from 72 h growth rate inhibition test with *R. subcapitata* and CFH-12®. Black diamonds represent observed values, black line shows the Log-normal distribution and light and dark grey lines represent the lower and upper 95% confidence interval at the concentration level, respectively.

For Fe_3O_4, the lack of any pattern could be explained by the stimulation of the algal growth due to the presence of dissolved Fe in the media (13 µg l^{-1}). It is very wellknown that, of all the trace metals, Fe is especially prominent in biochemical catalysis (Morel and Price, 2003; Shcolnick and Keren, 2006) and phytoplankton, with their Fe-rich photosynthetic apparatus, have significantly higher Fe demands as opposed to their heterotrophic counterparts (Raven et al., 1999). In the algal medium Fe is added to a final concentration of 64 µg l^{-1} which is the minimum to support an exponential growth of *R. subcapitata* for 72 h under the specified test conditions (ISO, 2012). Thus, the additional dissolved Fe present in the Fe_3O_4 tests (13 µg l^{-1}) represents a significant increase in available Fe to support enhanced algal growth. In relation to Phoslock®, no pattern of algal growth rate inhibition was observed in the tested concentration range (< 2 g l^{-1}). Our results are in disagreement with the results found by van Oosterhout and

Lürling (2013) in an experiment with Phoslock® in contact with *Scenedesmus obliquus* and *Microcystis aeruginosa*. These authors noted that growth rates of the green alga and of the cyanobacterium were negatively affected when Phoslock® concentration was higher than 0.5 g l^{-1}, which is actually much lower than that used in the present study. Again, these differences may be explained by differences in the medium used for each test as well as in the different test organisms. In fact, since the test medium used by van Oosterhout and Lürling (2013) has higher contents of macro- and micronutrients, these differences may be related to differences in species sensitivity to Phoslock®.

Finally, it is important to consider that the growth of microalgae is strongly affected by nutrient availability and accordingly, if P adsorbents cause a notable reduction in P concentration a potential limitation of primary production may occur. Although, in this study we have not been focused on monitoring changes in P concentrations when adding P adsorbents, a previous study (Álvarez-Manzaneda and de Vicente, 2017) noted that when adding MPs concentrations similar to those used in this study, P concentrations were still much higher than P threshold concentration (3 µg l^{-1}) proposed by Reynolds (1992, 1999) for identifying P as a limiting nutrient of the primary production.

3.2. Toxicity and uptake/depuration tests with Daphnia magna

3.2.1. Immobilization test by direct contact with adsorbents

Figure 2 shows the concentration-response curves obtained in the immobilization tests with *D. magna* in direct contact with the four P adsorbents. As expected, all adsorbents caused an increased immobilization with increasing concentrations and contact time. In relation to the EC_{50}, no statistically significant differences were found among the four studied adsorbents (Table 3).

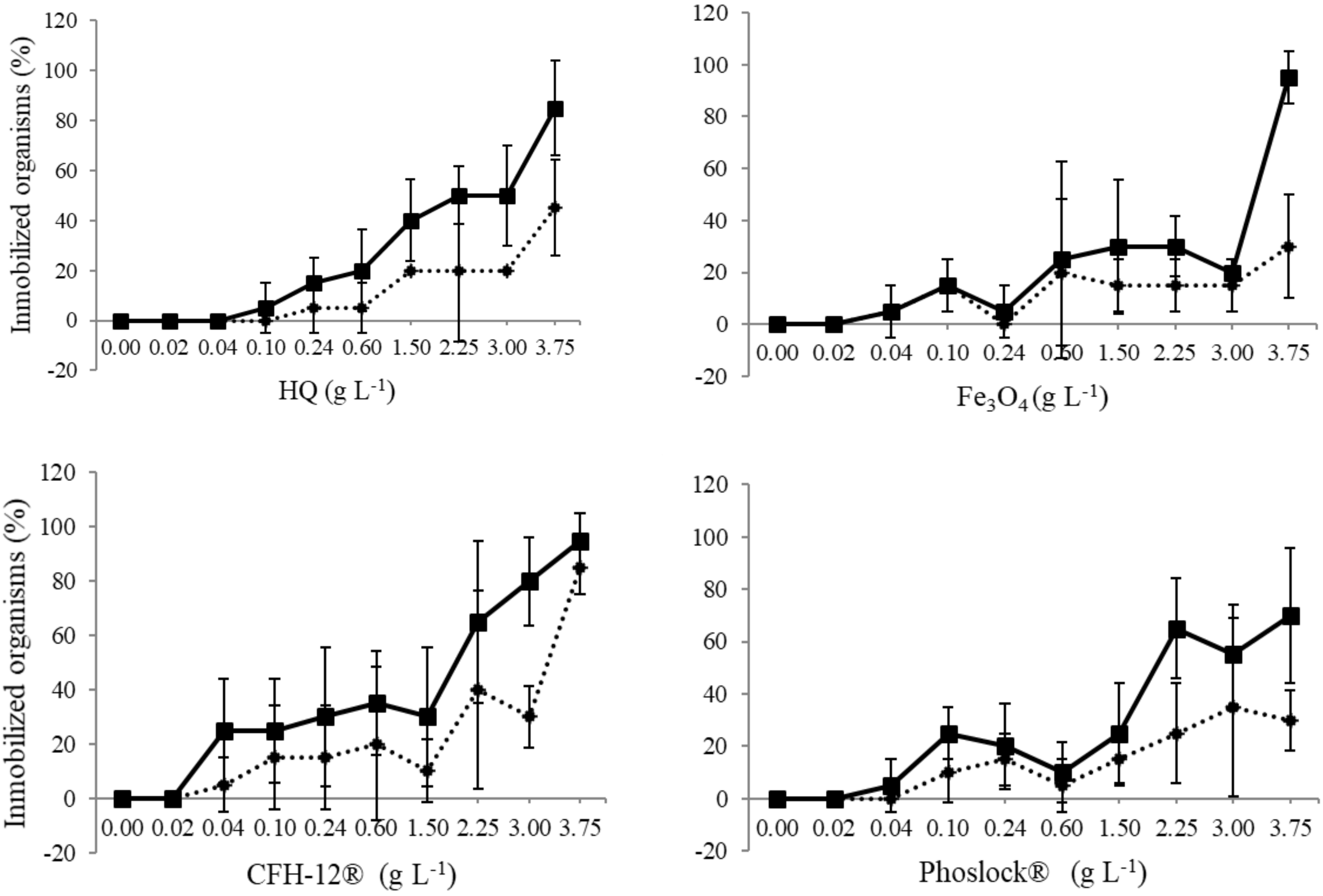

Figure 2. Individuals of *D. magna* immobilized (%) after direct contact with the adsorbents for 24 h (*dotted line*) and 48 h (*continuous line*). Vertical error bars show standard deviation (SD). n = 5.

Table 3. Results from 48 h *D. magna* immobilization test in direct contact with the studied adsorbents. Effect concentrations and corresponding 95 % confidence intervals are all expressed in g l^{-1}.

Adsorbent	**EC_{50} (48 h)**	**EC_{10} (48 h)**
HQ	1.86 (0.61; 3.03)	0.24 (0; 0.68)
Fe_3O_4	2.76 (1.45; 14.62)	0.5 (0; 1.11)
CFH-12®	2.26 (1.67; 2.64)	1.44 (0.64; 1.86)
Phoslock®	2.14 (-; -)*	0.09 (-; -)*

To deliver data to be used in a preliminary ecological risk assessment of the four studied adsorbents Table 4 lists the PEC, PNEC and $MAC\text{-}QS_{fw,\ eco}$. The hypertrophic Honda lake (Almería, Spain) was chosen as a realistic case to illustrate the possible order of magnitude of environmental concentrations of the four P adsorbents. It should be noted that the PECs for all adsorbents were lower than EC_{50} for all adsorbents (Table 3 and Table 4). On the other hand the PECs for the Honda lake scenario exceeded the limit value for a short-term exposure ($MAC\text{-}QS_{fw,\ eco}$). This indicates some risk for aquatic organisms during the treatment of the lake which is to be expected due to the nature of the treatment. It should however be noted that lake restorations are undertaken in aquatic ecosystems that are in unbalance and since longer term effects of the adsorbents in the pelagic phase are not expected, this risk may on a case-to-case basis be acceptable.

Table 4. Comparison between predicted no-effect concentration (PNEC), predicted environmental concentration (PEC) and environmental quality standard of relevance (MAC-QSfw,eco), for *R. subcapitata*[1] (algal growth inhibition test) and for *D. magna*[2] (immobilization test with direct contact).

Adsorbent	**PEC (mg L^{-1})**	**PNEC (mg L^{-1})**	**$MAC\text{-}QS_{fw}$ (mg L^{-1})**
HQ[1]	350	1.50	15
Fe_3O_4[2]	1120	2.76	27.6
CFH-12®[1]	430	0.42	4.2
Phoslock®[2]	480	2.14	21.4

The EC_{50} of HQ (1.86 g l^{-1}) was higher, but not statistically significant, than that found in a similar experiment with *D. magna* (0.913 g l^{-1}; Álvarez-Manzaneda et al., 2017). The slight differences may be caused by the different media used in the two studies (M7 in the present study and distilled water in Álvarez-Manzaneda et al., 2017). In fact, and similarly to our results, several authors have noted a lower toxicity in experiments with daphnids in contact with M7 medium as a consequence of the underestimation of the toxicity of metal compounds since M7 has chelators added (Guilhermino et al., 1997; Loureiro et al., 2011). ForFe_3O_4, and as expected, the EC_{50} (2.76 g l^{-1}) was much higher than EC_{50} and LC_{50} reported for much smaller Fe_3O_4 nanoparticles (5-6 nm; Baumann et al., 2014; García et al., 2011). This difference in toxicity may be related to particle sizes, but as pointed out by Hjorth et al. (2017) test technical issues like aggregation and sedimentation behavior in the different testing media makes it very challenging to obtain reliable and comparable results when testing Fe particles.

Lastly, it should be noted that the EC_{50} value for immobilization of *D. magna* when exposed to Phoslock® was 1.4-2.5 times higher than the EC_{50} values reported by Lürling and Tolman (2010) which were based on weight and length based growth rates of *D. magna*.

By visual inspection it was obvious that the particles might both have been ingested and been attached to the outer surfaces of the animals (Figure 3). As expected, daphnids present an empty gut and the carapace without any particle fixation in the control treatment (Figure 3a). However, when they are exposed to HQ they turned black-colored (Figure 3b) reflecting both the ingestion and the attachment of these particles on their carapace.

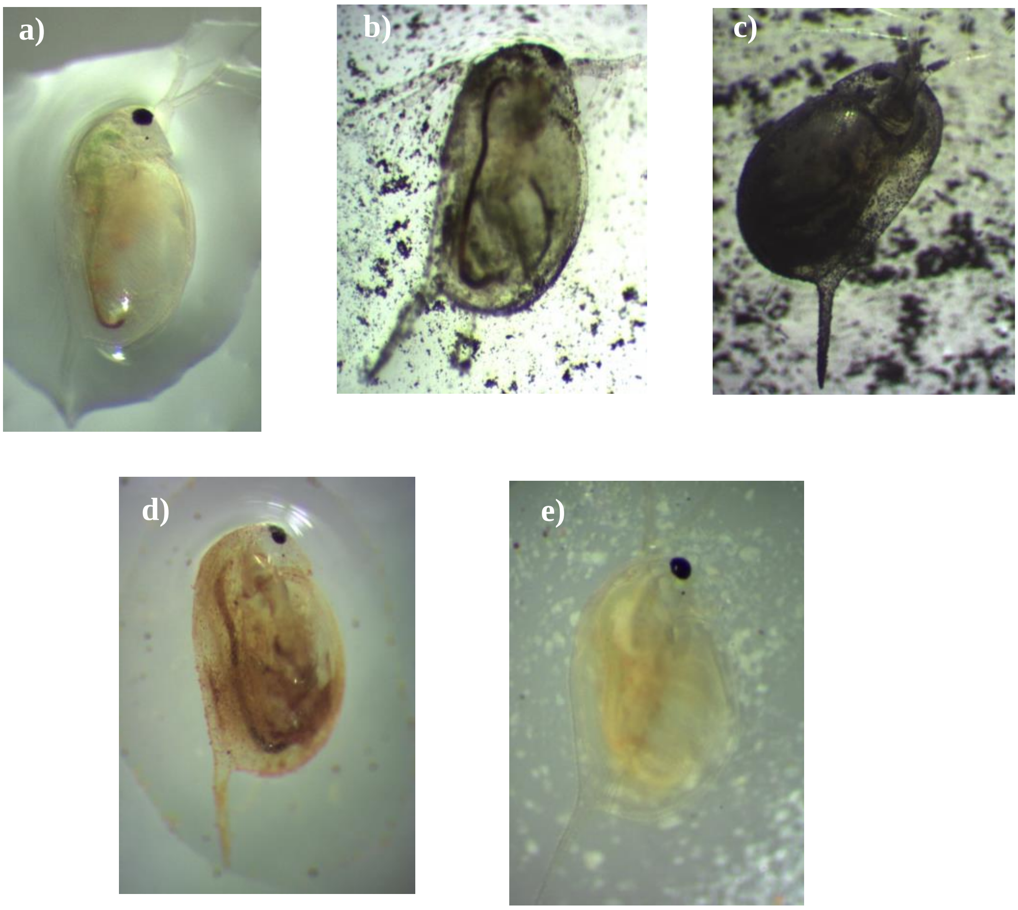

Figure 3. Photos of *D.magna* exposed, for 48 h, in the immobilization test to direct contact with the adsorbents (3.75 g l^{-1}). (a) Control; (b) HQ; (c) Fe_3O_4; (d) CFH-12® and (e) Phoslock®.

These results are in agreement with the concern of *D. magna* as a food-generalist organism with a mesh size in the filter appendices between 0.24 to 0.64 µm (Geller and Müller, 1981) which is correlated with the range of particle size that *D. magna* can ingest. Although the mean particle size of HQ is 805±10 nm (Table 1) would indicate no ingestion of HQ according to the previous statement, however since the mean value does not account for the particle size distribution it is still possible that the smaller particles in the distribution could have been be ingested by daphnids. On the other hand, the smaller size of Fe_3O_4 (50-100 nm; Table 1) could in theory have been too small for ingestion by daphnids however even smaller nanoparticles have been documented to be ingested by *D. magna* (e.g. Skjolding et al., 2014). Besides the possible ingestion of Fe_3O_4 visible body attachment was evident at the end of the testing period (Figure 3c). Although the size of CFH-12® is too high to be ingested by daphnids, the colored gut present in Figure 3d is likely to evidence both the filtration of a colored medium and the ingestion of the smaller particles by daphnids. Finally, the light color of Phoslock® particles makes laborious to distinguish between ingestion and attachment on daphnids carapace (Figure 3d). However and considering the particle size of diluted Phoslock® (22 µm, Haghseresht et al., 2009; Ross et al., 2008), it is likely that daphnids could ingest it.

3.2.2. Immobilization test by indirect contact with adsorbents

Apart from direct effect of MPs, it is essential to assess indirect effects on lake biota. These outcomes are especially relevant if we consider that in a practical application it is difficult to get 100% of MPs removal (Funes et al., 2017a) and accordingly, the remaining MPs are likely to interact for longer time and with complex mechanisms with lake biota.

Our results show much lower immobilization when *D. magna* was exposed in the indirect contact testing setup (Figure 4) than under direct contact (Figure 2). In fact, for all adsorbents less than 20% immobilization of the total number of animals was observed even at the highest concentration (3.75 g l^{-1}; Figure 4). These corresponding values were much higher in the direct contact testing setup (60-100%) reflecting the prevalence of physical effects of P adsorbents rather than chemical induced toxicity. Similarly, previous studies with platinum nanoparticles (1-10 nm) and *D. magna* have found that these particles resulted in immobilization of the organisms due to a physical "fixation" rather than a toxic action (Sørensen et al., 2014).

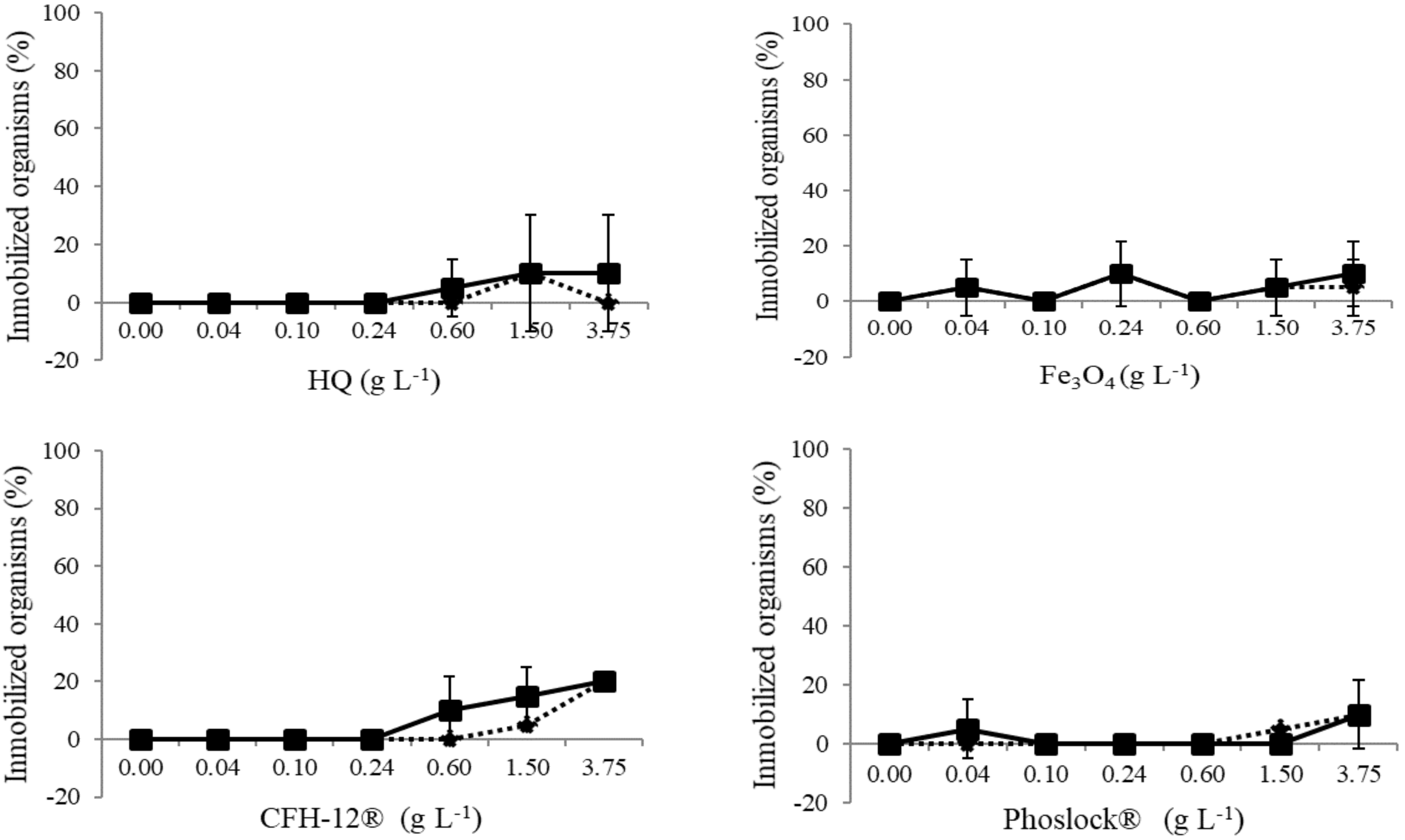

Figure 4. Individuals of *D. magna* immobilized (%) after indirect contact with the adsorbents for 24 h (*dotted line*) and 48 h (*continuous line*). Vertical error bars show standard deviation (SD). n = 5.

As expected, daphnia body burdens of Fe and La increased with increasing adsorbent concentration (Figure 5). Although at higher adsorbent concentrations similar Fe contents were found, for low and mid adsorbent concentrations Fe content was higher for CFH-12® than for HQ and Fe_3O_4. Although there are no data about La content in *D. magna* in the literature making difficult the comparison of our results, Waajen et al. (2017) measured La content in different body part of different fish species after being in contact with Phoslock®. These authors found the highest La concentration in the liver of eel while the lowest values were measured in the muscle of pike and perch.

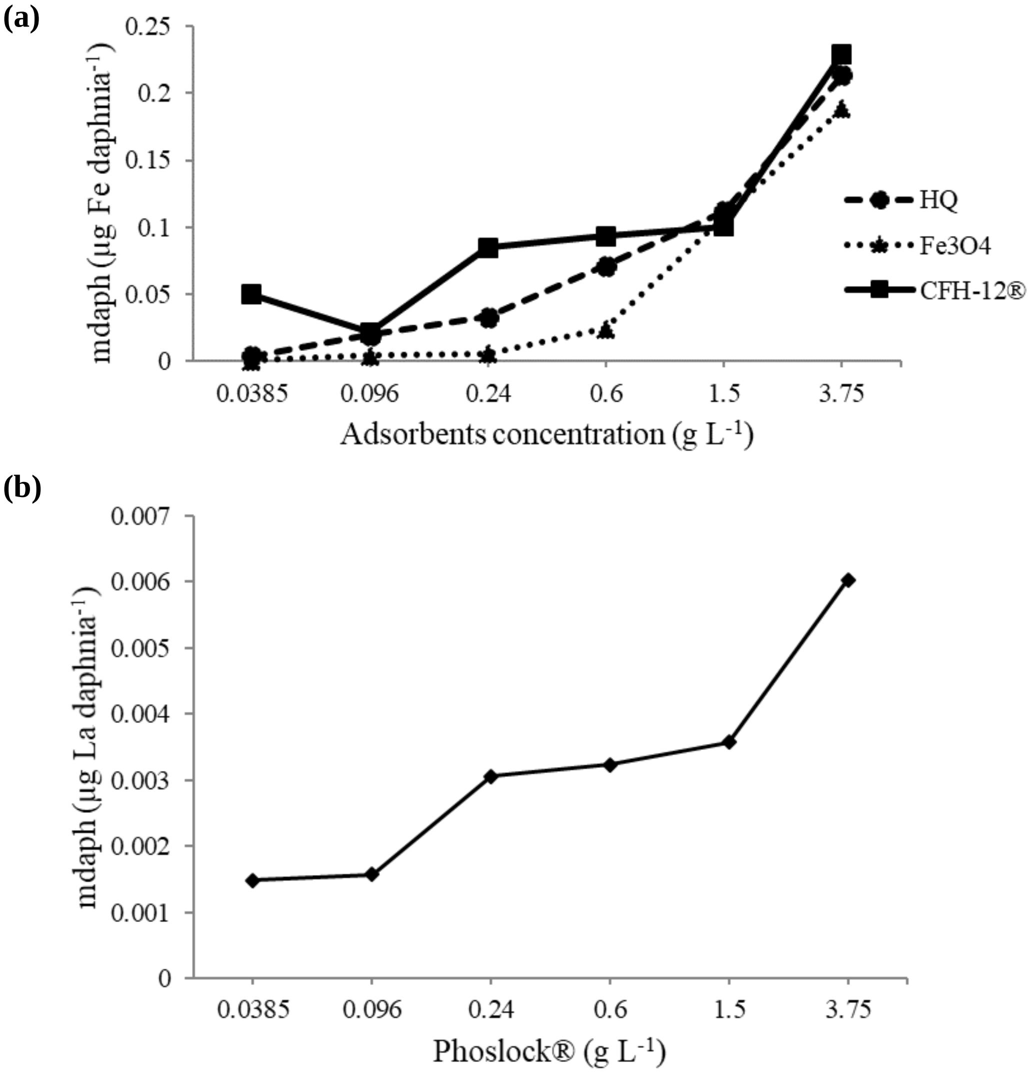

Figure 5. Body burdens of (a) Fe and (b) La in the *D. magna* immobilization test in indirect contact with (a) HQ, Fe_3O_4 and CFH-12® and (b) Phoslock®.

3.2.3. Uptake and depuration experiments

The uptake kinetic was quite different among the studied adsorbents (Figure 6). After 1 h of contact time, the lowest Fe concentration was measured in tests with Fe_3O_4 (0.04 µg daphnia^{-1}) while the highest was measured intests with HQ (0.31 µg daphnia^{-1}). A clear increase in body burdens with time was found for tests with Phoslock®. This tendency was also observed for HQ and Fe_3O_4. In contrast, Fe uptake by daphnids in the presence of CFH-12® followed a steady pattern over time and in just 1 h of contact time the same amountas at the end of the uptake experiment (24 h) was found (0.18 µg daphnia^{-1}). In order to compare the uptake dynamic among all adsorbents, uptake rate constants (K_u) were obtained (Table 5). From Table 5, it can be observed that although K_u for HQ could not be determined, K_u was highest for Phoslock® (5.80 h^{-1}) and lowest for CFH-12® (1.18 h^{-1}). Accordingly and in relation to Fe enriched adsorbents, it is important to remark that although K_u was highest for Fe_3O_4, reflecting a fast uptake kinetic, at the end of the experiment the body burden was low due to a fast depuration. At this point, it is striking that higher Fe content in daphnids were found in contact with CFH-12® and HQ, which are specifically characterized by larger particle size (Table 1). Therefore, these results suggest that Fe uptake measured in this experiment is mostly due to Fe attachment on daphnids carapace rather than ingestion.

Table 5. Uptake and depuration rate constants (K_u and K_d, respectively) and elimination half-life time ($t_{1/2}$) estimated in the uptake-depuration experiment with *D. magna*.

Adsorbent	**K_u (h^{-1})**	**K_d (h^{-1})**	**$t_{1/2}$ (h)**
HQ	-	-	-
Fe_3O_4	3.50	2.04	0.34
CFH-12®	1.18	2.50	0.28
Phoslock®	5.80	3.60	0.19

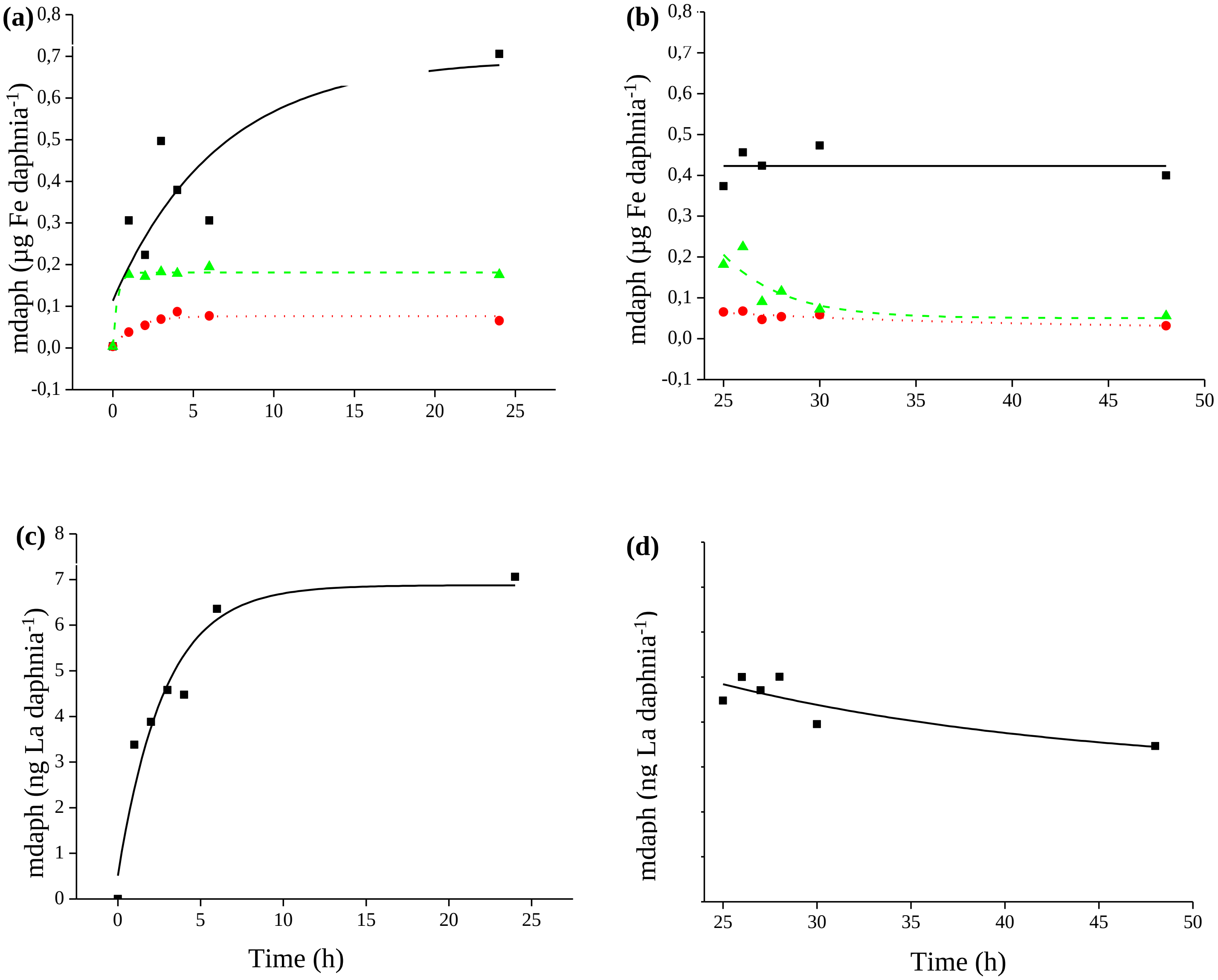

Figure 6. Temporal changes in the (a; c) uptake and (b; d) depuration tests in direct contact with the different P adsorbents. In Figures (a) and (b),squares, circles and triangles are refereed to HQ, Fe_3O_4 and CFH-12®, respectively while in (c) and (d) Phoslock® results are shown.

In the depuration test, when comparing the four P adsorbents, lowest depuration rate constant (K_d) was found for Fe_3O_4 (2.04 h^{-1}) while highest corresponded to Phoslock® (3.60 h^{-1}; Table 5). Similarly, elimination half-life time ($t_{1/2}$) was shortest for Phoslock® (0.19 h) and longest for Fe_3O_4(0.34 h). For the case of the residual body burden at the end of the depuration study, it was highest for HQ (0.40 µg Fe $daphnia^{-1}$; Figure 6) while very similar for Fe_3O_4 (0.03 µg Fe $daphnia^{-1}$) and CFH-12® (0.06 µg Fe $daphnia^{-1}$). These concentrations were higher than the measured Fe-content of non-exposed animals (0.003 µg Fe $daphnia^{-1}$). For the case of Phoslock®, residual body burden was similar to the background concentration (3.5 ng La $daphnia^{-1}$). It is important to consider that the uptake-depuration experiment was carried out without feeding, and in similar experiments it has been observed that both the uptake was lower and the depuration faster in presence of food (Kennedy et al., 2008; Skjolding et al., 2014). All in all, taking this into account, and comparing the uptake and depuration rates, accumulation of adsorbents is not expected in daphnids in realistic exposure scenarios.

4. Conclusions

Results from algal growth rate inhibition tests have shown large differences among the four P adsorbents. In relation to the EC_{50} estimated in the immobilization test of *D. magna* by direct contact, no statistically significant differences were found among the four studied adsorbents.

For discriminating between physical effects and chemical induced toxicity, immobilization tests with *D. magna* by using a double-beaker were also carried out. Results clearly revealed much lower immobilization when *D. magna* was exposed through indirect contact with the adsorbents than under direct contact, which reflect the prevalence of a physical “fixation” of P adsorbents on daphnids carapace.

Finally, when comparing uptake and depuration rates among all studied adsorbents (except for HQ which was not possible not be fitted), our results evidenced a faster uptake and depuration for Phoslock®, which was precisely the adsorbent with the highest particle size. However, if we consider only Fe enriched adsorbents, there is no a clear tendency as the faster uptake rate was found for the adsorbent characterized by the smallest size (Fe_3O_4) but it experienced the lowest depuration rates. Based on our findings, accumulation of adsorbents is not expected in daphnids in realistic exposure scenarios.

In a realistic worst-case scenario using data from Honda lake (Almería, Spain), some risk for aquatic organisms during the treatment of the lake was estimated, but most likely caused by physical effects of particles in the water phase. This risk may be on a case-to-case basis be acceptable, since lake restorations are undertaken in aquatic ecosystems that are in unbalance and neither accumulation nor longer term effects of the adsorbents in the pelagic phase are expected.

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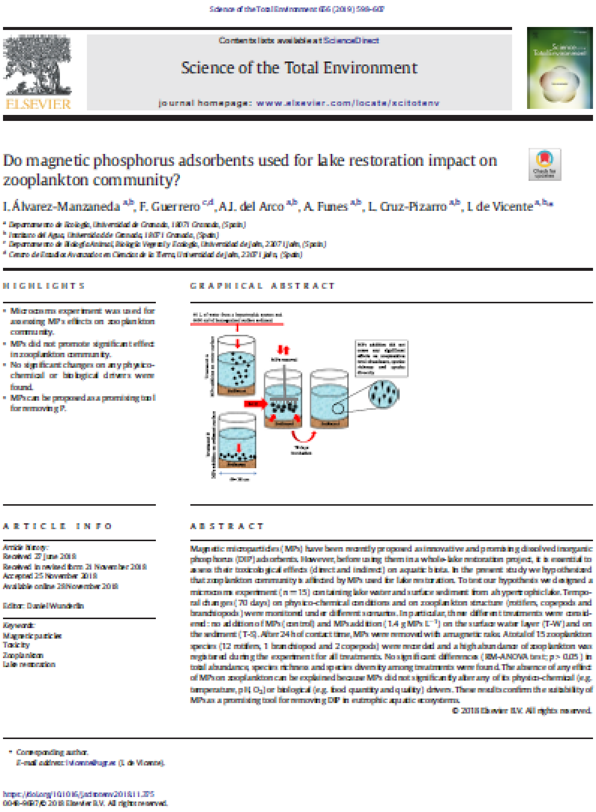
Chapter IV: Do magnetic phosphorus adsorbents used for lake restoration impact on zooplankton community?



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Do magnetic phosphorus adsorbents used for lake restoration impact on zooplankton community?

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HIGHLIGHTS

- Microcosms experiment was used for assessing MPs effects on zooplankton community.
- MPs did not promote significant effect in zooplankton community.
- No significant changes on any physico-chemical or biological drivers were found.
- MPs can be proposed as a promising tool for removing P.

GRAPHICAL ABSTRACT

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ABSTRACT

Magnetic microparticles (MPs) have been recently proposed as innovative and promising dissolved inorganic phosphorus (DIP) adsorbents. However, before using them in a whole-lake restoration project, it is essential to assess their toxicological effects (direct and indirect) on aquatic biota. In the present study we hypothesized that zooplankton community is affected by MPs used for lake restoration. To test our hypothesis we designed a microcosm experiment (n = 15) containing lake water and surface sediment from a hypertrophic lake. Temporal changes (70 days) on physico-chemical conditions and on zooplankton structure (rotifers, copepods and branchiopods) were monitored under different scenarios. In particular, three different treatments were considered: no addition of MPs (control) and MPs addition (1.4 g MPs L^{-1}) on the surface water layer (T-W) and on the sediment (T-S). After 24 h of contact time, MPs were removed with a magnetic rake. A total of 15 zooplankton species (12 rotifers, 1 branchiopod and 2 copepods) were recorded and a high abundance of zooplankton was registered during the experiment for all treatments. No significant differences (RM-ANOVA test; $p > 0.05$) in total abundance, species richness and species diversity among treatments were found. The absence of any effect of MPs on zooplankton can be explained because MPs did not significantly alter any of its physico-chemical (e.g. temperature, pH, O_2) or biological (e.g. food quantity and quality) drivers. These results confirm the suitability of MPs as a promising tool for removing DIP in eutrophic aquatic ecosystems.

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Abstract

Magnetic microparticles (MPs) have been recently proposed as innovative and promising phosphorus (P) adsorbents. However, before using them in a whole-lake restoration project, it is essential to assess their toxicological effects (direct and indirect) on aquatic biota. In the present study we hypothesized that zooplankton community is affected by MPs used for lake restoration. To test our hypothesis we designed a microcosms experiment (n=15) containing lake water and surface sediment from an hypertrophic lake. Temporal changes (70 days) on physico-chemical conditions and on zooplankton structure (rotifers, copepods and branchiopods) were monitored under different scenarios. In particular, three different treatments were considered: no addition of MPs (control) and MPs addition (1.4 g MPs l^{-1}) on the surface water layer (T-W) and on the sediment (T-S). After 24 h of contact time, MPs were removed with a magnetic rake. A total of 15 zooplankton species (12 rotifers, 1 branchiopod and 2 copepods) were recorded and a high abundance of zooplankton was registered during the experiment for all treatments. No significant differences (RM-ANOVA test; $p>0.05$) in total abundance, species richness and species diversity among treatments were found. The absence of any effect of MPs on zooplankton can be explained because MPs did not significantly alter any of its physico-chemical (e.g. temperature, pH, O_2) or biological (e.g. food quantity and quality) drivers. These results confirm the suitability of MPs as an ideal tool for removing P in eutrophic aquatic ecosystems.

1. Introduction

Eutrophication is one of the most striking problems affecting world water resources (OECD, 1982; Sas, 1989; Cooke et al., 2005). Although the over enrichment by nutrients occurs naturally over centuries as lake age (Carpenter, 1981), human activities have intensified the rate and extent of eutrophication through both point-sources and non-point discharges. The known consequences of cultural eutrophication include blooms of blue-green algae, tainted drinking water supplies, degradation of recreational opportunities and hypoxia (Chislock et al., 2013). Overall, eutrophication is responsible for not only a drastic impairment of ecosystem structure and function but also for economic losses causing annual costs just in the U.S. of approximately $2.2 billion (Dodds et al., 2009).

Due to the complexity of the phenomena, the lack of the consistent data sets and the lack of a harmonised approach to assess eutrophication, the severity and extent of the problem had not been adequately characterized at an international level. In Europe, the Water Framework Directive (WFD – 2000/60/EC) provides a common framework to protect, manage and restore surface and groundwater. WFD prescribed as a legal requirement by 2015 that all water bodies must have achieved "good status" (Heiskanen, 2004). However, for the case of Spain, the first national overview of the ecological, chemical and overall status of surface water bodies (rivers, lakes, transitional and coastal), evidenced that almost 50% of all surface waters in Spain are in poor status and 43% of surface water bodies have not yet been evaluated (Willaarts et al., 2014).

Despite some large-scale efforts to reduce nutrient enrichment, cultural eutrophication continues to be the leading cause of water pollution for many freshwater and coastal marine ecosystems and is a rapidly growing problem in the developing world (Smith and Schindler 2009; Chislock et al., 2013). Therefore, the endless deterioration of water quality together with the increasing demand for freshwater resources requires the implementation of imperative management and restoration measures. The essential and preliminary strategy for combating eutrophication by controlling algal biomass is the reduction of lake water P concentration, which is the main limiting nutrient for the primary production. Among all strategies, external load control is the first and necessary step for achieving the success of any restoration program of eutrophicated ecosystems (Jeppesen et al., 2009; Smith, 2009). However, and because internal P loading frequently has a considerable impact on lake water P concentrations (Phillips et al., 1994), additional methods are also recommended such as P inactivation techniques. This approach is based on increasing sedimentary P binding capacity by adding iron (Fe), aluminium (Al) or Phoslock® (Boers et al., 1992; Egemose et al., 2011; Spears et al., 2013, 2015). As the effectiveness to remove P of most P-sorbing materials is dependent on the pH, potential redox, and/or presence of other dissolved ions (Westholm, 2006; de Vicente et al., 2008; Vohla et al. 2011; Lürling et al. 2014), more research on novel P adsorbents is essential.

In this context, Fe magnetic particles (MPs) have been proposed as a new and innovative restoration tool (de Vicente et al., 2010a, 2011; Merino-Martos et al., 2011). Briefly, first, MPs adsorb P from aqueous solutions and later, by applying a magnetic gradient, MPs and therefore, P adsorbed onto MPs, are recovered from the solution. Finally, P can be desorbed from MPs in an alkaline solution allowing both MPs recovery for subsequent adsorption processes and P reuse as a fertilizer (Álvarez-Manzaneda et al., unpublished data). Among the most relevant advantages of using MPs for lake restoration, recorded in previous studies (de Vicente et al., 2010a; Merino-Martos et al., 2011; Funes et al., 2016, 2017; Álvarez-Manzaneda et al., 2017), we underline the next ones: (i) the high P:MPs molar ratio under both batch and flow conditions and oxic and anoxic conditions; (ii) the high P adsorption capacity in a wide pH range (5-9); (iii) the potential long-term decrease of P efflux from lake sediments as a consequence of the reduction of the sedimentary P_{Mobile} concentrations (even in anoxic conditions) and (iv) their low toxicological effects on both planktonic and benthic organisms.

As a result, the use of MPs as a restoration tool for eutrophic systems could tackle two current and coupled problems: the eutrophication of aquatic ecosystems and the worldwide depletion of P reserves for making fertilizers (e.g. Cordell et al., 2011; Mekonnen and Hoekstra, 2018). However, before using MPs in a whole-lake restoration project, it is essential to assess their toxicological effects on lake biota. Accordingly, basic acute and chronic experiments have been carried out in order to determine the risk assessment of MPs. Briefly, laboratory experiments to test the effects of MPs on immobilization and life-cycle of *Daphnia magna* and *Chironomus* sp. (Álvarez-Manzaneda et al., 2017); on the inhibition growth of *Chlorella* sp. and on mortality and cysts hatching of *Brachionus calyciflorus* (Álvarez-Manzaneda and de Vicente, 2017) have been already accomplished. However, it is important to consider that apart from single-species toxicity tests accomplished under laboratory conditions, it is critical to develop outdoor microcosms experiments which can be used to evaluate the fate and effects (both direct and indirect) of chemicals at many different levels of organization through appropriate endpoints (Caquet, 2013).

In this context, in the present paper we evaluate, by using outdoor microcosms, the effects of MPs addition on one of the main components of the biological communities of aquatic systems, which is the zooplankton. In short, the function of zooplankton in aquatic food webs is crucial as: (i) it acts as a link between phytoplankton and secondary consumers (fish and some birds; Conde-Porcuna et al., 2004) as herbivorous zooplankton control phytoplankton community but it also represents great value resources for planktivorous fish and other organisms (Christoffersen et al., 1993; Jeppesen et al., 1999); and (ii) zooplankton actively participates in nutrients (nitrogen and P) recycling (Sterner, 2009). Even more, zooplankton assemblages provide good elements for biogeographical studies (Xu et al., 2011) that may guide conservation polices needed to assess future shifts in biodiversity and community structure caused by anthropogenic impacts (Gilbert et al., 2014, 2015, 2017). As a result, zooplankton community has been proposed as an important criterion for wetlands conservation (Jeppesen et al., 2011). Despite of the key role of this community in aquatic ecosystems, there is a complete lack of studies focused on the assessment of the effect of P adsorbents used for lake restoration on zooplankton community by using microcosms. This is a relevant aspect as P adsorbents may direct (i.e. physical effects) or indirectly (i.e. by reducing phytoplankton biomass) affect to the zooplankton and the last effect of P adsorbents must be test by using more complex and realistic approaches than single-species toxicity tests. Therefore, in the present study we hypothesized that zooplankton community is affected by MPs used for lake restoration. To test our hypothesis we designed a microcosms experiment (n=15) containing lake water and surface sediment from an hypertrophic lake. This study is framed in a broader project focused on determining the consequences of MPs application on water quality and sediment phosphorus pools (Funes et al., 2017). In this experiment, MPs effects on physico-chemical conditions and on zooplankton composition and structure were monitored under different scenarios.

2. Material and methods

2.1. Study site

Honda lake is a well studied shallow (surface area=9 ha, Z_{mean}=1.3 m; Z_{max}=3.2 m) and hypertrophic wetland (Carrillo et al., 1987; Cruz-Pizarro et al., 2003; de Vicente and Cruz-Pizarro, 2003; Funes et al., 2016). It is included in Albufera de Adra Natural Reserve, a Ramsar site that represents one of most important wetland in south Spain (Figure 1). Its high trophic state is the result of both, the great extension devoted to intensive agricultural practices in its catchment area, which is responsible for the high external P load; and the relevance of P release from the lake sediment (internal P loading; de Vicente et al., 2006; 2010b).

Figure 1. Geographic location of Honda lake.

2.2. Experimental set-up

Water and sediment samples were collected on July 2015. Lake water was collected using a peristaltic pump and sediment was sampled using an Ekman dredge at the deepest site of the lake. Once in the laboratory, homogenized surface sediment (6600 cm^3; OECD, 2006) and lake water was distributed in 15 microcosms (PVC black containers; Ø=38 cm; h=58 cm), which were randomly placed in an outdoor roofed area. In addition, 25 litres of lake water with concentrated zooplankton (which was obtained from vertical and horizontal hauls with a plankton net of 30 µm) were homogenized and distributed in the microcosms for getting a final volume of 40 litres of lake water. The microcosms were kept oxygenated by using an aeration pump and they were covered with a mesh for avoiding the aerial colonization of flying insects or falling spores.

After a one-week stabilization period the experiment, which lasted for 70 days, started. Three treatments (five replicates per treatment) were considered: (i) no MPs addition (control, C); (ii) Treatment-Water (T-W), where MPs were added on the surface of the water and (iii) Treatment-Sediment (T-S), where MPs were added on the surface of the sediment. MPs were added to T-W and T-S in aqueous dispersion (120 g MPs l^{-1}) by using a peristaltic pump in order to obtain a final concentration of 1.4 g MPs l^{-1} in each microcosm. MPs supplied by BASF (Germany), have a composition of 97.5% Fe, 0.9% C, 0.5% O and 0.9% N and an average diameter of 800 nm (de Vicente et al., 2010a; Merino-Martos et al., 2011). MPs concentration in each microcosm was three-fold higher than that proposed by de Vicente et al. (2010a) in order to counteract any chemical interference (de Vicente et al., 2011).

On the first day (day 0), baseline physic-chemical and biological (zooplankton) data were obtained previously to the addition of the MPs. After 24 h of contact time (day 1), P loaded MPs were removed by fully immersing an especially designed magnetic rake in the microcosms down to the surface sediment. The efficiency of MPs removal by the magnetic rake was 91 and 32% for T-W and for T-S, respectively (Funes et al., 2017).

2.3. Monitoring of the microcosms

Temperature (T), pH, dissolved oxygen concentration (O_2; mg l^{-1}), conductivity (Cond, mS cm^{-1}) and total dissolved solids (TDS; g l^{-1}) were recorded on a weekly basis (days: 2, 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70) by using a multi-parameter probe (Hanna Instrument, HI 9829). In addition, total nitrogen (TN; APHA, 1995), total phosphorus (TP; APHA, 1995), total dissolved Fe ($Tot\text{-}Fe_{dis}$; Gibbs, 1979) and chlorophyll *a* (Chl *a*; Jeffrey and Humpfrey, 1975) concentrations were also measured. More details about chemical analysis can be found in Funes et al. (2017). Sampled and evaporated water in the containers was replaced, after each sampling, with filtered lake water.

Zooplankton community was studied by collecting integrated composite samples (3 litres) with a tubular sampler (Ø=5.4 cm; h=25 cm) at the beginning, mid-time and end of the experiment (days: 2, 21, 35 and 70). The sampled water volume was filtered through 30 µm-mesh size plankton net and preserved with 70% ethanol, until taxonomic identification. Organisms were counted and identified to species level (except for nauplii and copepodites which were merged in one category, hereafter N+C), according to Dussart (1969) and Bledzki and Rybak (2016) for copepods; Ruttner-Kolisko (1974) and Koste and Voight (1978) for rotifers and Alonso (1996) for branchiopods. At each sampling date, a minimum of 50 individuals of each species was counted and identified. Tests using the methodology proposed by Cain and Castro (1959) have shown that increasing the number of specimens did not imply an increase in the number of species.

Abundance (ind l^{-1}), taxonomic groups (% abundance) and several ecological indexes were calculated for each sampling time. These indexes were focussed on evaluating: (i) diversity [Margalef index (S_{mg}– Margalef, 1958); Shannon-Wiener index (H' (log_2) – Shannon, 1948) and Pielou evenness index (J – Pielou, 1967)]; (ii) dominance [Simpson index (D – Simpson, 1949)] and (iii) similarity [Jaccard index J_c – (Jaccard, 1908)].

In addition, trophic state indexes (TSI) were calculated for TP and Chl *a* concentrations (Carlson, 1977); for TN concentration (Kratzer and Brezonik, 1981), for rotifer abundance (Ejsmont-Karabin, 2012), for total crustaceans abundance ($TSI_{CR1} = 6.89$ Ln (N, ind l^{-1}) + 20.7 and cyclopoida biomass ($TSI_{CR2} = 3.48$ Ln (B, mg w.wt. l^{-1}) + 60.2 (Ejsmont-Karabin and Karabin, 2013).

Finally, to verify that MPs effects on the zooplankton community are not derived from an indirect action through the phytoplankton community, food availability was calculated. The method of Huntley and Boyd (1984) was used to determine if food concentration was limited during the experiment. For these calculations, critical food concentration (C_c) was obtained from mean temperature data of water and mean adult body size of individuals. Species biomass was estimated from abundance data according to the formula proposed by Dumont et al. (1975) for branchiopods; Bottrell et al. (1976) for copepods and McCauley (1984) for rotifers. Food concentration (C) was calculated from Chl *a* concentration data, assuming a carbon/Chl *a* ratio of 70 (Guerrero and Rodríguez, 1997; Mullin and Brooks, 1970).

2.4. Data analysis

Statistical analysis was done by using Statistica 7.1 software (Stat Soft Inc., 1997). Differences in physico-chemical and biological variables as well as in the estimated indexes among treatments were tested by using one-way ANOVA, repeated measures ANOVA (RM-ANOVA) and Friedman ANOVA. In particular, one-way ANOVA test was performed to identify if there exist significant differences in the total abundance of each single species among treatments for any particular sampling date. In addition, RM ANOVA was used to test if there exist significant differences over time among treatments. Residuals normality (Shapiro-Wilk test), sphericity assumption (Mauchly’s test) and homogeneity of variances (Levene test) were checked before performing the analysis and Fisher´s Least Significance Difference (LSD) was used as post hoc test. Finally, Friedman ANOVA was used for testing significant differences in the species abundances over time for those species that only appeared in some sampling dates, using Wilcoxon Sign test as post hoc test.

At last, differences in species composition (in terms of total abundance of each species) among treatments were tested by using Principal Response Curves analysis (PRC; Van den Brink and Ter Braak, 1999) with CANOCO software. PRC is especially designed for micro and mesocosms experiments for evaluating changes generated over time in the structure of a community, being a very useful tool for ecotoxicological experiments (Van den Brink et al., 2000; Pardal et al., 2004; Moser et al., 2007) .

3. Results

3.1. Effects of magnetic particles addition on environmental variables

Table 1 shows the mean values of all environmental variables monitored during the experiment and Table 2 exhibits the associated RM ANOVA results. No significant differences among treatments were found (RM ANOVA; $p>0.05$) except for TP and TN concentrations. In this sense, significantly higher TP and lower TN concentrations were measured in control than in T-W and T-S. Regarding to changes over time, significant differences were found for all parameters. A gradual reduction in T (from summer to autumn) was observed in all microcosms together with a reduction in Chl *a*, TP and TN concentrations. By contrast, pH, O_2, Cond and TDS values depicted an increase. Tot-Fe_{dis} concentrations (data not shown) were on all occasions (treatments and time) below the limit of detection.

Table 1. Environmental variables and ecological indexes recorded during the microcosms experiment (mean ± SD).

		Day 0	**Day 2**	**Day 21**	**Day 35**	**Day 70**
T (ºC)	C	26.1 ± 0.1	27.8 ± 0.1	22.0 ± 0.1	19.7 ± 0.2	16.9 ± 0.0
	T-W	26.3 ± 0.2	27.9 ± 0.2	22.5 ± 0.5	19.8 ± 0.1	16.9 ± 0.1
	T-S	26.2 ± 0.1	27.8 ± 0.1	22.0 ± 0.5	19.7 ± 0.2	17.5 ± 1.3
pH	C	8.7 ± 0.1	8.7 ± 0.1	8.9 ± 0.0	9.9 ± 0.0	9.1 ± 0.4
	T-W	8.7 ± 0.0	8.8 ± 0.1	8.9 ± 0.0	9.9 ± 0.1	8.9 ± 0.0
	T-S	8.7 ± 0.0	8.7 ± 0.1	8.9 ± 0.0	9.9 ± 0.0	8.9 ± 0.0
O_2 (mg/l)	C	6.5 ± 0.2	6.6 ± 0.2	7.5 ± 0.0	7.6 ± 0.1	8.4 ± 0.1
	T-W	6.5 ± 0.1	6.6 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	8.3 ± 0.1
	T-S	6.4 ± 0.2	5.8 ± 1.6	7.4 ± 0.1	7.6 ± 0.1	8.3 ± 0.1
Cond. (mS/cm)	C	5.98 ± 0.04	5.98 ± 0.05	6.71 ± 0.66	6.37 ± 0.96	6.91 ± 0.81
	T-W	5.96 ± 0.01	5.97 ± 0.03	6.30 ± 0.04	6.65 ± 0.06	7.10 ± 0.47
	T-S	5.94 ± 0.04	5.89 ± 0.20	6.25 ± 0.12	6.22 ± 0.85	6.55 ± 0.67
TDS (g/l)	C	2.99 ± 0.02	2.99 ± 0.02	3.19 ± 0.06	3.22 ± 0.44	3.38 ± 0.33
	T-W	2.98 ± 0.00	2.99 ± 0.01	3.15 ± 0.02	3.32 ± 0.03	3.47 ± 0.08
	T-S	2.97 ± 0.02	2.98 ± 0.01	3.11 ± 0.05	3.13 ± 0.43	3.28 ± 0.33
TP (µg/l)	C	776.4 ± 35.9	715.5 ± 44.5	464.9 ± 28.7	393.3 ± 26.9	437.3 ± 59.7
	T-W	733.7 ± 61.4	549.4 ± 94.7	298.4 ± 25.5	261.6 ± 74.1	209.5 ± 24.6
	T-S	763.9 ± 59.4	574.2 ± 141.4	277.3 ± 26.4	252.2 ± 54.7	190.0 ± 48.0
TN (mg/l)	C	4.42 ± 0.22	3.45 ± 0.40	3.14 ± 0.29	3.22 ± 0.41	2.75 ± 0.46
	T-W	4.15 ± 0.47	3.77 ± 0.33	3.46 ± 0.36	3.13 ± 0.71	3.02 ± 0.53
	T-S	4.08 ± 0.28	3.82 ± 0.28	4.18 ± 0.42	3.45 ± 0.45	3.23 ± 0.38
Chl *a* (µg/l)	C	63.1 ± 27.6	48.0 ± 15.9	27.9 ± 14.4	30.9 ± 10.5	26.6 ± 22.3
	T-W	62.8 ± 10.1	39.1 ± 16.7	57.4 ± 20.2	45.0 ± 26.2	28.1 ± 19.4
	T-S	71.6 ± 19.5	44.8 ± 35.1	43.6 ± 33.0	29.7 ± 13.5	34.9 ± 35.9
S_{mg}	C	0.50 ± 0.17	0.42 ± 0.09	0.66 ± 0.16	0.48 ± 0.05	0.35 ± 0.09
	T-W	0.29 ± 0.19	0.26 ± 0.12	0.58 ± 0.12	0.52 ± 0.10	0.52 ± 0.13

	T-S	0.42 ± 0.06	0.36 ± 0.02	0.57 ± 0.04	0.49 ± 0.13	0.26 ± 0.08
H’	C	1.83 ± 0.50	1.53 ± 0.41	1.67 ± 0.61	1.73± 0.37	1.36± 0.64
	T-W	1.40 ± 0.81	1.44 ± 0.47	1.51 ± 0.51	1.97 ± 0.42	1.86 ± 0.42
	T-S	1.91 ± 0.33	1.45 ± 0.53	1.45 ± 0.35	1.94 ± 0.43	1.32 ± 0.29
J	C	0.71 ± 0.13	0.66 ± 0.09	0.56 ± 0.19	0.66 ± 0.15	0.55 ± 0.23
	T-W	0.59 ± 0.29	0.77± 0.07	0.52 ± 0.17	0.69 ± 0.13	0.68 ± 0.13
	T-S	0.80 ± 0.13	0.66 ± 0.26	0.51 ± 0.13	0.68 ± 0.18	0.66 ± 0.07
D	C	0.39 ± 0.15	0.44 ± 0.14	0.48 ± 0.21	0.42 ± 0.13	0.56 ± 0.23
	T-W	0.48 ± 0.24	0.46 ± 0.15	0.52 ± 0.17	0.35 ± 0.11	0.36 ± 0.15
	T-S	0.31 ± 0.09	0.48 ± 0.22	0.54 ± 0.13	0.31 ± 0.06	0.48 ± 0.11
J_c	C *vs* T-W	0.71	0.75	0.61	0.92	0.75
	C *vs* T-S	0.71	0.67	0.75	1.00	0.73
	T-W *vs* T-S	1.00	0.86	0.69	0.92	0.54

Table 2. Results of the repeated-measures ANOVA. df= degrees of freedom.

	TREATMENT				TIME				TREATMENT*TIME			
	df1	df2	F	p	df1	df2	F	p	df1	df2	F	p
T	2	12	0.6	ns	1.6	18.8	2015.4	<0.001	3.1	18.8	1.3	ns
pH	2	12	1.4	ns	1.1	13.7	281.5	<0.001	2.3	13.7	0.8	ns
O_2	2	12	0.8	ns	1.1	13.1	66.5	<0.001	2.2	13.1	1.1	ns
Cond	2	12	1.4	ns	1.3	15.9	9.8	<0.001	2.7	15.9	0.6	ns
TDS	2	12	0.5	ns	1.0	12.5	14.6	<0.001	2.1	12.5	0.4	ns
TP	2	12	25.8	<0.001	1.9	22.6	224.5	<0.001	3.8	22.6	3.5	<0.005
TN	2	12	4.4	ns	4.0	48.0	18.9	<0.001	8.0	48.0	2.0	ns
Chl *a*	2	7	0.7	ns	4.0	28.0	4.3	<0.005	8.0	28.0	1.7	ns
Zooplankton abundance	2	7	2.1	ns	4.0	28.0	4.7	<0.005	8.0	28.0	0.9	ns
S_{mg}	2	7	1.5	ns	4.0	28.0	10.3	<0.001	8.0	28.0	3.2	<0.05
H’	2	7	0.0	ns	4.0	28.0	1.6	ns	8.0	28.0	0.7	ns
J	2	7	0.2	ns	4.0	28.0	1.4	ns	8.0	28.0	0.5	ns
D	2	7	0.1	ns	2.1	14.7	1.4	ns	4.2	14.7	0.6	ns
Dubois	2	7	0.1	ns	4.0	28.0	1.4	ns	8.0	28.0	0.6	ns
TSI_{TP}	2	12	25.3	<0.001	2.4	28.3	190.5	<0.001	4.7	28.3	7.7	<0.001
TSI_{TN}	2	12	4.0	<0.05	2.5	30.0	15.8	<0.001	5.0	30.0	1.5	ns
$TSI_{Chl\ a}$	2	7	0.4	ns	4.0	28.0	4.8	<0.005	8.0	28.0	2.1	ns
TSI_{ROT}	2	6	0.7	ns	4.0	24.0	10.8	<0.001	8.0	24.0	1.7	ns
TSI_{CR1}	2	8	0.4	ns	4.0	32.0	1.0	ns	8.0	32.0	1.4	ns
TSI_{CR2}	2	8	0.6	ns	4.0	32.0	0.9	ns	8.0	32.0	1.6	ns

3.2. Effects of magnetic particles addition on zooplankton community composition and structure

A total of 15 zooplankton species (12 rotifers, 1 branchiopod and 2 copepods) were recorded (Table 3). A high abundance of zooplankton was registered during the experiment for all treatments.

Table 3. List of zooplankton species recorded along the experiment. C: Common species (present 100% of the experimental time); F: Frequent species (present 80% of the experimental time); O: Occasional species (present 60% of the experimental time); and R: Rare species (present ≤ 40% of the experimental time).

	C	**T-W**	**T-S**
***Anuraeopsis* sp.**	C	C	C
B. angularis	C	C	C
B. calyciflorus	C	C	C
***B. plicatilis* L**	C	C	C
***B. plicatilis* SM**	C	C	C
***Cephalodella* sp.**	R	R	R
***Colurella* sp.**	O	O	O
***Lecane* sp.**	R	R	R
H. oxyuris	O	R	R
K. quadrata	C	C	C
***Testudinella* sp.**	R	R	R
***Trichocerca* sp.**	R	O	O
A. rectangula	C	C	F
N + C	C	C	C
***Acanthocyclops* sp.**	C	C	C
M. gigas	F	C	C

In general, rotifers were the most abundant group, being *Brachionus* the predominant genus and *B. angularis* the most abundant species. This predominance is even more evident at the end of the experimental period (days 35 and 70; Figure 2). Copepods were the second majority group in abundance, being the developmental stages (N+C) the most abundant and *Acanthocyclops* sp. the predominant species. At last, branchiopods had the lowest total abundance in all treatments.

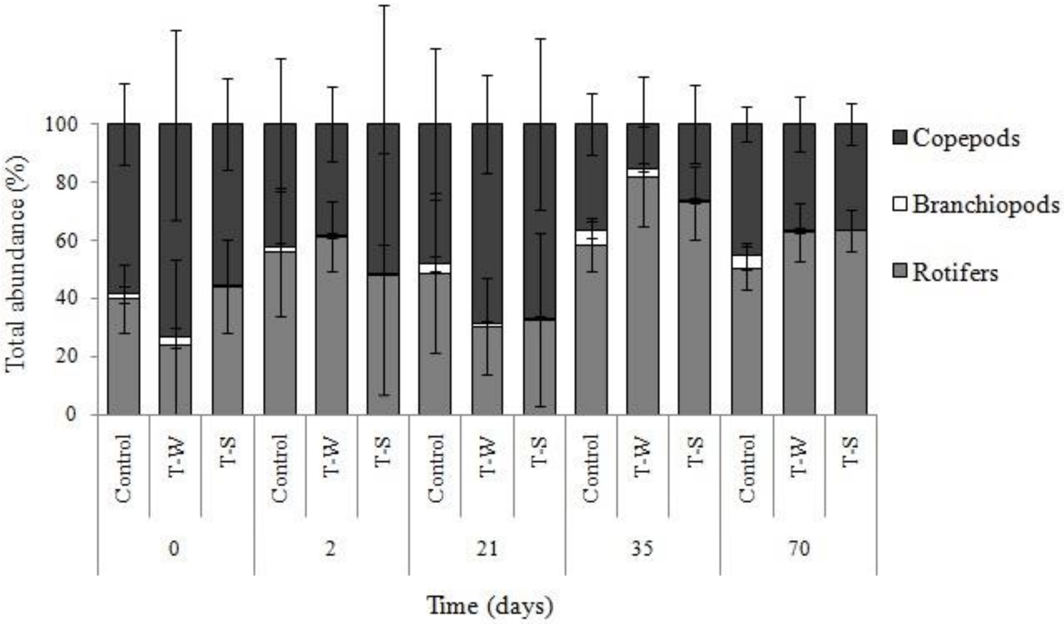

Figure 2. Temporal changes in the abundance of the different zooplankton groups.

The results for total zooplankton abundances (Table 2) evidenced no significant differences neither among treatments (p=0.17) nor the interaction time x treatment (p = 0.53), but significant differences were found over time (<0.005). Even more, for all species no significant differences in their abundances were found among treatments. When analyzing changes over time, most of species (*B. angularis*, *B. plicatilis* L, *K. quadrata*, *A. rectangula*, *Trichocerca* sp., *Cephalodella* sp., *Colurella* sp., *Lecane* sp., *H. oxyuris* and N+C) evidenced significant changes but no interaction between treatment x time were found except for N+C.

Table 1 also shows mean values of the different ecological indexes. Species richness reflected by the Margalef index (S_{mg}) was quite low, ranging from 0.26 to 0.66 for all treatments and times, which is likely to be a consequence of both the low species number and the high abundance values. The Shannon-Wiener diversity index (H´) ranged from 1.36 to 1.83 in C; from 1.40 to 1.97 in T-W and from 1.32 to 1.94 in T-S. Accordingly, intermediate values of evenness and dominance were found. Pielou evenness index (J) was always lower than 0.8 while values of the Simpson dominance index (D) were lower than 0.6 in any treatment and time. For comparing community structure among treatments, Jaccard index (J_c) was estimated denoting that all treatments shared similar species at all sampling times (values close to 1). RM ANOVA evidenced that no significant differences in any of the ecological indexes was found among treatments (Table 2) reflecting that MPs addition did not cause any significant effect in the structure of zooplankton community. However, significant changes over time and treatment x time interaction were found just for S_{mg}.

The results for the TSI drastically differ when considering different chemical and biological variables (Figure 3). TSI_{TP} showed the highest value, with a mean value ranging from 88.7 for T-S to 94.7 for C, while TSI_{Chla} and TSI_{CR2} exhibited the lowest values (mean value 42.7 and 44.6 for 42.0 for TSI_{Chla} and $TSI_{CR2,}$ respectively). In general, all indexes evidenced an hypereutrohic state of the study lake except for TSI_{Chla} and TSI_{CR1} which denoted a meso-eutrophic condition. Again, no significant differences among treatments were found except for TSI_{TP} and TSI_{TN} (Table 2). Changes over time were significant for TSI_{TP}, TSI_{TN}, TSI_{Chla} and TSI_{ROT} while treatment x time interaction was only significant for TSI_{TP}.

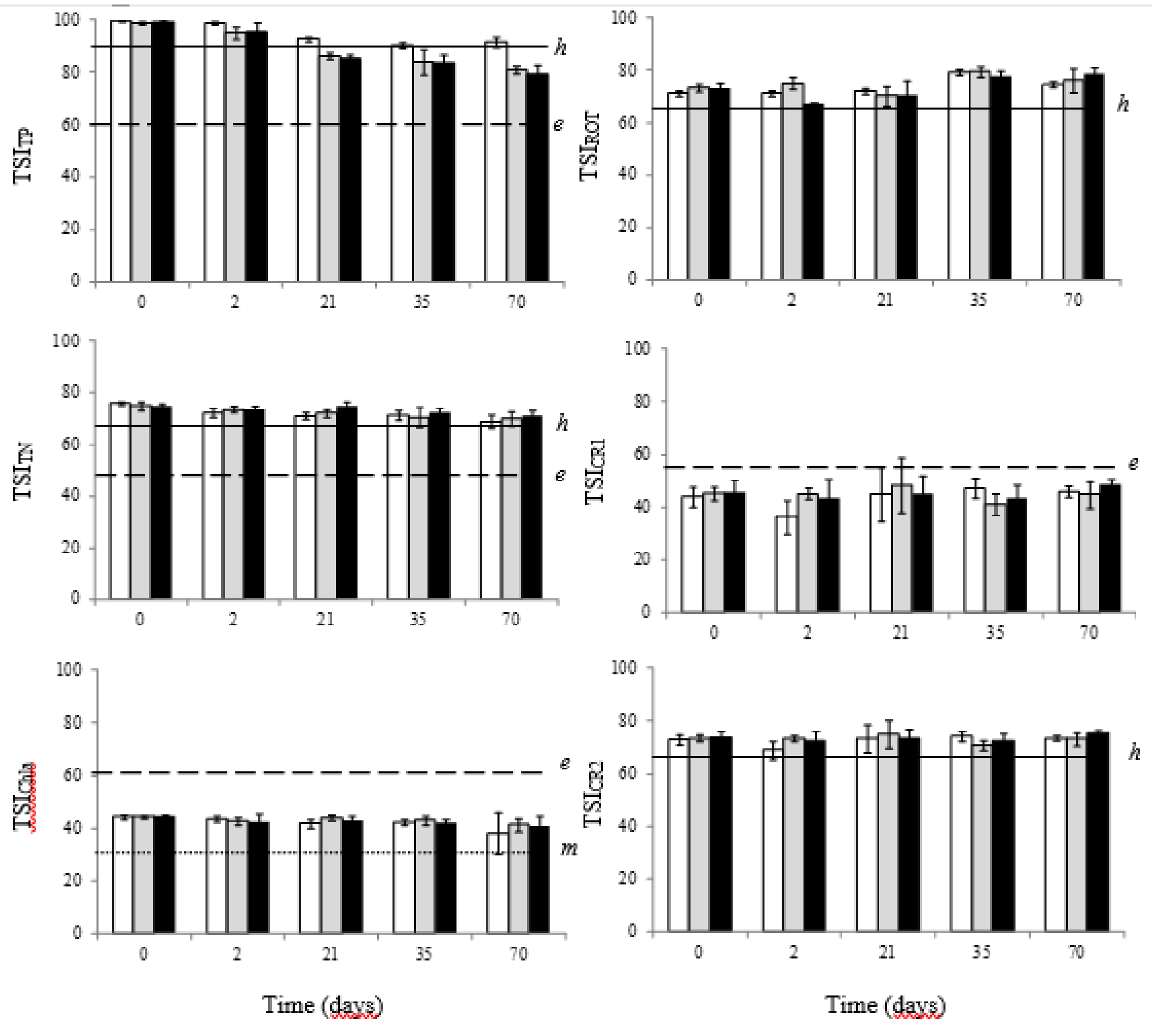

Figure 3. Time evolution of trophic state indexes estimated from different chemical and biological variables. White, grey and black vertical bars represent C, T-W and T-S, respectively. Horizontal lines represent trophic states (h, e and m correspond to hypertrophy, eutrophy and mesotrophy, respectively).

Next and considering that one of the most essential variables for zooplankton community growth is food availability, we explore if there was a lack of food resources for any species. The results evidenced that there were not food limitation ($C>C_c$) in any treatment at any sampling date.

Finally, PRC revealed no significant effects of treatment or its interaction with time according to Monte Carlo permutation test (Figure 4; F=3.0, p=0.43). Most species (33%) have shown weights between -0.5 and +0.5 evidencing either a weak response or a response that is unrelated to that shown in Figure 4. *A. rectangula* has the highest high positive weight and is thus inferred to decrease most strongly in abundance relative to the controls. In contrast, *B. angularis* has a negative weight, indicating an increase in abundance relative to the controls.

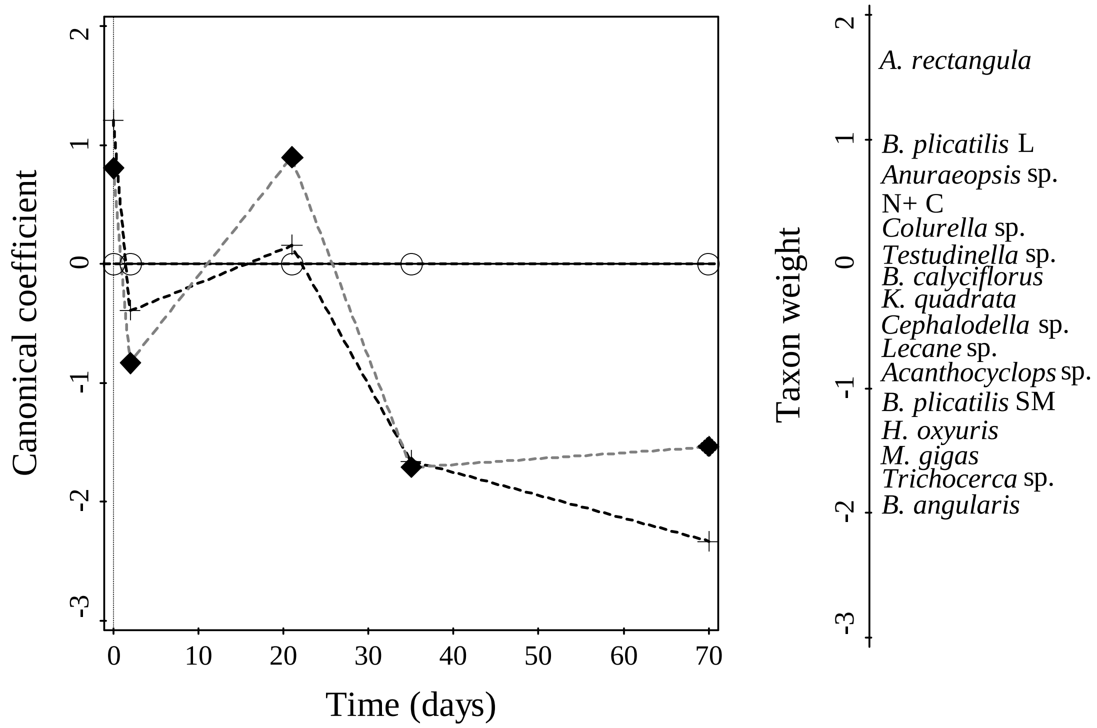

Figure 4. Principal Responses Curves (PRC) showing the response of zooplankton community to different environmental variables. Empty circles represent Control while asteriks and diamonds symbolize T-W and T-S, respectively.

4. Discussion

Although there exist a growing tendency for using microcosms experiments when testing chemical consequences of novel P adsorbents used for lake restoration (Lin et al., 2015; Yamada-Ferraz et al., 2015; Funes et al., 2017; Wang et al., 2017); up to date, there are scarce toxicological studies focused on using this methodological approach (Waajen et al., 2017). In this context, our results based on using microcosms have evidenced that MPs addition did not promote significant direct or indirect effect neither in composition nor structure of zooplankton community. The absence of any effect of

MPs on zooplankton organisms can be explained because MPs did not significantly alter any of its physico-chemical (e.g. temperature, pH, O_2) or biological (e.g. food quantity and quality) drivers.

Although zooplankton communities are known to be highly susceptible to a wide range of environmental factors, during the experiment temperature, pH and O_2 concentrations were in their tolerance range. As a way of illustration, it has been found that for *B. calyciflorus*, one of the most abundant species in the study site, the mean number of offspring per female shows an optimum at 20 °C (Halbach, 1970), which is just the average temperature recorded along the study period. Additionally, pH played a secondary role in our study due to both the consistent pH values (pH ranges just from 8.7 to 9.3) and the absence of any significant pH change after MPs addition. In fact, pH values were very close to 9 in all experimental units and according to Mitchell (1992) the highest net reproductive rate of the *B. calyciflorus* occurs at pH 8.5 and 9.5.

Apart from abiotic factors, zooplankton drastically depends on food quantity and quality (DeMott, 1986; Gulati and DeMott, 1997; Persson et al., 2008). At this point is important to consider that although MPs addition caused a notably reduction (65%) in Dissolved Inorganic P (DIP) concentrations (Funes et al., 2017), no significant effect on Chl *a* concentrations were found. The most likely explanation is that although DIP concentrations were much lower after MPs addition, they remained very high (TP >0.2 mg L^{-1}) and previous studies found that significant and sustained changes in the biological community and the water transparency of shallow, temperate freshwater lakes may not appear unless TP concentrations are reduced below 0.05–0.1 mg L^{-1} (Jeppesen et al., 2000). Hence, as Funes et al. (2017) suggested, achievement of a significant reduction in Chl *a* concentrations may involve higher MP doses, either in the first application or by repeated additions, and requires long-term follow-up of changes in water quality. Accordingly, and as a result of the high food availability, zooplankton abundance did not change after MPs addition. In fact, available C (estimated from Chl *a* concentrations) for zooplankton was always, for all treatments, much higher than C_c evidencing the lack of any food limitation for zooplankton.

In relation to the food quality, it has been found that MPs did not change phytoplankton composition, being cyanobacteria (95.2%), the most dominant group in all treatments before and after adding MPs (del Arco et al., unpublished data). Despite cyanobacteria are known for being inadequate as a food source for zooplankton, whether by their toxicity, large size, lack of essential compounds or due to feeding inhibitors (Lürling, 2003; Martin-Creuzburg and Von Elert, 2009); in eutrophic systems, higher abundance of rotifers is often observed with higher abundance of cyanobacteria (Starkweather, 1981; Bouvy et al., 2001; Leonard and Paerl, 2005) which indicates that not all cyanobacteria inhibit rotifer growth. However, contrasting results are reported in the literature. More recently, Soares et al. (2010) found, in 2-day life-cycle assays, that *B. calicyflorus* was capable of ingesting *Microcystis aeruginosa* although diets consisting only of *Microcystis* caused death of the animals. However, Starkweather and Kellar (1987) reported a similar median survivorship for *Brachionus* fed *Microcystis* and unfed animals, while in another study, *B. calyciflorus* showed the ability to utilize *M. aeruginosa* as food or to possess tolerance to the toxins (Fulton and Paerl, 1988). These results are specially striking if we consider that *M. aeruginosa* represents one of the most dominant phytoplankton species (1.94% of the total phytoplankton abundance; del Arco et al., unpublished data) in the study lake. In any case, it is important to take in account that natural waters are complex mixtures of different phytoplankton species and accordingly, rotifers will encounter a variety of food items of different quality. In this sense, a significant increase in population growth rate of *B. calicyflorus* was found when it was fed with a combination of *M. aeruginosa* and *Scenedesmus obliquus* compared to cyanobacteria based diet (Soares et al., 2010).

When analyzing the potential toxic effects of any P adsorbent used for lake restoration, both direct and indirect effects on lake biota may be take in account. Here it is important to take in account that there exist a continuum of experimental approaches and tools which ranges from single-species toxicity tests to the intentional contamination of natural ecosystems through experimental laboratory food chains and indoor microcosms (Caquet, 2013). In this continuum of experimental approaches, outdoor microcosms are characterized by a high complexity-ecological realism (including both direct and indirect effects) and by the possibility of replicability. In our study, no significant direct effects of MPs addition on zooplankton abundance and composition have been found. Contrarily to our results, del Arco et al. (2018) observed that, during MPs removal, *Daphnia magna* abundances drastically reduced regardless the concentration of MPs, competition or habitat structure. Discrepancies between our results and those obtained by del Arco et al. (2018) can be explained because of differences in both methodology and zooplankton species composition. Briefly, our study is based on microcosms (40 l) filled with a natural aquatic ecosystem while 1 l glass jars filled with 0.8 l of mineral water were used by del Arco et al. (2018) and accordingly, a much complex habitat is obtained in the present study. Even more, del Arco et al. (2018) used *D. magna* for evaluating MPs effects while the only branchiopod present in our study was *A. rectangula*. In this sense, previous studies have reported contrasting results when comparing the susceptibility to toxicants of *D. magna* and *Alona* sp. In particular, Bossuyt and Janssen (2005) found a major sensibility of *Alona* sp. to copper (immobilization test), while Sarma et al. (2007) did not observe consistent differences in the LC_{50} to methyl parathion (a pesticide) and mercury ($HgCl_2$), between *Alona* sp. and *Daphnia* sp.

Indirect effects on zooplankton community were also negligible and only significant changes in *A. rectangula* were found at the end of the experiment. It is important to state that Álvarez-Manzaneda et al. (2017) found in chronic toxicity tests that Tot-Fe_{dis} (even at very low concentrations) had a negative effect on reproductive output in *D. magna* as it significantly reduced the number of female offspring but no effect on the number of male offspring was observed. Accordingly, it is likely that the late disappearance in *A. rectangula* population dynamics may be caused by an affection of its reproduction as similar MPs concentrations were used in both experiments. However, the absence of any general long-term effect of MPs addition on the others zooplankton groups may be explained by the high percentage MP recovery (Funes et al., 2017) and by the extremely low Tot-Fe_{dis} concentrations.

More in detail, rotifers are the dominant group in the zooplankton community in Honda lake as it was expected due to the extremely high trophic state (Gannon and Stemberger, 1978). The predominance of rotifers in the study site has been previously reported (Carrillo et al., 1987; Martínez-Vidal and Castro, 1990; Cruz-Pizarro et al., 2003). Among rotifers, *B. calyciflorus* was one of the most dominant species in the study lake. In relation to this species, when comparing our results with single-specie toxicological experiments reported in the literature for the same adsorbents (MPs), we found that the LC_{50} was higher (1.63 g l^{-1}; Álvarez-Manzaneda and de Vicente, 2017) than MPs concentration used in this experiment (1.4 g l^{-1}) and accordingly, our results confirmed the expected minor effect of MP addition on *B. calyciflorus.*

Finally, in the context of any successful restoration project for combating eutrophication, it is essential to keep in mind that the final goal is to reduce the lake trophic state. Changes in the trophic state of the study lake after adding MPs were assessed by estimating different trophic indexes. Although a wide outcome was observed depending of the variable, the general trend was that no significant differences were found among treatments except for the one based on TP concentrations (TSI_{TP}). The ultimate effect of MPs on TSI_{TP} (promoting a change from hypereutrophy to eutrophy) was observed as TP concentrations were drastically reduced after MPs addition. In view of $TSI_{Chl\mathit{a}}$, and TSI_{CR1} lake trophic state was mesotrophic while a much higher state (hypereutrophic) was obtained when considering TP and TN concentration as well as rotifers (TSI_{ROT}) and crustacean abundance (TSI_{CR2}). The extremely low $TSI_{Chl\mathit{a}}$ could be explained by the high inorganic turbidity which ultimately may cause a primary production limitation by light. In fact, de Vicente et al. (2010b) measured, by using a combination of field measurements, modelling and laboratory experiments, large resuspension fluxes in the study lake promoted by the lake morphometry and sediment grain size distribution.

All in all, the lack of any significant effects of MPs addition on zooplankton community is especially striking in the context of restoration programs for Mediterranean endorheic ponds where zooplankton is naturally the highest aquatic trophic level. In this sense, it is important to consider that Mediterranean endorheic ponds are the most abundant systems in the Iberian Peninsula (Dantín, 1929; 1940; 1942). More research before applying MPs in a whole-lake restoration project should be focused on the assessment of their effects on (i) other trophic levels by using microcosms (e.g. phytoplankton) and (ii) in artificial aquatic ecosystems (e.g. farm ponds).

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Chapter V: Magnetic particles as new adsorbents for reducing phosphorus inputs from a wastewater treatment plant to a Mediterranean Ramsar wetland (South of Spain)

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Abstract

Eutrophication of inland waters is still the most important challenge for water resources managers. The keystone of lake restoration is the reduction of external phosphorus (P) load by, among others, increasing nutrient removal during wastewater treatment. Although recent studies are actually focused on assessing P removal from wastewaters by using novel adsorbents; we are still far for recognizing the best P adsorbent. In this study, and based on promising previous results obtained for inland eutrophicated ecosystems, the convenience of using magnetic microparticles (MPs) for reducing P concentration in treated wastewaters is assessed. The working hypothesis is that MPs addition increases P removal in artificial wastewater treatment ponds. To get this purpose, water samples were collected at the inlet (sample A) and outlet (sample B) of a semi-natural pond receiving secondary municipal effluents that lastly discharge in a Ramsar site (Fuente de Piedra, Málaga, Spain). In the laboratory, batch experiments were run for (i) assessing the effect of adding MPs on the chemical composition (major cations, major anions and metal concentration) of treated wastewaters; (ii) identifying the number of adsorption cycles (by reusing MPs) which are able to still trapping a high percentage of P (>50%) and (iii) selecting the optimum ratio between MPs mass and initial dissolved inorganic P (DIP) concentration. The results firstly evidenced that the semi-natural pond acts as a continuous green filter operating as a tertiary treatment of the outflow coming from the wastewaters treatment plant. Accordingly, this pond facilitates water self-purification acting as a reliable countermeasure to reduce the impact of wastewater effluents in the Fuente de Piedra Ramsar site. Secondly, the results have shown the suitability of using MPs for removing P in treated wastewaters. This statement is based on both the high equilibrium adsorption capacity (q) and P removal efficiency of MPs. In particular, average value for P removal efficiency was 93% (sample A) and 95% (sample B) during the 1st adsorption cycles. Finally, and considering that, from a practical point of view, it is essential to identify the optimum dose of MPs to be added to treated wastewaters, both advantages (P removal efficiency, %) and disadvantages (economic price, € l^{-1}) of using MPs have been considered as a

function of MPs dose. We can conclude that the optimum ratio is ≥0.16 g MPs mg^{-1} P for achieving a high P removal efficiency (>50%).

1. Introduction

Wetlands are among the world´s most productive and valuable ecosystems (Mitsch and Gosselink, 2015). Despite covering only 1.5% of the Earth´s surface, wetlands provide a disproportionately high 40% of global ecosystem services such as provisioning, regulating, habitat and cultural services (Zedler and Kercher, 2005). One likely explanation for supporting such high-value ecosystem services is their position in landscape as recipients, conduits, sources and sinks of biotic and abiotic resources (Keddy, 2000; Mitsch and Gosselink, 2015). Based on The Economics of Ecosystems and Biodiversity for water and wetlands (Russi et al., 2012), which translate the values of ecosystem services into dollar terms, wetlands have one of the highest value per hectare per year among the 10 biomes considered, exceeding temperate and tropical forest and grasslands. Despite the existence of international, national and regional legislation for wetland protection, many wetlands continue to degrade through reduced water availability, eutrophication (increase in primary production) and impacts from weeds and pests (Clarkson et al., 2013).

In particular, Mediterranean wetlands which represent unique repositories of biodiversity holding exclusive communities of aquatic organisms (Gascón et al., 2009; Gilbert et al., 2014; García-Muñoz et al., 2016) are actually undergoing a strong detrimental human disturbance (Berny et al., 2002; López-Flores et al., 2003; Vázquez-Roig et al., 2011). Their general features such as shallowness and high catchment: lake area ratio (Álvarez-Cobelas et al., 2005) makes them especially sensitive (as receiving ecosystem) to the activities generated in their drainage basins (i.e. agriculture, cattle; Ortega et al., 2006; García-Muñoz et al., 2010; Gilbert et al. 2017). Accordingly, structure and function of Mediterranean wetlands is being strongly affected by cultural eutrophication (de Vicente et al., 2003; Poquet et al., 2008; Conde-Álvarez et al., 2012). Nutrients coming from the catchment and from internal recycling are largely transformed as a result of the intensive biogeochemical processes occurring in wetlands. In fact, wetlands are worldwide considered as "hotspot" sites for biogeochemical transformation (Richardson and Vaithiyanathan, 2009) as a consequence of their intrinsic characteristics (i.e., shallowness, high water level fluctuations and anoxic sediment). Based on the effective role of wetlands as natural water purifiers, constructed wetlands technology has been developed since 1980s (Kadlec and Knight, 1996; Kadlec, 2009). By definition, treatment wetlands are constructed ecosystems designed primarily to enhance physical, chemical and biochemical processes with the goal of reducing specific contaminants to acceptable levels (Kadlec, 2009). Although the range of usage of wetland treatment technology is large and still expanding, the largest number and the longest history belong to the class that threats municipal and domestic wastewaters. Because a large proportion of phosphorus (P), the main nutrient limiting primary production in inland waters, inputs come from domestic wastewater (Gakstatter et al., 1978; Egle et al., 2016), increasing the efficiency of treatment wetlands for trapping P is essential for combating eutrophication of receiving aquatic ecosystems. Recent studies are actually focused on assessing P removal from wastewaters by novel adsorbents such as zeolites (Ping et al., 2008) and struvite (de-Bashan and Bashan, 2004); but we are still far for recognizing the best P adsorbent. Accordingly it is necessary to continue developing new techniques in order to achieve an environmentally acceptable level with affordable costs.

Apart from lake eutrophication, we are actually facing another problem that is the exhaustion of P reserves for fertilizer production (Cordell et al., 2011). In fact, both problems are convincingly connected as P is anthropogenically mobilized from its land reserves to the aquatic environment. Therefore, two of the most important challenges in the field of natural resources are to combat the next coupled and worldwide increasing problems: (i) the global reduction of P reserves essential for making P fertilizers (Gilbert, 2009) and (ii) the eutrophication, nutrient enrichment, of aquatic ecosystems (OECD, 1982; Sas, 1989; Cooke et al., 2005).

In this study, the use of magnetic particles (MPs) for removing P in semi-natural wastewaters ponds and therefore combating both eutrophication and the exhaustion of global P reserves is proposed. In this sense, previous studies have assessed the convenience of using MPs in inland eutrophicated ecosystems as MPs adsorb P and later P loaded MPs can be efficiently removed from solution by applying a magnetic separation gradient (de Vicente et al., 2010, 2011; Merino-Martos et al., 2011, 2015; Funes et al., 2016, 2017). Although the chemical composition of natural and wastewater drastically differ, the working hypothesis is that MPs addition increase P removal in wastewater treatment ponds. In this context, the general aim of the present study is to achieve the efficiency of MPs for trapping P in secondary municipal effluents that lastly discharge in a Ramsar site (Fuente de Piedra, Málaga, Spain). The specific goals are: (i) to assess the effect of adding MPs on the chemical composition (major cations, major anions and metal concentration) of treated wastewaters; (ii) to identify the number of adsorption cycles (by reusing MPs) which are able to still trapping a high percentage of P (>50%) and (iii) to select the optimum ratio between MPs mass and initial dissolved inorganic P (DIP) concentration.

2. Material and methods

2.1. Site description

Fuente de Piedra (37° 6′ N, 4° 44′ W) is a hypersaline wetland of great extension (13.5 km^2; NE-axis: 6.8 km; SE-axis: 2.5 km and a perimeter of 18 km; Linares and Rendón, 1998). It became the third Spanish wetland to be included in the Ramsar convention (in 1983) and it was declared as regional Nature reserve in 1984. Its catchment is situated in the water divide of the Atlantic Ocean (Guadalquivir river) and Mediterranean Sea (Guadalhorce river) near Antequera (Málaga, Spain; Höbig et al., 2016; Figure 1). The present-day lake has an average water depth of 70.5 cm (Rodríguez-Rodríguez, 2002) and several small ponds around the margin. The main fluvial supply is located in the north, and minor streams in the east and west (Höbig et al., 2016). Due to its shallow water, the site is an ideal habitat for migrating and non-migrating birds supporting one of the most important breeding colonies of the greater flamingo (e.g., *Phoenicopterus roseus*; Rendón et al., 2014). Despite its protection, one of the major problems of this ecosystem is the discharge of treated wastewater from the nearby Fuente de Piedra town and Humilladero town, even though it fulfills the standards for the treatment of urban wastewater (RD 509; de-los-Ríos-Mérida et al., 2017). Since 1999, secondary municipal effluents go through several small semi-natural ponds: Laguneto (27801 m^2 and a maximum depth of 1.42 m), Laguna de los Juncares (9222 m^2 and a maximum depth of 0.39 m), and Los Juncares (19828 m^2 and a maximum depth of 0.20 m; de-los-Ríos-Mérida et al., 2017). A recent study has quantified a notable reduction in nutrient (N and P) concentrations in the water along it flows through the semi-natural ponds system (de-los-Ríos-Mérida et al., 2017).

2.2. Materials and reagents

All chemicals used in this study were analytical reagents of high purity. MPs (HQ grade, BASF, Germany) were micron-sized magnetic spherical particles with negligible coercive field and remanent magnetization (i.e., zero magnetization upon removal of the magnetic field). Further details on these particles have been reported by de Vicente et al. (2010) and Merino-Martos et al. (2011).

2.3. Experimental set-up

On May 2018, watersamples at the inlet (station A) and at the outlet (station B) of Laguneto pond were collected (Figure 1) and named sample A and sample B respectively. Once in the laboratory, an initial characterization of water samples was carried out. Firstly, pH and conductivity were measured with a pH meter (pH 196, WTW, Germany) and a conductivity meter (InoLab Con Level 1, WTW, Germany), respectively. Later, water samples were filtered through glass microfiber filters (Whatmann GF/F, 0.7 mm) and dissolved inorganic P (DIP; Murphy and Riley, 1962) and total dissolved Fe (Tot-Fe$_{dis}$; Gibbs, 1979) concentrations were spectrophotometrically quantified (BiochromLibra S50).

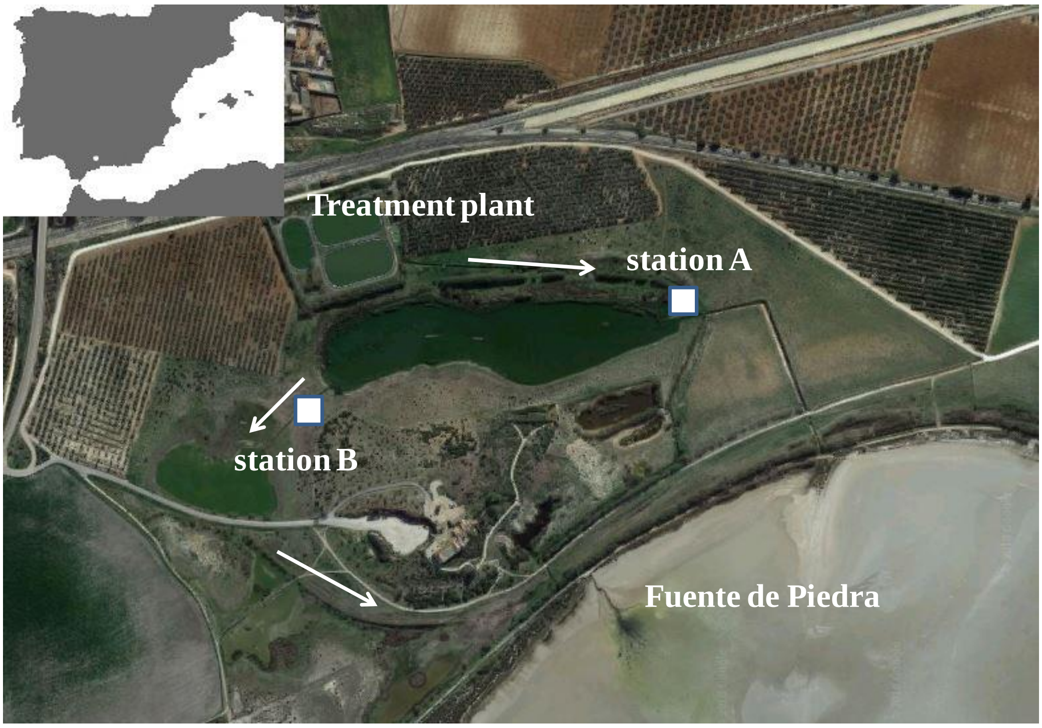

Figure 1. Location of the sampling sites.

For the experimental set up, a standard suspension (50 g l^{-1}) of MPs was prepared with 5 g of magnetic adsorbent were mixed with distilled water in a polypropylene container (100 mL). To ensure homogeneity of the sample, the suspension was sonicated for 5 min prior to its use in the adsorption experiments.

For the batch adsorption experiments, five different treatments (with four replicates) were considered: X/2; X; 2X; 3X and 5X, where X was the estimated MPs concentration as a function of both the initial DIP concentration in the sample and the maximum P adsorption capacity of MPs (18.83 mg P g^{-1} MPs; de Vicente et al., 2010). Accordingly, the next MPs concentrations were added: 0.08; 0.16; 0.32; 0.48 and 0.8 g l^{-1} for sample A and 0.045; 0.09; 0.18; 0.27 and 0.45 g l^{-1} for sample B. Briefly, the adsorption experiment consisted on adding the different adsorbent concentrations to 50 mL of treated wastewater. Samples were then agitated for 24 h in a horizontal shaker (150 rpm). After this time, the supernatant was separated from the adsorbents by using a variable gap magnet for 5 min (PASCO scientific; EM-8641). Finally, the supernatant was filtered for DIP analysis. The equilibrium adsorption capacity (q, mg g^{-1}) was calculated as follows:

$$q = \frac{C_0 - C_e}{M_a} V$$

where C_0 is the initial DIP concentration (mg l^{-1}); C_e is the equilibrium DIP concentration (mg l^{-1}); M_a is the mass of adsorbent (g) and V is the total volume of the suspension (L).

Next, a new adsorption experiment was performed to identify the number of possible adsorption cycles for achieving a high P removal efficiency by MPs. To get this purpose, once the 1st adsorption experiment (1st adsorption cycle) was carried out, following the methodology described above, MPs were separated and later washed twice (each time for 24 h) with 0.1M NH_3. Resulting particles were then reused for a 2nd adsorption experiment (2nd adsorption cycle) following the above mentioned adsorption procedure. The same scheme (adsorption and desorption) was again repeat to finally get a total of four adsorption cycles.

For samples A and B, DIP and Tot-Fe_{dis} concentrations were quantified in all adsorption cycles. Additionally, for sample A, major cations [calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and potassium (K^{+})] and major anions [chlorides (Cl^{-}), bromide (Br^{-}), fluoride (F^{-}), sulphates (SO_4^{2-}) as well as silica (Si)] were measured in the supernatants from the 1st and 4rd adsorption cycles. Major cations and Si were measured in filtered lake waters (Millipore 0.22 mm filters) by Ion Cromatography (IC; 940 Professional IC Vario, Metrohm) and the rest of anions were analyzed by inductively coupled plasma-mass spectroscopy (ICP-MS; Perkin Elmer NexION300D). Although Al^{3+}, Cr^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, NO_3^{-} and NO_2^{-} were also measured, their concentrations were lower than the detection limits (<0.01 mg l^{-1}) and accordingly, results are not shown.

2.4. Data analysis

Differences in water chemical parameters between sample A and sample B were tested by using t-test (for pH) or Mann–Whitney U test (for conductivity and DIP), according to the satisfaction of the normality assumption (Shapiro-Wilk test). Effect of MPs addition on pH and conductivity (for all adsorption cycles) and on major cations and anions concentrations, during the 1st and 4th adsorption cycle, were also tested by using Repeated Measures ANOVA (RM-ANOVA) with Fisher's least significance difference (LSD) post-hoc test. Differences in Tot-Fe_{dis} concentrations and equilibrium adsorption capacity (q) for the different MPs concentrations (treatments) and adsorption cycles (time) were tested by using non-parametric analysis of variance as our data did not satisfied homocedasticity assumptions (Levene test, $p < 0.05$). For this reason, Kruskal-Wallis was carried out to test the differences among treatments and a Friedman ANOVA by ranks was performed to test the significant differences among adsorption cycles. For testing if there exist significant differences in these response variables (Tot-Fe_{dis} and q) paired t-test analysis between adsorption cycles was performed after the normality was checked by the Shapiro-Wilk test. Statistica 7.1 software (StatSoft Inc., Tulsa, USA) was used for the statistical analysis.

3. Results and Discussion

3.1. Efficiency of the semi-natural pond for the treatment of wastewater

Table 1 shows changes in physico-chemical parameters at the inlet (station A) and at the outlet (station B) of the semi-natural Laguneto pond. In general, an improvement in water quality along it passes through the Laguneto pond, has been observed. More in detail, pH did not significantly change between station A and B (t-test; $p>0.05$) evidencing a slight decrease (1.32%). Although the pH values were in the range of those measured by de-los-Ríos-Mérida et al. (2017) in the same study site, the tendency was opposite as these authors found a general increase in pH from station A to station B. As it was expected, a significant reduction in conductivity values has been noticed along water passes through the Laguneto pond (Mann-Whitney U test; $p<0.001$). Similarly, a notable reduction in conductivity values was also measured by de-los-Ríos-Mérida et al. (2017) reporting an even higher reduction (21%) in this parameter.

Table 1. Changes in some selected chemical features of water at the inlet (station A) and at the outlet (station B) of Laguneto.

	station A	station B	Reduction (%)
pH	7.98 ± 0.06	7.88 ± 0.26	1.2
Conductivity(mS cm^{-1})	5.89 ± 0.04	5.55 ± 0.03	5.8
DIP (mg l^{-1})	1.86 ± 0.04	0.75 ± 0.01	59.7

As expected, if we compare DIP concentrations at the inlet and at the outlet of Laguneto, a significant (Mann-Whitney U test; $p<0.05$) reduction (59.5%) is detected. These results are in agreement with previous studies evidencing the efficiency of constructed wetlands for nutrients removal (Rao et al., 2013; Lu et al., 2015; Guo et al., 2016). Moreover, it has been demonstrated that the presence of aquatic vegetation in ponds drastically enhances P removal rate (Breen, 1990; Busnardo et al., 1992; Quan et al., 2016). For the case of Laguneto, de-los-Ríos-Mérida et al. (2017) described up to six different helophytic species, one of them was *Phragmites australis* that has been recently proved to have the maximum DIP removal efficiency in a mesocosm experiment (Afrous et al., 2013). All in all, the obtained results confirm that there exists a marked improvement in treated wastewater quality along it goes through the semi-natural Laguneto pond supporting the convenience of constructed wetlands for achieving an enhancement in water quality of the final effluent.

3.2. *Effects of magnetic particles addition on wastewater physico-chemical and chemical composition*

The effects on pH and conductivity of adding different MPs concentration at different adsorption cycles are shown in Figure 2. For station A, and similarly to previous studies carried out with the same MPs (Funes et al. 2017), no significant effect of MPs addition was observed on pH while a significant increase with increasing adsorption cycles was found (MR ANOVA; $p<0.005$). As an illustration, for 0.8 g MPs l^{-1}, pH changed from 7.79 (1st adsorption cycle) to 8.37 (4th adsorption cycle). For sample B, pH was significant and positive related to adsorption cycles (MR ANOVA; $p<0.005$) but it was also significantly related to MPs concentration (MR ANOVA; $p<0.05$). In relation to conductivity values, a significant difference when adding MPs was only observed for sample A, with a general decrease in conductivity values with MPs. For both water samples, conductivity experienced a significant increase with subsequent adsorption cycles.

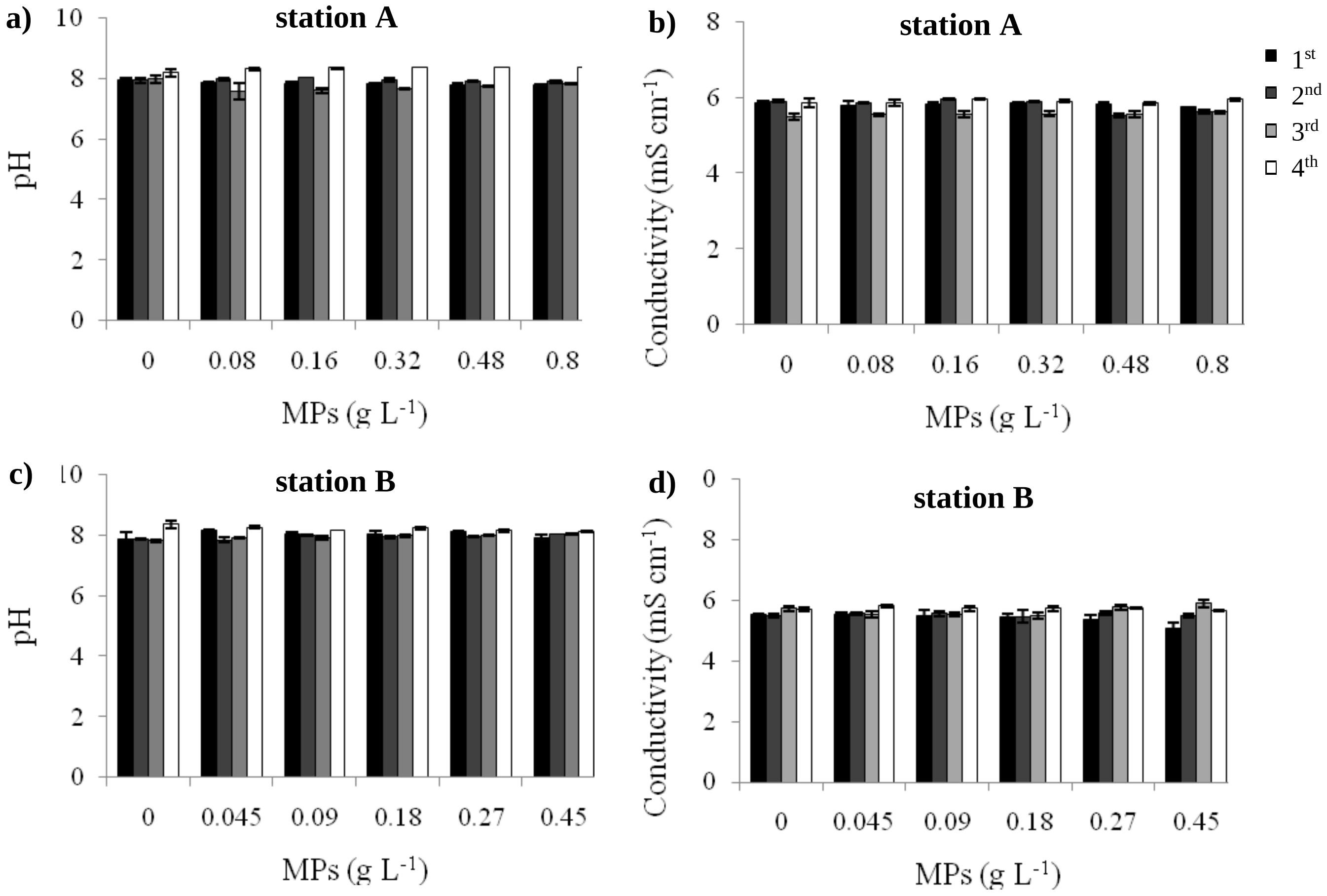

Figure 2. Changes in pH and conductivity values among treatments and among adsorption cycles.

Next, and based on the fact that wastewaters always contain lots of coexisting ions which could potentially compete with DIP for adsorption sites, we aim on achieving if MPs addition cause changes in major cation and anions concentrations (Figure 3; Table 2). Significant differences among treatments (MPs concentration) have been found for

Ca^{2+}, K^+, Cl^-, F^-, SO_4^{2-} and Si. Among them, it is especially remarkable the sharp reduction in Ca^{2+} (22%) and Si (55%) concentrations when adding the highest MPs concentration in the 1st adsorption cycle. In relation to Ca^{2+}, Stachowicz et al. (2008) studied the interaction between Ca^{2+} and DIP on goethite. Specifically, they found that DIP adsorption existed, as well as Ca^{2+}adsorption in a pH range 3-11, in spite of the fact that Ca^{2+} had a much lower affinity for goethite than DIP. This result was explained by the electrostatic interactions, as the negative charges of the adsorbed P ions on goethite stimulated the binding of the positively charged Ca^{2+} ion. However, contrary to these observations, Merino-Martos et al. (2015) using single-ion solutions and the same adsorbents (MPs) did not found any Ca^{2+} removal by MPs in the presence of P while a notable Ca^{2+} removal was observed when DIP was not present. For Si, the results are consistent with previous findings stating that Si and DIP had the same specific adsorption behavior, and competition for adsorption sites should occur (Sigg and Stumm, 1981; Cornell and Schwertmann, 1996; Hartikainen *et al.*, 1996). Briefly, Si concentrations have also been notable reduced when adding DIP adsorbents such as MPs (Merino-Martos et al., 2015); $Al(OH)_3$ (de Vicente et al., 2008; Egemose et al., 2011) or goethite (Sigg and Stumm,1981). Similarly, Hartikainen et al. (1996), analyzing sediment samples from Lake Vesijärvi (southern Finland), found that P oxyanions competed directly with Si compounds for sorption sites, but their results indicated that Si retention on the oxide surface was less effective than that of P. The obtained results are consistent with these statements as Si concentrations were drastically reduced in the 1st adsorption cycle.

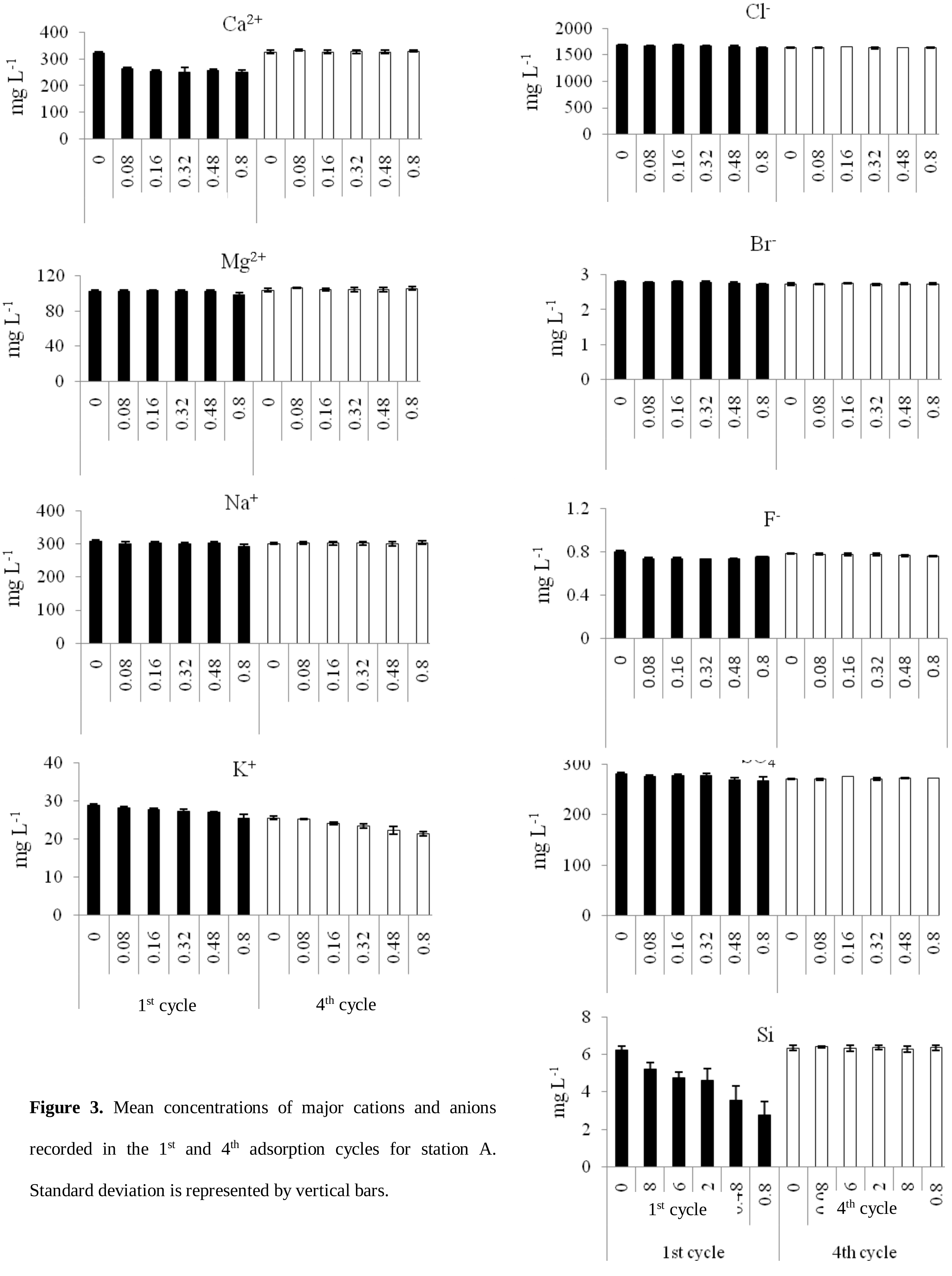

Figure 3. Mean concentrations of major cations and anions recorded in the 1st and 4th adsorption cycles for station A. Standard deviation is represented by vertical bars.

Table 2. Results of the repeated-measures ANOVA for major cations and anions in sample A. df= degrees of freedom; ns (no significant differences); * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.0005$).

	TREATMENT				**TIME**				**TREATMENT*TIME**			
	df1	**df2**	**F**	**p value**	**df1**	**df2**	**F**	**p value**	**df1**	**df2**	**F**	**p value**
Ca^{2+}	5	12	13.53	***	1	12	974.21	***	5	12	34.37	***
Mg^{2+}	5	12	0.6	ns	1	12	19.5	**	5	12	2.2	ns
Na^{+}	5	12	1	ns	1	12	0.2	ns	5	12	1.7	ns
K^{+}	5	12	29.10	***	1	12	508.62	***	5	12	2.37	ns
Cl^{-}	5	12	4.4	*	1	12	95.9	***	5	12	10.3	**
Br^{-}	5	12	2.8	ns	1	12	33.6	***	5	12	2.8	ns
F^{-}	5	12	38.8	***	1	12	46.6	***	5	12	6.1	**
SO_4^{2-}	5	12	3.1	*	1	12	4.8	*	5	12	5.4	*
Si	5	12	30.63	***	1	12	86.70	***	5	12	6.45	**

Regarding to the Tot-Fe_{dis} concentrations, notable differences were found between sample A and B (Table 3). In station A, much higher values were measured (even in the control) and the addition of MPs caused a significant increase in Tot-Fe_{dis} during the 1st adsorption cycle. It is striking that for the other adsorption cycles, in general, no effect of MPs addition was found. In station B, the Tot-Fe_{dis} concentrations were quite alike among treatments and adsorption cycles, reaching the highest value, as expected, when adding the highest MPs concentration (0.45 g l^{-1}). It is worth to remark that results obtained for station A are opposite to those found by previous studies using the same adsorbents where no changes in Tot-Fe_{dis} concentrations were detected even in anoxic conditions (Funes et al., 2017). In fact, these authors found that after adding much higher MPs concentrations (1.4 g l^{-1}) than those used in the present study (0.8 g l^{-1}), Tot-Fe_{dis} concentrations were always lower than the detection limit of the analytical method. In any case, and as Funes et al. (2017) suggested, there is no evidence of negative effects of increasing Fe concentrations on the maximum achievable ecological quality for fish, macrophyte and diatom communities although it has been reported a decrease of macroinvertebrate populations in contact with high total Fe concentrations (WFD-

UKTAG, 2012). In fact, some thresholds have been proposed to guarantee the good state of these communities such as 1 mg l^{-1} total Fe (EPA, 1976) that is actually in the range of the higher values measured in the present study.

Table 3. Changes in mean ± SD of total dissolved Fe(Tot-Fe_{dis}) concentrations along the experiment.

		Tot-Fe_{dis} (mg l^{-1})			
	MPs (g l^{-1})	**1st**	**2nd**	**3rd**	**4th**
station A	Control	0.04 ± 0.04	0.03 ± 0.02	0.00 ± 0.00	0.03 ± 0.01
	0.08	0.04 ± 0.03	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	0.16	0.33 ± 0.11	0.04 ± 0.06	0.03 ± 0.02	0.01 ± 0.01
	0.32	0.44 ± 0.19	0.21 ± 0.19	0.00 ± 0.00	0.04 ± 0.02
	0.48	1.54 ± 1.17	0.20 ± 0.04	0.01 ± 0.02	0.07 ± 0.05
	0.8	1.88 ± 0.96	0.17 ± 0.16	0.04 ± 0.04	0.06 ± 0.03
station B	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
	0.045	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.27	0.07 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02
	0.45	0.48 ± 0.46	0.02 ± 0.04	0.06 ± 0.12	0.00 ± 0.00

3.3. Changes in the equilibrium adsorption capacity along subsequent adsorption cycles

In general, significant differences among treatments were found in the equilibrium adsorption capacity (q) for all adsorption cycles although these differences were weaker along the subsequent adsorption cycles (Figure 4; Table 4). In fact, as expected, it strongly decreased when MPs concentrations increase. Regarding to changes in q values along the different adsorption cycles, there exists a significant reduction for the lowest MPs concentration in both sampling sites (Table 5). However, for the subsequent adsorption cycles, predominantly, there is no a significant pattern.

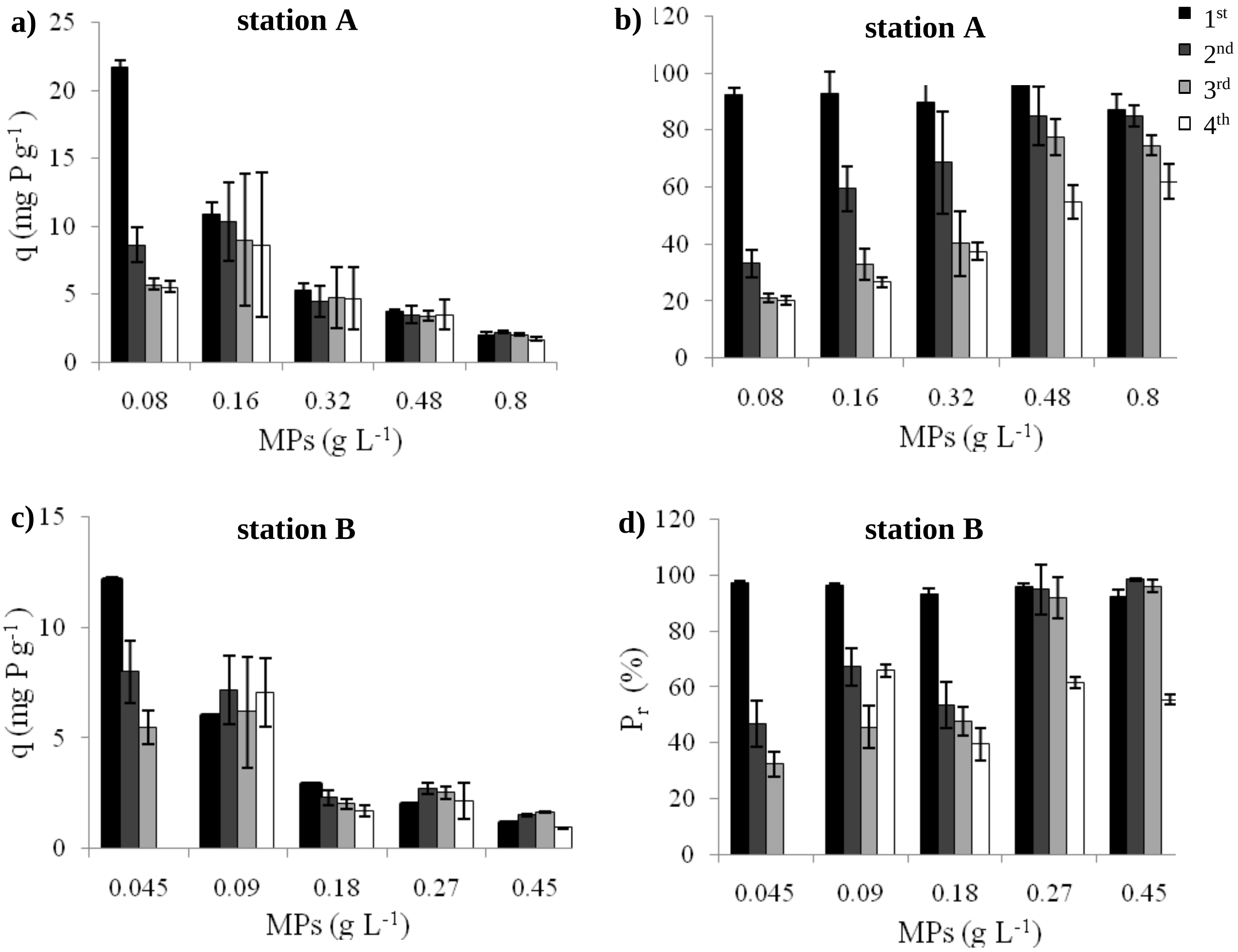

Figure 4. Equilibrium adsorption capacities (q) and phosphorus removal efficiency (P$_r$; %) for each MPs concentration in station A (a, b) and station B (c, d). Standard deviation is represented by vertical bars.

Table 4. Results of the t-test for independent variables; ns (no significant differences); * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.0005$).

			q (mg g^{-1})					**Pr (%)**				
	MPs (g l^{-1})		**0.08**	**0.16**	**0.32**	**0.48**	**0.8**	**0.08**	**0.16**	**0.32**	**0.48**	**0.8**
station A	1st	0.08		***	***	***	***		ns	ns	*	ns
		0.16			***	***	***			ns	ns	ns
		0.32				***	***				ns	ns
		0.48					***					**
	2nd	0.08		ns	***	***	***		**	**	***	***
		0.16			***	***	***			ns	**	**
		0.32				ns	**				ns	ns
		0.48					**					ns
	3rd	0.08		ns	ns	***	***		*	*	***	***
		0.16			*	*	*			-	***	***
		0.32				ns	*				***	**
		0.48					***					-
	4th	0.08		ns	ns	*	***		**	***	***	***
		0.16			ns	*	*			**	***	***
		0.32				ns	*				**	***
		0.48					*					ns

	MPs (g l^{-1})		**0.045**	**0.09**	**0.18**	**0.27**	**0.45**	**0.045**	**0.09**	**0.18**	**0.27**	**0.45**
station B	1st	0.045		***	***	***	***		ns	*	ns	*
		0.09			***	***	***			**	ns	**
		0.18				***	***				ns	ns
		0.27					***					*
	2nd	0.045		ns	***	***	***		*	ns	***	***
		0.09			***	***	***			*	***	***
		0.18				*	**				***	***
		0.27					***					ns
	3rd	0.045		ns	***	***	***		*	**	***	***
		0.09			*	**	*			ns	***	***
		0.18				*	*				***	***
		0.27					***					ns
	4th	0.045		***	**	***	*		***	***	***	***
		0.09			***	***	***			***	*	***
		0.18				ns	**				**	**
		0.27					*					*

Table 5. Results of the t-test for dependent variables; ns (no significant differences); * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.0005$).

			q (mg g^{-1})					Pr (%)			
	MPs (g^{-1})		1st	2nd	3rd	4th		1st	2nd	3rd	4th
station A	0.08	1st		**	***	***	1st		***	***	***
		2nd			*	**	2nd			*	**
		3rd				ns	3rd				ns
	0.16	1st		ns	ns	ns	1st		*	***	***
		2nd			ns	ns	2nd			***	***
		3rd				ns	3rd				ns
	0.32	1st		ns	ns	ns	1st		*	*	**
		2nd			ns	ns	2nd			*	ns
		3rd				ns	3rd				ns
	0.48	1st		ns	ns	ns	1st		*	**	***
		2nd			ns	ns	2nd			ns	**
		3rd				ns	3rd				**
	0.8	1st		ns	ns	ns	1st		ns	*	*
		2nd			*	*	2nd			*	*
		3rd				*	3rd				*
station B	0.045	1st		*	***	***	1st		**	***	***
		2nd			**	**	2nd			**	**
		3rd				**	3rd				**
	0.09	1st		ns	ns	ns	1st		**	**	***
		2nd			*	ns	2nd			**	ns
		3rd				*	3rd				*
	0.18	1st		*	***	***	1st		**	***	***
		2nd			**	**	2nd			ns	**
		3rd				**	3rd				**
	0.27	1st		ns	ns	ns	1st		*	ns	**
		2nd			ns	ns	2nd			ns	**
		3rd				ns	3rd				**
	0.45	1st		**	***	**	1st		*	*	***
		2nd			*	***	2nd			*	***
		3rd				***	3rd				***

Concerning changes in P removal efficiency along the different adsorption cycles, as expected, maximum values were reported for the 1st adsorption cycle for all treatments and for both water samples. More in detail, average value for P removal efficiency decreased from 93% (sample A) and 95% (sample B) during the 1st adsorption cycles to

35% (sample A) and 44% (sample B) during the 4th adsorption cycle. In general, our results evidence that in both water samples, station A and B, significant differences in P removal efficiency were recorded as a function of MPs concentration (Table 3) and as a function of adsorption cycle (Table 4). Even more, it is relevant to notice that for MPs concentrations ≥0.48 g l^{-1} for sample A and ≥0.27 g l^{-1} for sample B, which lastly correspond to a ratio ≥0.16 g MPs mg^{-1} P, MPs can be reused up to 4 times and they still exhibit a P removal efficiency higher than 50%.

When comparing our results with those reported in the literature, it is striking that q values found in the present study are in the range of previous studies carried out with the same MPs but using synthetic solutions (18.83 mg P g^{-1}; de Vicente et al., 2010) instead of wastewaters, which therefore confirm the suitability of using MPs for trapping P even in wastewaters. Considering other magnetic particles, contrasting results have been reported. While several authors have found much lower adsorption capacities and P removal efficiencies for magnetite than those measured in the present study (Xiao et al., 2017; Augusto et al., 2019), other authors have shown high P removal efficiency (>90%) when using zirconium ferrite and magnetic Fe oxide nanoparticles (7-10 nm) in treated wastewaters (Ishiwata et al., 2010; Lakshmanan et al., 2014).

Next, we compare our results with other P adsorbents frequently used for lake restoration. Recently, Kasprzyk et al. (2018) found a much lower adsorption capacity when using Phoslock© (a lanthanum-modified bentonite) for removing P from wastewaters. These authors also found that q values were, as expected, higher in synthetic wastewaters (4.31 mg P g^{-1}) than in real effluents from wastewater treatment plants (2.09 mg P g^{-1}). In relation to P removal efficiency, a variety of lanthanum-based adsorbents have shown efficiencies higher than 95% in experiments with wastewaters (Zhang and Tian, 2015; Zuo et al., 2016). For aluminum based adsorbents, a much higher q value has been found for $Al_2(SO_4)_3$ (23 mg P g^{-1}; Urano and Tachikawa, 1992) than for activated Al_2O_3 (7-10 mg P g^{-1}; Brattebø and Ødegaard, 1986) in synthetic wastewaters. Despite of other authors have obtained P removal efficiencies even higher than ours (Ashekuzaman and Jiang, 2013), it is essential to point out that these adsorbents have exhibited marked changes in their efficiencies as a function of pH (Brattebø and Ødegaard, 1986; Zhang et al., 2012; Li et al., 2014). Similarly, it is worth to remark that other new promising adsorbents such as zeolites have also shown a high P adsorption capacity in wastewaters (Kallo, 2001; Ping et al., 2008) although the maximum adsorption capacity was measured at a short pH range (4-6; Ping et al., 2008).

3.4. Identifying the optimum dose of magnetic particles: an economic approach

From a practical point of view, it is essential to identify the optimum dose of MPs to be added to treated wastewaters which will lastly depend on the initial DIP concentration (g MPs mg^{-1} P). To identify it, both advantages (P removal efficiency, %) and disadvantages (economic price, € l^{-1}) of using MPs have been considered as a function of MPs dose (Figure 5). In this approach, we have considered that MPs can be reused as many times as they can still reduce ≥50% of initial DIP concentrations. Accordingly, the final costs will the initial cost divided by the number of adsorption cycles that can be carried out assuring a final P removal ≥50%. A general pattern for both water samples (A and B) has been observed and the best conditions are likely to be obtained for ratios ≥0.16 g MPs mg^{-1} P. In other words, for those ratios, a maximum benefit (P removal efficiency) and a lowest economic cost are achieved as MPs can be reused up to 4 times.

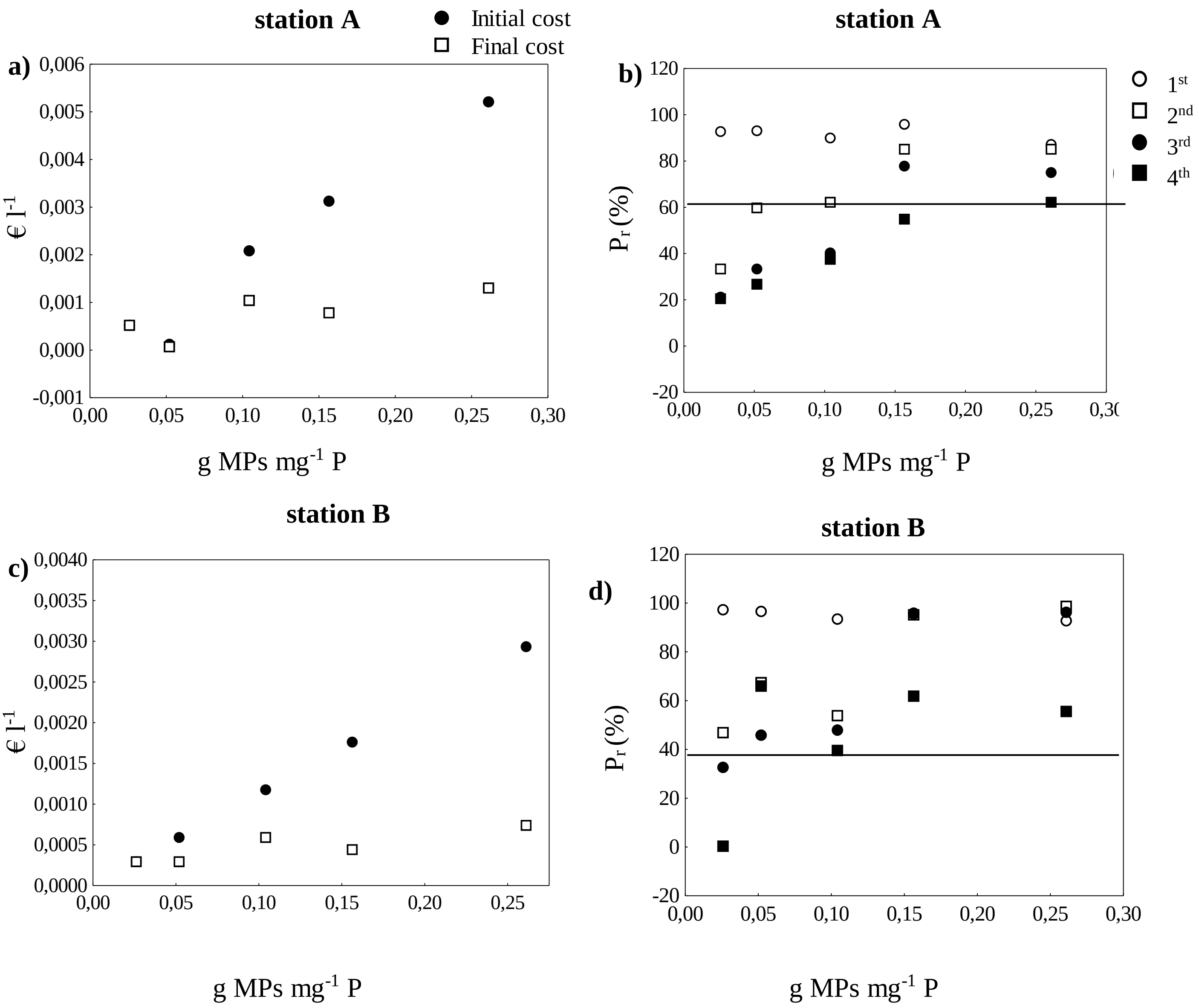

Figure 5. Cost (€ l^{-1}) and benefits (P_r; %) of MPs application in station A (a, b) and station B (c, d). Horizontal lines (in b and d) indicate a P removal efficiency corresponding to 50%.

As expected, given the chemical composition of wastewaters, this ratio (≥0.16 g MPs mg^{-1} P) is much higher (3 times) than the maximum P adsorption capacity estimated for the same adsorbent by previous studies (18.83 mg P g^{-1} MPs; de Vicente et al., 2010).

Finally, and despite of the inherent limitations of comparing our results with those obtained with other P sorbents that have been only used in eutrophicated waters but not in wastewaters, we compare the final cost of adding four different adsorbents (MPs, Phoslock®, $AlCl_6$ and an Fe oxide, CFH-12®) for trapping DIP in Laguneto pond. First, we have quantified the total mass of P-DIP present in lake water considering mean DIP concentration and morphometric features (de-los-Ríos-Mérida et al., 2017). Next, the final cost has been estimated based on the maximum P adsorption capacity (except for MPs, 0.16 g MPs mg^{-1} P) and the price of each product (Funes, 2016). The obtained results have shown the highest cost when using $AlCl_6$ (7526 €) while lowest cost has been found for CFH-12® (4020 €). Similar and intermediate costs have been estimated when adding Phoslock® and MPs (reused up to four times). Again, it is important to reflect the difficulties in making such comparisons as real P adsorption capacities in wastewaters are likely to be much lower than maximum P adsorption capacities estimated in laboratory conditions with artificial solutions.

4. Conclusions

Firstly, it is worth noting that when treated wastewaters pass along the Laguneto semi-natural pond, there is a notable improvement in water quality. This pond acts as a continuous green filter operating as a tertiary treatment of the outflow coming from the wastewaters treatment plant. Accordingly, this pond facilitates water self-purification as nutrients concentration in the treated wastewaters remains too high for the receiving waters. As a result, it appears that this semi-natural pond results a reliable countermeasure to reduce the impact of wastewater effluents in the Fuente de Piedra Ramsar site.

Secondly, our results evidenced the suitability of using MPs for removing P in treated wastewaters. This statement is based on both the high equilibrium adsorption capacity (q) and P removal efficiency of MPs. In this sense, it is relevant to consider that P removal efficiency decreases along the subsequent adsorption cycles and, as expected, maximum values were reported for the 1st adsorption cycle for all treatments and for both water samples. More in detail, average value for P removal efficiency decreased from 93% (sample A) and 95% (sample B) during the 1st adsorption cycles to 35% (sample A) and 44% (sample B) during the 4th adsorption cycle. Even more, it is relevant to notice that for ratios ≥0.16 g MPs mg^{-1} P, MPs can be reused up to 4 times and they still exhibit P removal efficiencies higher than 50%.

Finally, and considering that, from a practical point of view, it is essential to identify the optimum dose of MPs to be added to treated wastewaters, both advantages (P removal efficiency, %) and disadvantages (economic price, € l^{-1}) of using MPs have been considered as a function of MPs dose. We can conclude that the optimum ratio is ≥0.16 g MPs mg^{-1} P for achieving a high P removal efficiency (>50%). If we compare the optimum dose for MPs identified in the present study with that recommended by the manufacturer of Phoslock® (100:1), which is a typical P adsorbent used for lake restoration, we can conclude that both doses are in the same range. It finally confirms the convenience of using MPs for removing P from treated wastewaters as they can be recovered from the aqueous solutions and later be reused up to 4 times which lastly reduces the economic cost.

Acknowledgements

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Chapter VI: Phosphorus recovery and reuse from eutrophicated aquatic ecosystems

This article is in preparation

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Abstract

One of the most important worldwide challenge that we are facing at present is the depletion of Phosphorus (P) mineral reserves. Magnetic microparticles (MPs) have been recently proposed for trapping P in natural eutrophicated ecosystems as well as in wastewaters. One of main advantages of using MPs is that both P and MPs can be recovered from the treated water. Then, our working hypothesis is that P can be desorbed from P loaded MPs and recovered P can be later used as a fertilizer. To test this hypothesis, first, the best working conditions for desorbing P were identified and secondly, an experiment with different plant nutritive solutions (e.g. commercial fertilizer and recovered P) was carried out with three different species: *Ocimum basilicum*, *Cucumis sativus* and *Cucumis melo*. Finally, germination, height and biomass were compared among treatments. Firstly, our results evidenced that the best conditions for P desorption from P loaded MPs occurred when using 0.1 M NH_4OH and using H_3PO_4 for neutralizing pH. Fertirrigation experiments showed that, in general, heights and growth rates were higher in treatments than in control. It is worth to note that for *O. basilicum* and *C. sativus*, similar height values were recorded when using the commercial P and when using recovered P amended with micro and macronutrients.

O. basilicum germination was much lower than the other two species tested probably due to the different nutritional requirements of the different species. On the other hand, significant differences were obtained at the end of the experiment due to treatment for *C. sativus* and *C. melo* although there were not significant differences for *O. basilicum*.

According to the balanced growth hypothesis, it seems as any species suffered nutrient deficiency since the highest biomass was obtained for the shoot part of the plant. Finally, all the total P concentrations recorded were similar for all the species and parts of the plants.

1. Introduction

Since 2009, planetary boundaries is a concept involving Earth system processes which contain environmental boundaries (Rockström et al., 2009). Rockstrom et al. (2009) and Steffen et al. (2015) selected control variables for nine critical earth system processes and assigned environmentally safe planetary boundaries to these variables (Conijn et al., 2018).

Actually, one of the planetary boundaries is the alteration of biogeochemical phosphorus (P) cycle. It is accepted that transgressing one or more planetary boundaries may be highly damaging or even catastrophic, due to the risk of crossing thresholds that trigger non-linear, abrupt environmental change within continental- to planetary-scale systems. In this sense, current value for anthropogenic P going into the oceans (millions of tonnes per year) is 8.5-9.5 while the boundary value is 11 (Rockström et al., 2009). Accordingly, much more efforts must be done for not crossing that value. A recent proposal for avoiding a drastic deterioration in a reference scenario of 2050 was focused on reduction of waste and reduction of P losses from agricultural land, among others (Conijn et al., 2018).

P biogeochemical cycle is being dramatically altered in two ways. By one hand, modern human society depending on a large, continuous supply of mined P to sustain the global food supply (Schroder et al., 2011) is responsible for the global exhaustion of P reserves (Cordell et al., 2011; Gilbert, 2009). Phosphorus has no substitute in food production and in a world of 9 billion people by 2050, securing sufficient P will be critical for future food security (Cordell et al., 2011). Actually, Cordell et al. (2009) estimated that peak P will occur by 2035, after which demand would outstrip supply. However, there are some reasons for being optimistic as the current food production and consumption system is highly inefficient with respect to P use and accordingly, some action for reverting this tendency can be still done. As Cordell et al. (2011) stated there is no single solution for achieving a P-secure future. Among others, it is necessary more investments for increasing P use efficiency and for recovering and reusing P from all current waste streams throughout the food production and consumption system.

By the other hand, mobilized P is moved to the hydrosphere causing the eutrophication of aquatic ecosystems, which actually is the primary worldwide water quality issue (OECD, 1982; Sas, 1989; Cooke et al., 2005; Hupfer and Hilt, 2008). Eutrophication promotes negative effects both in the ecological and economical dimension (i.e. annual costs just in the U.S. of approximately $2.2 billion; Dodds et al. 2009). As a result of eutrophication, the unbalanced ecosystem and changed chemical composition make the water body unsuitable for recreational and other uses, and the water becomes unacceptable for human consumption.

For combating both coupled problems (exhaustion of global P reserves and eutrophication), new technologies for capturing P from enriched P systems (i.e. wastewaters and eutrophicated ecosystems) and reusing it as a fertilizer are imperative. Similarly, de-Bashan and Bashan (2004) pointed out that the contemporary focal issue is not eliminating P *per se* but recycling it. In fact, P recycling is converting an acute disposable problem into a raw material (for making fertilizer) that will benefit industry and society.

At this point, it is important to consider that P removal from P enriched waters and its later reuse, would perfectly fit in the framework of the recent concept of bioeconomy. The European Commission defines the bioeconomy as "*the production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and bioenergy. Its sectors and industries have strong innovation potential due to their use of a wide range of sciences, enabling and industrial technologies, along with local and tacit knowledge*" (EC, 2012).

In this context, although recent studies are actually focused on assessing P removal by using novel adsorbents from eutrophicated ecosystems (e.g. Phoslock®, aluminum) and wastewaters [e.g. zeolites (Ping et al., 2008) and struvite (de-Bashan and Bashan, 2004)], we are still far for recognizing the best P adsorbent. Accordingly, it is necessary to continue developing new techniques in order to achieve an environmentally acceptable level with affordable costs.

Therefore, in this study, we propose the use of magnetic particles (MPs) as they have several outstanding advantages. Firstly, MPs adsorb P from aqueous effluents and after that, P loaded MPs are recovered from the medium by applying a simple high gradient magnetic separation process (de Vicente et al., 2010; de Vicente et al., 2011; Merino-Martos et al., 2011). Our working hypothesis is that P can be desorbed from P loaded MPs and recovered P can be later used as a fertilizer. To test this hypothesis, first, the best working conditions for desorbing P are identified and secondly, an experiment with different plant nutritive solutions (e.g. commercial fertilizer and recovered P) was carried out with three different species: *Ocimum basilicum*, *Cucumis sativus* and *Cucumis melo*. Finally, germination, height and biomass were compared among treatments.

2. Material and methods

2.1. Phosphorus loaded magnetic particles

P loaded MPs were obtained from a previous experiment (Funes et al., 2017). Briefly, on July 2015, surface sediment and lake water were collected in Honda lake which is a shallow (surface area=9 ha, Z_{mean}=1.3m; Z_{max}=3.2 m), hypertrophic, and brackish water (6000 µS cm^{-1}) wetland on the coast of Almeria in Southeast Spain (de Vicente et al., 2003; Funes et al., 2016). Once in the laboratory, lake water and sediment were distributed in 15 PVC microcosms (Ø=38cm; h=58 cm) containing 6600 cm^3 of surface sediment and 40 L of lake water. Three different treatments were considered and to two of them, MPs were added to get a final concentration of 1.4 g MPs l^{-1}. After a contact time of 24 h (day 1), P loaded MPs were recovered by fully immersing a magnetic rake in the microcosms down to the sediment surface.

MPs (97.5 % Fe, 0.9 % C, 0.5 % O and 0.9 % N) used as P adsorbents (HQ grade, BASF, Germany) in the microcosm experiment were micronsized (800 nm) soft magnetic spherical particles with negligible coercive field and remanent magnetization (i.e., zero magnetization upon removal of themagnetic field). Further details on these particles have been reported by de Vicente et al. (2010) and Merino-Martos et al. (2011).

Once P loaded MPs were recovered from microcosms, they were washed and sonicated (1 min) three times with distilled water. After each wash, P loaded MPs were recovered by applying a magnetic field gradient exerted by a permanent magnet (volume ¼ 25.6 cm^3; NB032, Aiman GZ, Spain). Finally, after the last wash, the supernatant was removed and P loaded MPs were dried for 24 h at 60ºC.

2.2. *Optimizing phosphorus desorption*

P loaded MPs were washed by using three different basic solutions (NaOH, KOH and NH_4OH) at four different concentrations (0.1; 0.5; 1 and 3 M). The methodological procedure was based on shaking in a rotating shaker (at 20 rpm for 24 h), 1 g of dried P loaded MPs with 50 ml of each solution in centrifuge tubes. Later, suspensions were centrifuged at 4000 rpm for 5 minutes (Hettich® ROTOFIX 32A centrifuge). After their centrifugation, the pellets were used for the subsequent wash while the pH of the supernatants was neutralized with two different acids: HCl and H_3PO_4. This extraction procedure was repeat up to four times, with each concentration of every basic solution, for assuring the complete P desorption. Finally, the chemical composition of all supernatants was determined by using ICP-MS (Al, B, Ca, Cu, Co, Fe, K, Mg, Mn, Mo, Na, S and Zn), ion chromatography (F^-, Cl^-, Br^-, NO_3^-, PO_4^{3-} and SO_4^{2-}) and an elemental analyzer (C, N and H).

2.3. Preparation of the plant nutritive solutions

A total of four plant nutritive solutions (Treatments) were considered (Table 1).

Table 1. Treatments applied to each species. T1: Comercial fertilizer; T2: Recovered P; T3: Recovered P and macronutrients; T4: Reovered P and macro and micronutrients. (In the case of Control only irrigation water was added, no nutrients added)

		T1	**T2**	**T3**	**T4**
Microelements (mg L^{-1})	Fe^{2+}	0.84	-	-	0.84
	Mn^{2+}	0.55	-	-	0.55
	Zn^{2+}	0.33	-	-	0.33
	B^{3-}	0.35	-	-	0.35
	Cu^{2+}	0.05	-	-	0.05
	Mo^{2+}	0.05	-	-	0.05
Macroelements (mg L^{-1})	NH_4^+	21.25	-	21.25	21.25
	K^+	273	-	273	273
	Ca^{2+}	180	-	180	180
	Mg^{2+}	34	-	34	34
	NO_3^-	992	-	992	992
	SO_4^{2-}	134	-	134	134
	$H_2PO_4^-$	122.5	-	-	-
Recovered P (mg L^{-1})		-	38.7	38.7	38.7

One of them was prepared by reproducing the chemical composition of a typical commercial fertilizer (T1) while the others (T2; T3 and T4) were prepared by using P desorbed from P loaded MPs (extracted by using 0.1 M NH_4OH and by neutralizing with H_3PO_4). Considering both the concentration in the stock nutritive solution obtained by merging the four subsequent washes when using 0.1 M NH_4OH and neutralized with H_3PO_4 and nutrient concentrations used in previous studies (Stradiot, 2002; Table 2), T2, T3 and T4 supernatants were diluted up to 68 times with irrigation water (tap water; Table 3). While T2 only contains P desorbed from P loaded MPs; T3 was enriched in macroelements and T4 was amended with both micro and macroelements.

Table 2. Chemical composition of the suitable nutritive solution (T1) (Stradiot, 2002; Casas, 2005).

	Macroelements (mmol L^{-1})							**Microelements (mg L^{-1})**					
EC	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	NO_3^-	SO_4^{2-}	$H_2PO_4^-$	Fe^{2+}	Mn^{2+}	Zn^{2+}	B^{3-}	Cu^{2+}	Mo^{2+}
1.2-2.0	1.25	7.00	4.50	1.4	16.0	1.4	1.25	0.84	0.55	0.33	0.35	0.05	0.05

Table 3. Concentration of macro and micronutrients in the fertilizer, the concentration of them diluted 68 times and the required concentration (Stradiot, 2002; Casas, 2005).

	Chemical elements	**Concentration (mg L^{-1})**	**Diluted concentration (/68)**	**Required concentration**
Microelements	Na	0.472	0.01	
	Al	0.109	0.00	
	Fe	1.863	0.03	0.84
	Mn	0.026	0.00	0.55
	Zn	0.087	0.00	0.33
	B	0.407	0.01	0.35
	Cu	0.070	0.00	0.05
	Mo	0.163	0.00	0.05
Macroelements	K	1.667	0.02	273
	Ca	3.286	0.05	180
	Mg	0.429	0.01	34
	NH_4^+			22.5
	NO_3^-			992
	P	2633	38.7	38.7
	S	13.350	0.20	44.8

All reagents were of analytical grade and used without further purification. Macroelements were added by using the next reagents: NH_3; KNO_3; $Ca(NO_3)_2$; $MgSO_4 \cdot 7H_2O$ and H_3PO_4. Similarly, microelements were added by using the next ones: $Fe(NO_3)_3$; $Mn(NO_3)_2$; $Zn(NO_3)_2$; $CuSO_4$; $MoO_4Na_2 \cdot 2H_2O$ and H_3BO_3.

2.4. Fertirrigation experiments

Three different species were considered: *Ocimum basilicum, Cucumis sativus* and *Cucumis melo.* These species were selected based on their rapid growth and on their high values both as aromatic medicinal herb (*O. basilicum*, Chiang et al., 2005; Politeo and Milos, 2007) and as food (*C. sativus* and *C. melo,* Whitaker and Davis, 1962; McCreight et al., 1993; Lester, 1997; Huang et al., 2009).

A total of 330 horticultural nurseries, 110 for each species, were used. The seeds (three of each species), belonging to the same batch, were placed at 2 cm depth in a nutrient poor composite substrate of black peat (15%), yellow peat (70%) and pearlite (15%). Each plant species was exposed to four treatments (already mentioned in section 1.3). A control with no addition of any nutrient was also considered. All treatments were run in 22 replicates. The experiment was run in a canopy area located in the Andalusian Institute of Agricultural Research and Training (IFAPA).

The experiment lasted for 31 days. Fertirrigation was done four times per day during 30 min. Since plantation, germination was daily recorded and when it was higher than 70%, fertirrigation with plant nutritive solutions started. It consisted on adding nutritive solutions (25 ml) by foliar and substrate application to each seedling twice a week. For the case of the control, irrigation water was used. After germination, plant height was recorded three days a week. Finally, at the end of the experiment, seedlings were collected for measuring root and leaf biomass and for quantifying P content.

More in detail, roots and leaves were washed with distilled water and dried at 70ºC for 48 h. Then, plant biomass was determined by using a weighting scale (Precisa Gravimetrics AG 360 ES). Additionally, P content in roots and leaves was also quantified. To get it, ten replicates of each treatment and species were combusted at 450ºC for 1 h and digested in 5 ml of HCl, following a slight modification of the method proposed by Flindt and Lillebo, (2005). After samples digestion, P concentration was measured by spectrophotometry (Murphy and Riley, 1962).

2.5. Statistical analysis

All the statistical analyses were performed by using Statistica 7.1 software (Stat Soft Inc., Tulsa, USA). Differences in P concentration among different basic solutions (treatments) and adsorption cycles (time) were tested by using non-parametric variance analysis because these data did not satisfied homocedasticity assumptions (Levene test,

$p < 0.05$). For this reason, Kruskal-Wallis was carried out to test the differences among treatments and a Friedman ANOVA by ranks was performed to test the significant differences among adsorption cycles. The same method was carried out to test if there exist significant differences in plant height among different treatments and along the experiment (time) for each of the three plants species, as these data did not either satisfied homocedasticity assumptions (Levene test, $p < 0.05$). Then, significant differences in growth rates among treatments and over time were also tested by using Repeated Measures ANOVA (RM-ANOVA) with Fisher's least significance difference (LSD) post-hoc test. For testing if there exist significant differences in shoot and root biomass and the rate among these variables, Kruskal-Wallis was used for testing differences among treatments as, again, these data did not satisfied homocedasticity assumptions (Levene test, $p < 0.05$). Finally, in order to test the differences in TP concentration in shoots and roots among treatments, one-way ANOVA was performed.

3. Results and discussion

3.1. *Optimizing phosphorus desorption and chemical characterization of the supernatants*

The first step was to identify the best working conditions for desorbing P from P loaded MPs. Figure 1 shows P concentration for the different concentrations (0.1; 0.5; 1 and 3 M) of every basic solution (NaOH; KOH and NH_4OH) during the four subsequent extractions (washes).

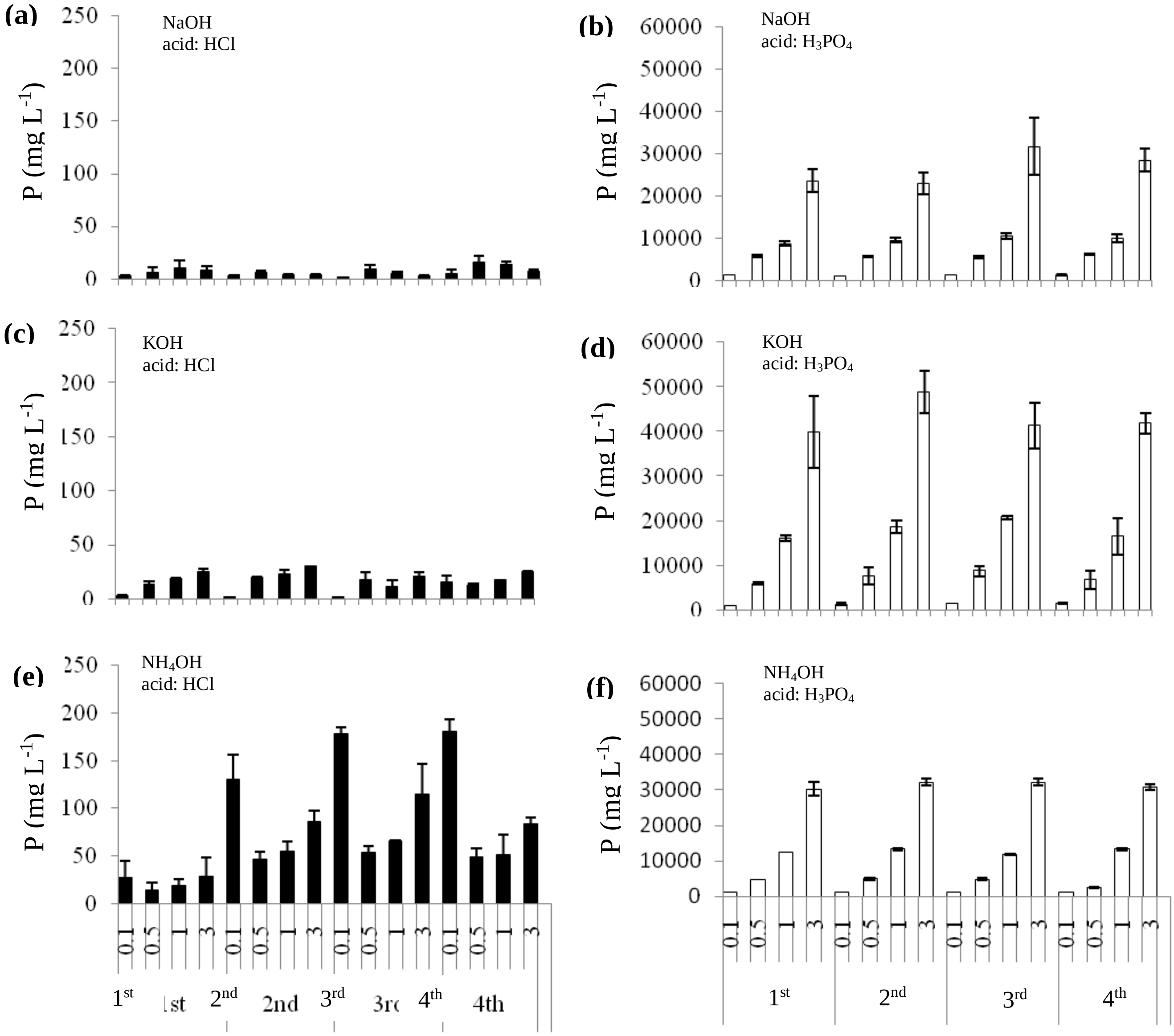

Figure 1. P concentration measured in the supernatants at the four different subsequent extractions (1st, 2nd; 3rd and 4th) by using four concentrations (0.1; 0.5; 1 and 3 M) of three basic solutions: NaOH; KOH and NH_4OH; neutralized by using HCl (left) and H_3PO_4 (right). Please note the different vertical scale used for figures at the right and at the left sides.

As expected, much higher P concentrations were recorded when using H_3PO_4 instead of HCl for samples neutralization. Actually, P concentrations clearly increased with H_3PO_4

molarity. Even more, and considering that Cl^- could cause lesions on the plants according to Terrón (1992), solutions neutralized by using HCl were excluded. Additionally, it is also well-known that high Na^+ concentrations could have negative effects on plants (Phocaides, 2007; Terrón, 1992), and therefore NaOH as a basic solution was also excluded for the experiment. At this point, and based on P concentrations desorbed when using KOH and NH_4OH, and neutralized with H_3PO_4, both solutions could have been selected. However, if we consider that nutrient concentration in standard solutions are used to be higher for N than for K (Stradiot, 2002; Casas, 2005), it is clear that the perfect basic solution is NH_4OH. It is also worth to note that when comparing P concentrations among different NH_4OH concentration, there exists a clear increasing tendency with basic solution concentration. This result would conduct to select 3 M NH_4OH. However, taking into account P concentrations used by previous studies in similar fertilization experiment (38.7 mg L^{-1}; Stradiot, 2002; Casas, 2005), P concentration in all supernatants is overpassed even when using the lowest molarity. Accordingly, and considering that using a high concentration is not cost effective in long term, the best working condition for desorbing P is when using 0.1 M NH_4OH neutralized with H_3PO_4.

More in detail, Table 2 shows the chemical composition of the merged solution containing the four subsequent washes, of the 0.1 M NH_4OH. neutralized with H_3PO_4. Among all elements, we may remark that P concentration (2633 mg L^{-1}) was much higher than P concentration used in similar fertilization experiments (Stradiot, 2002; Casas, 2005), and accordingly, the final nutritive solution used for the fertirrigation experiment, was diluted up to 68 times in order to optimize the solution and to do not add more P than it is necessary for the plants.

3.2. Temporal patterns in the germination

On day 11, 70% of seeds had germinated and therefore, fertirrigation started (Figure 2).

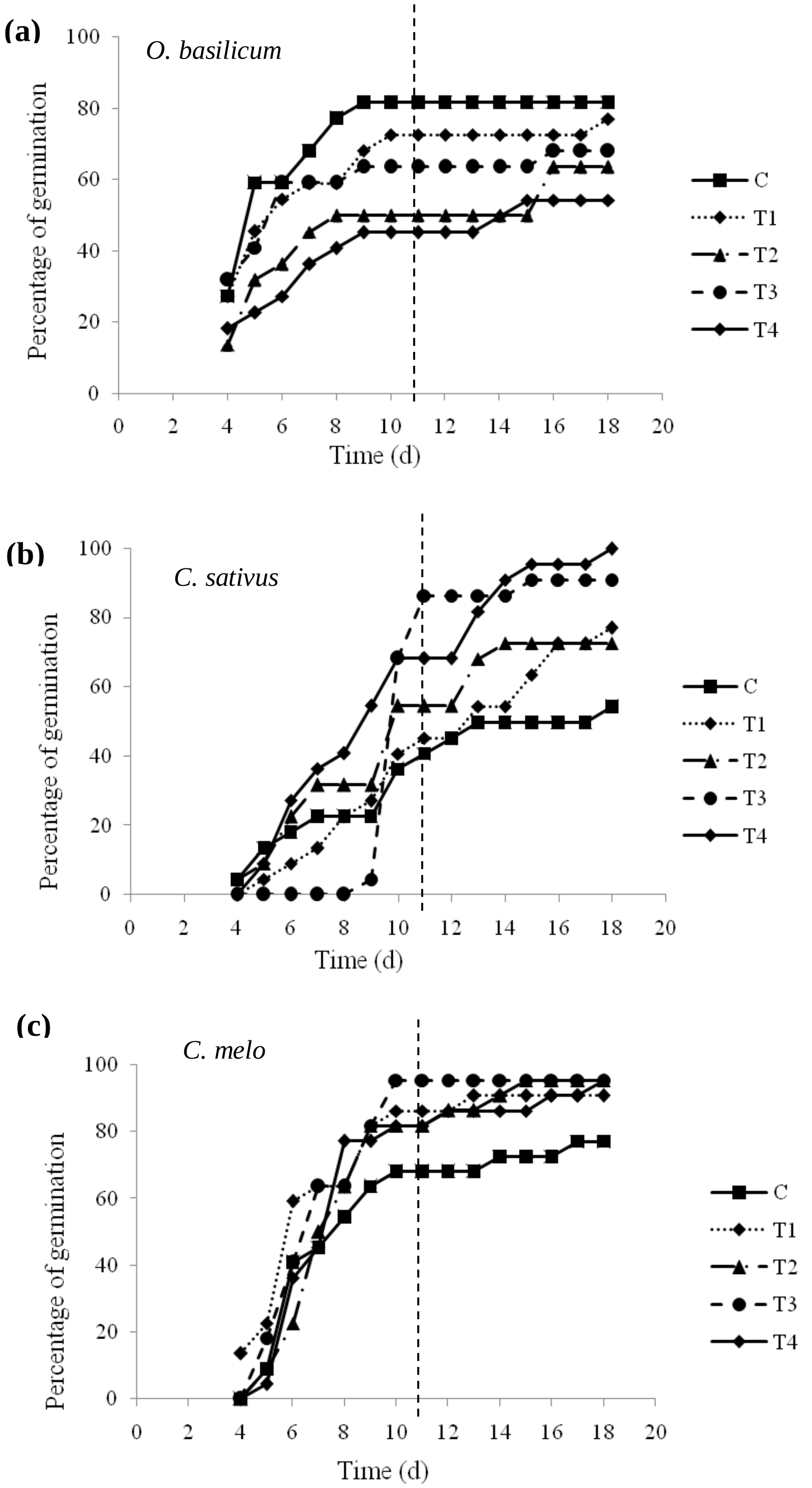

Figure 2. Percentage of germination recorded along the experiment. Dotted vertical line shows the time when fertirrigation started.

Marked differences in the germination patterns were found among the three plants species. The first species in germinating was *O. bassilicum* but it later experienced a slower tendency and at the end of the experiment the highest percentage of germination was 80%, which corresponded to the control. The germination of the other two species, *C. sativus* and *C. melo*, started later (from day 4) and it generally reached values up to 100% at the end of the experiment. Additionally, germination in the different treatments experienced notable differences among plants species. More in detail, while in *O. bassilicum,* the germination in the Control was also higher than in the other treatments for any time of the experiment; for *C. sativus* and *C. melo*, the percentage of germination in all treatments was higher than in Control, evidencing the key role of adding nutrients for the germination of these species. One likely explanation may be the different nutritional requirements among the study species. Our experiment was designed based on the nutritional requirements of *C. sativus* (Stradiot, 2002; Casas, 2005). For the case of *C. melo*, Alarcón and Egea (1999) used P concentrations similar to that used in this experiment pointed out. However, the composition of the nutritional solution notably differed from that recommended by Moncayo-Luján; (2015) to *O. basilicum*. As a result, it is clear that plant requirements drastically depend on plant species but also on the age (Haynes, 1986; Yamada 2003).

An additional reason behind such differences in plant germination is a low quality of *the O. basilicum* seeds compared to the other two species, since germination depends on homogeneity of seeds lots, genetic purity, longevity or quality among others characteristics (San Martín, 2002).

3.3. Changes in the height of the three species throughout the experiment

For all plant species, there were significant differences over time ($p<0.0005$) reaching, as expected, the maximum height on day 31 (Figure 3).

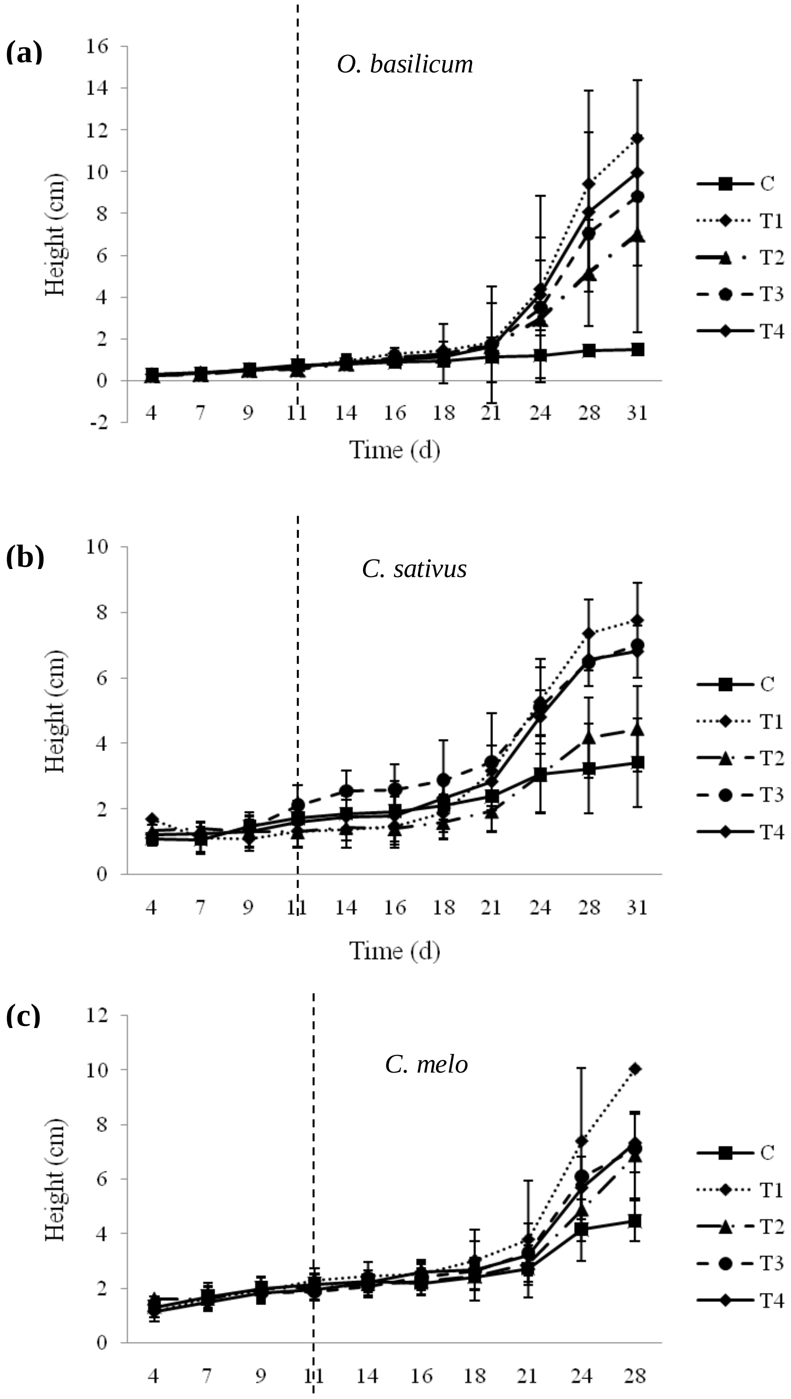

Figure 3. Temporal changes in plant height along the experiment. Dotted vertical line shows the time when fertirrigation started.

Regarding the results achieved for *O. basilicum*, significant differences between control and treatments were obtained from day 18 in advance ($p<0.05$) and the highest height was reached for T1, although between T1 and T3, T2 and T3 and between T3 and T4 significant differences were not found. So, *O. basilicum* grew interchangeably when using the commercial nutrient solution or the recovered P enriched with macronutrients as well as the addition of macro and micronutrients did not significantly increase the growth of *O. basilicum*. These results contrast with several authors that observed that P is fundamental for growth and development of seedlings of *O. basilicum* (Ichimura et al., 1995; Borges et al., 2016) and with Prakasa et al. (2007) whom observed that N increased the herbs and the essential oil in *O. basilicum*.

For the case of *C. sativus*, significant differences were obtained from day 16 in advance among all the treatments ($p<0.05$) except between control and T2. Control had the lowest height on days 28 and 31, while previously, the lowest height was recorded for T2. So, it seems like P is not crucial in the growth of *C. sativus* until the end of the experiment while Ciereszko et al. (2002) noted a decrease in the growth of *C. sativus* in medium with a low concentration of P.

The height of *C. melo* significantly differed among treatments ($p<0.0005$) from day 16 although between T2 and T3 and between T3 and T4, significant differences were only obtained in one sampling day (day 18 and day 24 respectively).

It is important to point out that *C. sativus* and *C. melo* are fruit plants and they were harvesting much time before fruit growing. For other species with fruits such as *Cucurbita pepo* it was observed that the fruit had the highest nutritional demands (N, P and K; Segura-Pérez and Fernández-Fernández, 2016).

The use of the struvite as a fertilizer, it has been proposed as a promising method to recover nutrients from wastewater. Several authors have shown similar effects by using struvite as a fertilizer in comparison with commercial fertilizers (Johnston et al., 2003; Plaza et al., 2007; Kataki et al., 2016). Even more, Barak and Stafford (2006) obtained better fertilization results for struvite than for ammonium phosphate.

The nutritional solutions were added to every seedling following a fertirrigation method by adding the fertilizer both on the plant leaves and on the substrate. This kind of fertilization enhances the development and production of crops (Trinidad-Santos and Aguilar-Manjarrez, 1999). Also, although leaves are not specialized in nutrients absorption, Franke (1986) showed that nutrients in solution can be absorbed in certain specific areas of the leaves.

Moreover, the substrate used to carry out the experiment was nutrient poor with the aim to assess the fertilizers effects on the plants. On the other hand, both black and white peat have a high cation exchange capacity (CEC) which have as a consequence the retention or storage of the cationic nutrients (Alarcón and Egea, 1999). This characteristic of the substrate type coupled with the fertirrigation had as a consequence the growth of the three plants species.

The results obtained in relation with the low height in the control treatment in all the species are in agreement with several authors that pointed out the high importance of the P in the growth of the plants and for photosynthesis, respiration or cellular division (Flindt and Lillebø, 2005; Rajasekar et al., 2017).

3.4. Temporal evolution of the growth rate

Growth rates increased with experimental time, reaching the highest values around day 28 (Figure 4).

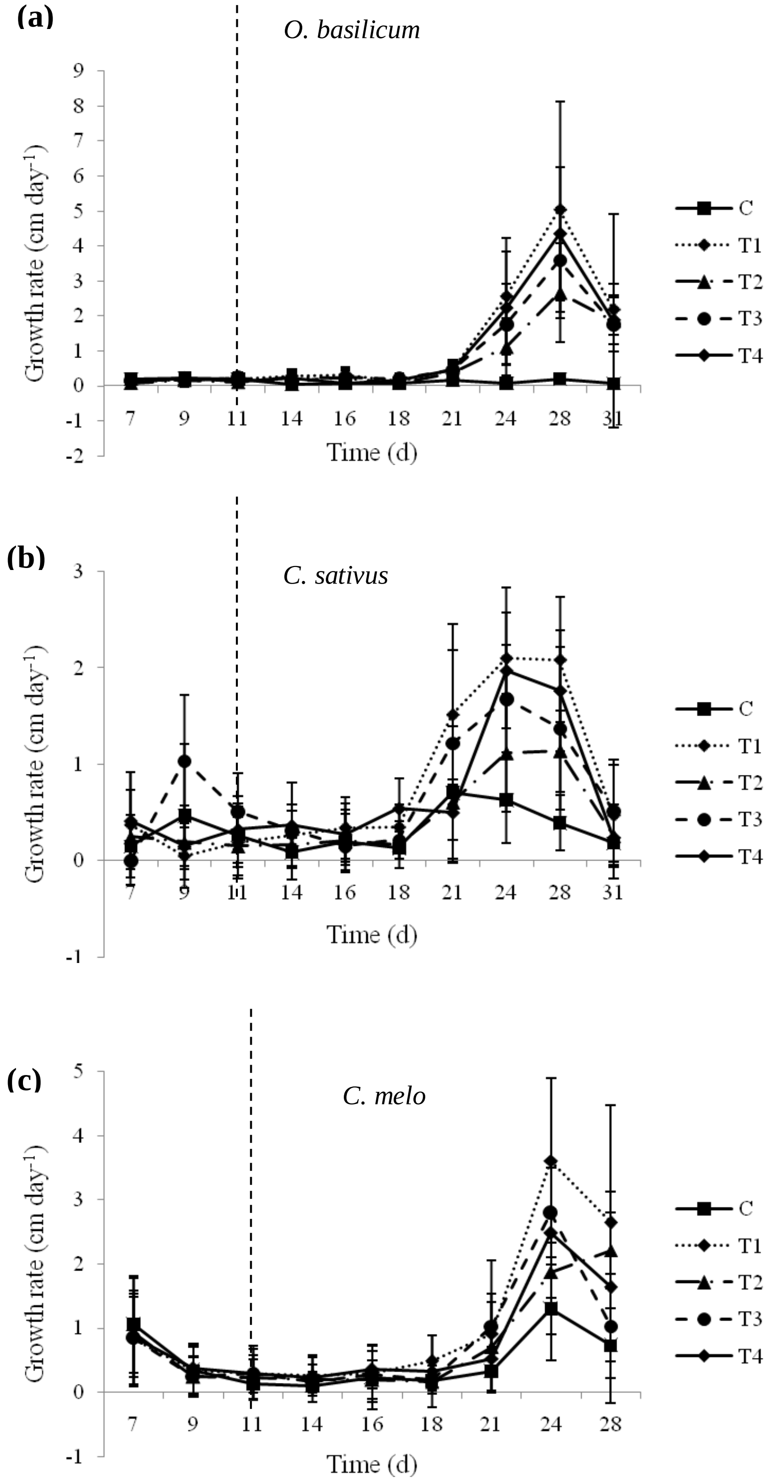

Figure 4. Temporal changes in growth rates along the experiment. Dotted vertical line shows the time when fertirrigation started.

Significant differences due to the treatment ($p<0.0005$) were obtained only from day 24 for *O. basilicum* and from day 21 for *C. sativus* and *C. melo* when the highest growth rates were obtained. These results are in contradiction with these obtained by Colorado et al. (2013) for *O. basilicum* and Sánchez et al. (1998) for *C. melo* whom observed the highest growth rates at the beginning of their experiments. At the end of the experiment, it is observable that the growth rates decreased for all the treatments and species and significant differences were obtained over time ($p<0.005$).

3.5. Shoot and root biomass at the end of the experiment

Comparing shoot and root biomass, Figure 5 shows that higher values were measured for shoots. Also, in the three species used control had the lowest value for both shoot and root biomass except for the root biomass of *C. melo*. However, in any case there exist significant differences between control and T2.

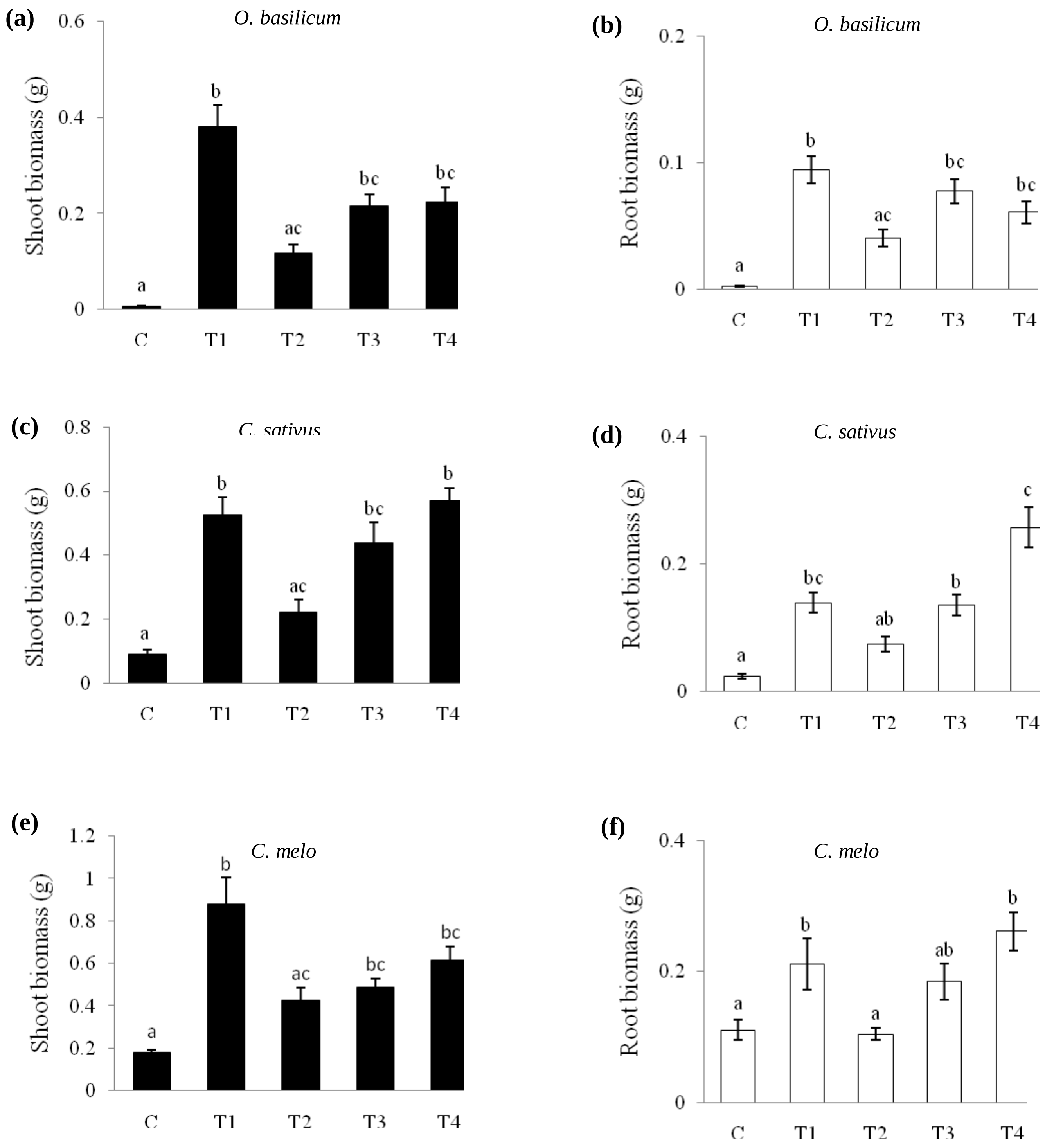

Figure 5. Shoot (left) and root (right) biomass at the end of the experiment. Significant differences ($p < 0.05$) between treatments are indicated by letters.

For *O. basilicum* the highest biomass values were recorded for T1 in both shoot and root although there were not significant differences between the biomass obtained in T1 and T3 and T4 in any case. The very low values obtained for both shoot and root biomass are in agreement with the results obtained by Borges et al. (2016) who observed a limitation in shoot and root dry weight in *O. basilicum* seedlings with lack of P. Also, Souza et al. (2007) observed a lower shoot biomass in *Mentha piperita* in plants with low doses of P.

Regarding *C. sativus*, the highest biomass was found for T4 which did not have significant differences with T1 in neither shoot nor root. Moreover, although in shoot and root biomass there were not significant differences between T1 and T3, in root biomass significant differences were found between T3 and T4 (p=0.046).

In the case of *C. melo* (Fig. 5c) the results were different between shoot and root biomass, while for shoot biomass the highest value was for T1, for root biomass that occurred in T4. Moreover, significant differences were not found between T1 and T3 nor T1 and T4 neither for shoot nor root biomass. However, Navarro et al. (1999) observed that *C. melo* seedlings suffered a significant effect in its shoot weight due to P concentration. Moreover, Fita et al. (2011) observed that P scarcity had consequently a high decrease in the biomass root of *C. melo* after 40 days. However, our results did not show this trend since control and T2 root biomass had similar response, so the lack of micronutrients may be a higher effect on root biomass than P lack in a shorter time.

Moreover, our results are in contradiction with the hypothesis of balanced growth that points that plants in situations with low nutrient availability assigned more biomass to roots with the aim to increase their uptake capacity (Müller et al., 2000; Shipley and Meziane, 2002). Also, these authors observed that as the plants grew up, the biomass assigned to roots was higher in an experiment with herbaceous plants. On the other hand, Shipley and Meziane, (2002) observed that herbaceous species than grow up in optimal conditions assigned more biomass to shoot than root.

3.6. Shoot and root biomass ratio of the three species treated

(a) oot biomass was higher tha *O. basilicum* , the ratio between shoot and root biomass was always higher than

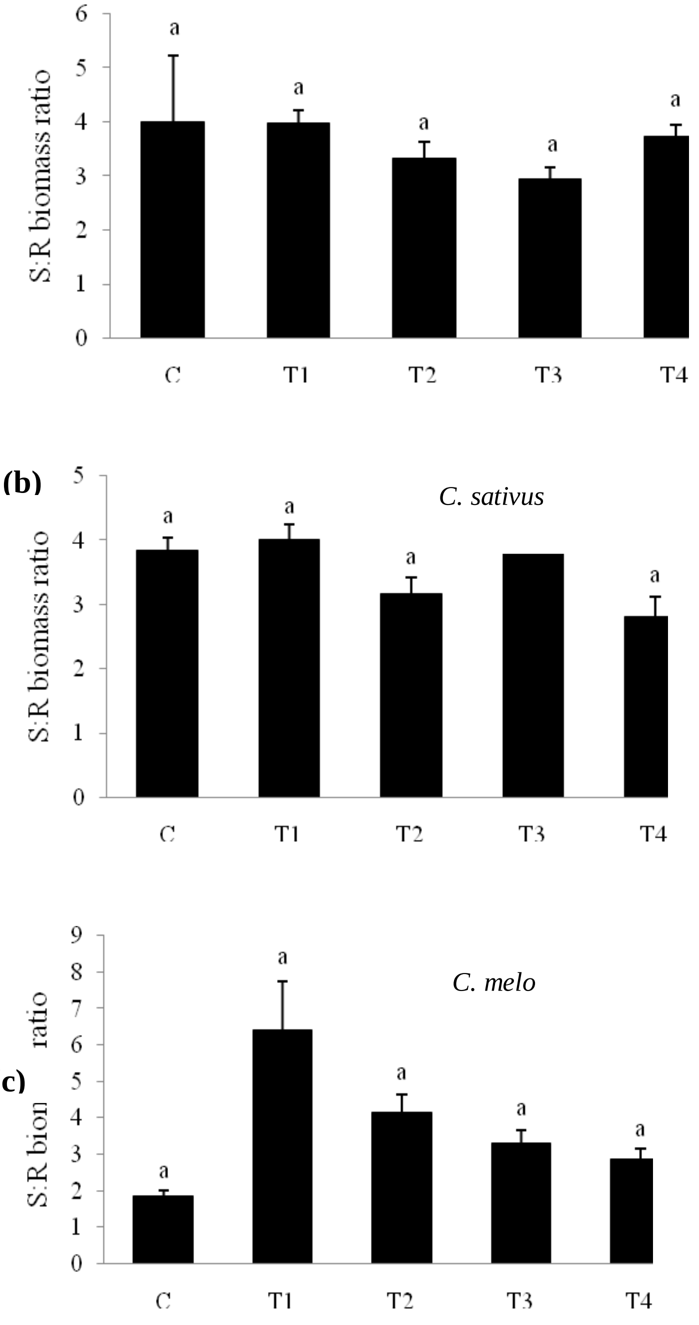

Figure 6. Shoot and root biomass ratio (S: R ratio) at the end of the experiment. Significant differences ($p < 0.05$) between treatments are indicated by letters.

Contrarily to our results where no Ciereszko et al. (2002) observed a decrease in the S:R ratio of plants of *C. sativus* in medium with scarcity of P, something that we did not observe in our experiment since control did not have the lowest S:R ratio for *C. sativus*.

The results obtained for *O. basilicum* and *C. sativus* are opposite with Ericsson, (1995) who observed a decreased in the S:R ratio in *Betula pendula* under P-limiting growth conditions. In the case of *C. melo* the lowest S:R ratio was obtained for control, the only treatment with scarcity of P, since P seems to be essential for production of new structures that has a consequence the decrease of S:R ratio (Ericsson, 1995). Bolinder et al. (1997) observed in a experiment with cereal species that the S:R ratio in experiments carried out under the same conditions, are affected because of the physiological differences between cultivars.

On the other hand, the S:R ratio of T2 was not notably lower in any species in spite of their lack in K, Mg and Mn, in contradiction with Ericsson (1995) who observed that a low concentration of these elements had as a consequence a decrease in the S:R ratio in *Betula pendula*.

3.7. Phosphorus concentration in shoot and root of the species

Regarding the concentrations of TP obtained in the three species in shoot and root (Figure 7), the values were in the same order of magnitude for all the species and part of the plants.

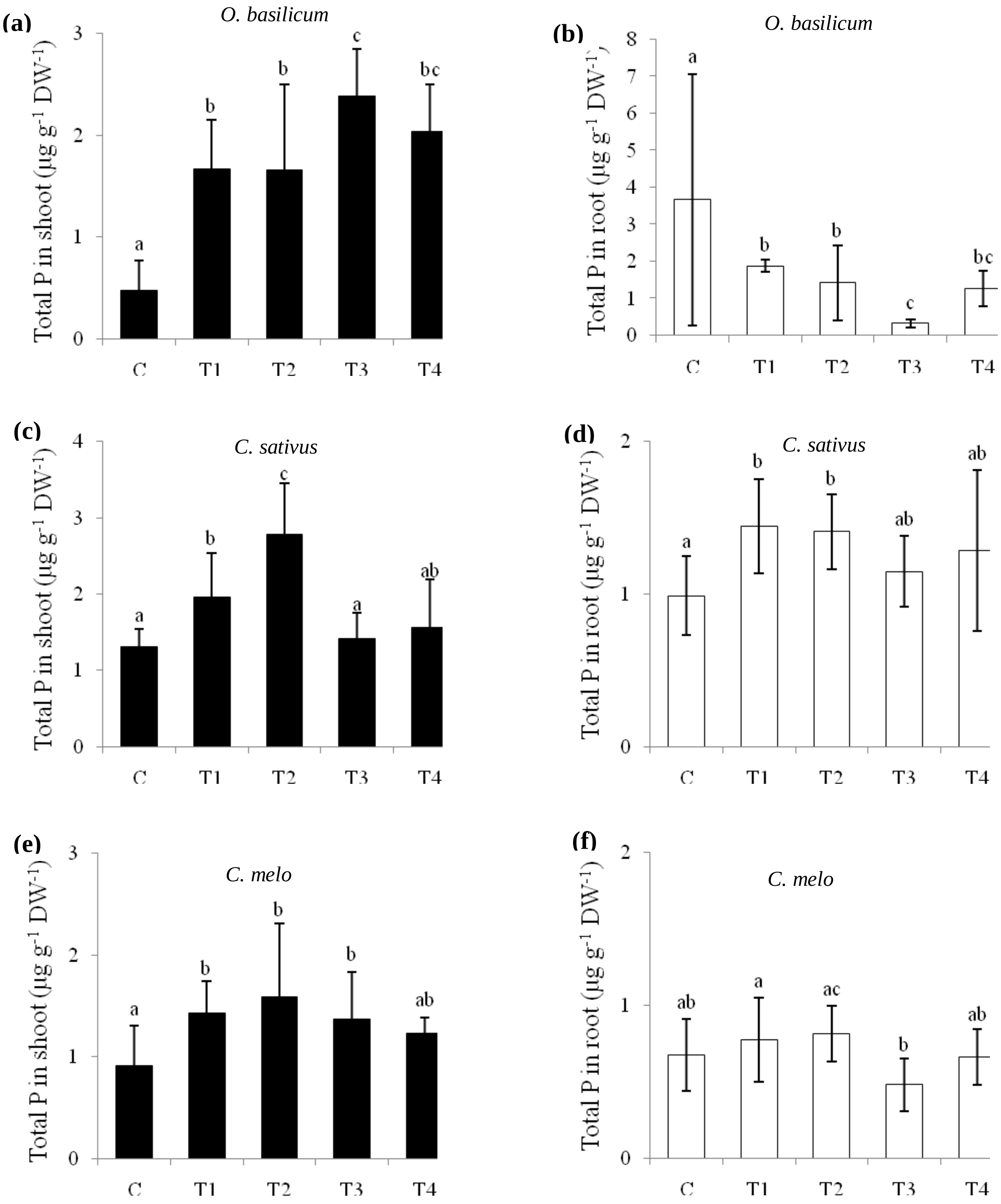

Figure 7. Final total Phosphorus concentrations measured in shoots (left) and roots (right).

We can observe that for *O. basilicum* TP concentration of shoot was the lowest for control and the highest for T3 with significant differences between control and all the treatments ($p<0.05$) and without significant differences between T1 and T2 and between T1 and T4. In the case of the TP in root the situation was the opposite, the highest value was obtained in control and the lowest in T3 without significant differences between T1 and T2 and T4. P is also important for the accumulation of other nutrients in shoot and root, as Borges et al. (2016) observed in an experiment with *O. basilicum* where a drastic reduction in the content of nutrients in both shoot and root was observed when there was a lack of P in the fertilizer used.

For the case of *C. sativus*, in both shoot and root the lowest TP concentration was obtained in control. Also, Ciereszko et al. (2002) observed a decrease in the P content in both shoot and root in seedlings treated without P. The highest concentration was for T2 in shoot and T1 in root. In both cases, control did not have significant differences with T3 and T4 and significant differences were not found between T1 and T4.

Finally, for *C. melo*, both shoot and root had the highest TP concentration for T2, while in shoot the lowest value was obtained in control and T3 in root. For shoot only were observed significant differences among control and T1, T2 and T3 ($p<0.05$). These results are in contradiction with Fita et al. (2011) who observed that *C. melo* had similar P concentrations in shoot when P was abundant. On the other hand, for root the significant differences were obtained between T3 and T1, T3 and T2.

4. Conclusions

Considering nutrient concentrations used in previous studies and the cost-effectiveness of the method in a long-term, the best option for desorbing P from P loaded MPs was based on using 0.1 M NH_4OH (neutralized with H_3PO_4). Actually, P concentration in the selected conditions was so high that it was necessary to dilute the supernatants, making profitable to use recovered P as a fertilizer.

Marked differences in the germination patterns were found among the three plants species. The first species in germinating was *O. bassilicum* but it later experienced a slower tendency and at the end of the experiment the highest percentage of germination was 80%, which corresponded to the control. The germination of the other two species, *C. sativus* and *C. melo,* started later (from day 4) and it generally reached values up to 100% at the end of the experiment. Even more, germination in the different treatments experienced notable differences among plants species. While in *O. bassilicum,* the germination in the Control was also higher than in the other treatments for any time of the experiment; for *C. sativus* and *C. melo*, the percentage of germination in all treatments was higher than in Control, evidencing the key role of adding nutrients for the germination of these species.

In general, height and growth rate for the three plants species was significantly lower in control than in treatments. Additionally, it is worth to note that no significant differences were found when using commercial fertilizer or recovered P from the P loaded MPs. Similarly, shoot and root biomass as well as P concentration in shoot was significantly lower in control than in treatments while no significant differences when using a commercial fertilizer and recovered P were found. These results suggest that P desorbed from P loaded MPs can be used as a liquid fertilizer. As a result, our results are promising in order to counteract the widespread and coupled problems of the exhaustion of the P reserves and the eutrophication of aquatic ecosystems.

Acknowledgments

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GENERAL CONCLUSIONS/
CONCLUSIONES GENERALES

CONCLUSIONS

1. In the acute toxicity test (immobilization), the concentration of MPs that caused 50% of immobilized individuals (EC_{50}) was much higher in *D. magna* (0.913 g MPs l^{-1}) than in *Chironomus* sp. (0.445 g MPs l^{-1}) which is likely to be the result of their different behavior (pelagic *vs* benthic). The results of chronic toxicity tests in *D. magna* showed that in presence of dissolved Fe (Tot-Fe_{dis}), parthenogenetic reproduction was significantly affected, while no significant effect on mortality of larvae and pupae and on adult emergence was detected in *Chironomus* sp. Anyway, these MPs concentrations are far above those required in a whole-lake restoration project based on the P adsorption capacity (53 mg MPs: mg P) reported in previous studies.

2. In the algal growth test when using *Chlorella* sp., no inhibition occurred at the two lowest MPs concentrations (0.01 and 0.05 g l^{-1}). For higher MPs dose (EC_{50} for *Chlorella* sp. was 0.15 g l^{-1}), no nutrient limitations but high turbidity (“shading effect”) and Tot-Fe_{dis} values cause negative effects on algal growth.

3. For the case of *Brachionus calyciflorus*, MPs concentration causing 50% organisms death (LC_{50}) was 1.63 g MPs l^{-1} which is in the range of EC_{50} (immobilization; at 24 h) values reported for other planktonic and benthic organisms. When analyzing Tot-Fe_{dis} effects on hatching rate, no significant effects were found. To sum up our results for *B. calyciflorus* lethal and sublethal toxicity tests, it is extremely unlikely the mortality of adult organisms in contact with MPs as well as an affectation of the rotifer egg bank.

4. Results from algal growth rate inhibition tests with *Raphidocelis subcapitata* have shown large differences among the two magnetic (HQ; Fe_3O_4) and the two non-magnetic P adsorbents (Phoslock® and CFH-12®).

5. In relation to the EC_{50} estimated in the immobilization test of *D. magna* by direct contact, no statistically significant differences were found among the four studied

adsorbents. For discriminating between physical effects and chemical induced toxicity, immobilization tests with *D. magna* by using a double-beaker were also carried out. Results clearly revealed much lower immobilization when *D. magna* was exposed through indirect contact with the adsorbents than under direct contact, which reflect the prevalence of a physical “fixation” of P adsorbents on daphnids carapace. Finally, when comparing uptake and depuration rates among all studied adsorbents except for HQ, our results evidenced a faster uptake and depuration for Phoslock®, which was precisely the adsorbent with the highest particle size.

6. In the microcosm experiments carried out with lake water and lake sediment from the hypertrophic Honda lake, no significant differences in total abundance, species richness and species diversity among treatments were found. The absence of any effect of MPs on zooplankton can be explained because MPs did not significantly alter any of its physico-chemical (e.g. temperature, pH, O_2) or biological (e.g. food quantity and quality) drivers.

7. Based on our results from both single-species toxicity tests and microcosm experiments, we can conclude that using MPs for reducing P concentration in lake water and lake sediment is a risk-less (no toxic effect) and efficient (high P adsorption capacity) tool for lake restoration although more research on toxicological effects on other plankton and benthic organisms is required.

8. Our results evidenced that Laguneto semi-natural pond acts as a continuous green filter operating as a tertiary treatment of the outflow coming from the wastewaters treatment plant. Accordingly, this pond facilitates water self-purification acting as a reliable countermeasure to reduce the impact of wastewater effluents in the Fuente de Piedra Ramsar site.

9. We have proved the suitability of using MPs for removing P in treated wastewaters. This statement is based on both the high equilibrium adsorption capacity (q) and P removal efficiency of MPs. Considering both advantages (P removal efficiency, %) and disadvantages (economic price, € L-1) of using MPs, we have identified an optimum ratio ≥ 0.16 g MPs mg^{-1} P.

10. When comparing P concentrations extracted from P loaded MPs by using three different basic solutions at different concentrations, we have selected as the best option

the use of 0.1 M NH_4OH (neutralized with H_3PO_4). Actually, P concentration in the selected conditions was so high that it was necessary to dilute the supernatants, making profitable to use recovered P as a fertilizer.

11. Marked differences in the germination patterns were found among the three plants species. The first species in germinating was *O. bassilicum* but it later experienced a slower tendency and at the end of the experiment the highest percentage of germination was 80%, which corresponded to the control. The germination of the other two species, *C. sativus* and *C. melo*, started later (from day 4) and it generally reached values up to 100% at the end of the experiment.

12. In general, height and growth rate for the three plants species was significantly lower in control than in treatments. Additionally, it is worth to note that no significant differences were found when using commercial fertilizer or recovered P from the P loaded MPs. Similarly, shoot and root biomass as well as P concentration in shoot was significantly lower in control than in treatments while no significant differences when using a commercial fertilizer and recovered P were found. These results suggest that P desorbed from P loaded MPs can be used as a liquid fertilizer. As a result, our results are promising in order to counteract the widespread and coupled problems of the exhaustion of the P reserves and the eutrophication of aquatic ecosystems.

CONCLUSIONES

1. En la prueba de toxicidad aguda (inmovilización), la concentración de partículas magnéticas (MPs) que causó la inmovilización en el 50% de los individuos (EC_{50}) fue mucho mayor en *D. magna* (0.913 g MP 1^{-1}) que en *Chironomus* sp. (0.445 g MPs l^{-1}) lo que puede ser resultado de su diferente comportamiento (pelágico *vs* bentónico). Los resultados de las pruebas de toxicidad crónica en *D. magna* mostraron que, en presencia de Fe (Tot-Fe_{dis}) disuelto, la reproducción partenogenética se vio afectada significativamente, mientras que no se detectó un efecto significativo sobre la mortalidad de larvas y pupas ni sobre la emergencia de adultos en *Chironomus* sp. De todos modos, la concentración de MPs es muy superior a la concentración estimada de MPs que se aplicaría en un proyecto de restauración real teniendo en cuenta la relación óptima entre la masa de P y masa de MPs reconocida en estudios previos.
2. En el test de toxicidad algal con *Chlorella* sp, no se observó ninguna inhibición para las dos concentraciones más bajas de MPs (0.01 y 0.05 g l^{-1}). Para dosis más altas (EC_{50} para *Chlorella* sp. fue de 0.15 g l^{-1}), la inhibición del crecimiento algal pudo deberse a los mayores valores de turbidez ("efecto de sombreado") así como de Tot-Fe_{dis}, si bien no se observó ninguna limitación por nutrientes.
3. Para el caso de *Brachionus calyciflorus*, la concentración de MPs que causó la mortalidad en el 50% de los individous (LC_{50}) fue 1.63 g MPs l^{-1}, que está en el rango de EC_{50} (inmovilización; a las 24 h) registrados para otros organismos planctónicos y bentónicos. En cuanto al efecto de la presencia de Tot-Fe_{dis} sobre la tasa de eclosión de huevos, no se encontraron efectos significativos. En base a nuestros resultados de los tests de toxicidad letal y subletal de *B. calyciflorus*,

podemos afirmar que la adición de MPs es inocua tanto para los organismos adultos como para el banco de huevos de rotíferos.

4. Los resultados de las pruebas de inhibición de la tasa de crecimiento algal con *Raphidocelis subcapitata* han mostrado grandes diferencias entre los dos adsorbentes magnéticos (HQ; Fe_3O_4) y los dos no-magnéticos (Phoslock® y CFH-12®).

5. En relación con la EC_{50} estimada en la prueba de inmovilización de *D. magna* con contacto directo, no se han encontrado diferencias estadísticamente significativas entre los cuatro adsorbentes estudiados. Los resultados de los ensayos para discriminar entre los efectos físicos y la toxicidad química inducida sobre *D. magna* revelaron el predominio de una inmovilización causada básicamente por procesos físicos. Finalmente, al comparar las tasas de asimilación y depuración entre todos los adsorbentes estudiados, excepto para HQ, nuestros resultados evidenciaron una asimilación y depuración más rápidas para Phoslock®, que fue precisamente el adsorbente con el mayor tamaño de partícula.

6. En los experimentos de microcosmos realizados con agua y sedimento superficial de la laguna Honda de la Albufera de Adra (Almería) han mostrado que la adición de MPs no ha causado efectos significativos ni en la abundancia total, ni en la riqueza de especies ni en la diversidad de especies. La ausencia de efectos de la adición de MPs sobre la comunidad zooplanctónica puede explicarse por la no alteración ni de los condicionantes físico-químicos (por ejemplo, temperatura, pH, O_2) ni biológicos (por ejemplo, cantidad y calidad de alimento).

7. En base a nuestros resultados, tanto de las pruebas de toxicidad con una única especie como de los experimentos de microcosmos, podemos concluir que el uso de MPs para reducir la concentración de P en ecosistemas acuáticos presenta una toxicidad reducida sobre los organismos acuáticos, si bien es necesario continuar la investigación de los posibles efectos toxicológicos sobre otros organismos planctónicos y bentónicos.

8. Nuestros resultados han mostrado que el Laguneto actúa como un tratamiento terciario del agua tratada por la depuradora. En consecuencia, este humedal semi-natural facilita la auto-purificación del agua, actuando como una medida adecuada para reducir el impacto de los efluentes de aguas residuales tratadas sobre la laguna de Fuente de Piedra.

9. Hemos constatado la conveniencia de utilizar MPs para eliminar P en aguas residuales tratadas. Esta afirmación se basa tanto en la elevada capacidad de adsorción de P en equilibrio (q) así como en la eficiencia de retirada de P por las MPs. Teniendo en cuenta tanto las ventajas (eficiencia de retirada de P) como las desventajas (coste económico) de usar MPs, hemos identificado que la dosis óptima a utilizar es ≥ 0.16 g MPs mg^{-1} P.

10. De la comparación de la concentración de P medida en los extractos cuando se utilizan tres diferentes soluciones básicas (NaOH; KOH y NH_4OH), podemos concluir que la mejor opción para desorber P es la utilización de 0.1 M NH_4OH (neutralizado con H_3PO_4). De hecho, la concentración de P en las condiciones seleccionadas fue tan alta que fue necesario diluir los sobrenadantes, lo que optimiza el uso del P recuperado como fertilizante.

11. En los experimentos de fertirrigación se encontraron marcadas diferencias en los patrones de germinación entre las tres especies de plantas estudiadas. La primera especie en germinar fue *O. bassilicum*, si bien experimentó una tendencia más lenta a largo plazo y, al final del experimento, el mayor porcentaje de germinación para esta especie fue del 80%, que correspondió al control. La germinación de las otras dos especies, *C. sativus* y *C. melo*, comenzó más tarde (a partir del día 4) pero se alcanzaron valores de hasta el 100% al final del experimento

12. La altura y la tasa de crecimiento para las tres especies de plantas fue significativamente menor en el control (sin adición de nutrientes) que en los tratamientos. Más aún, es importante destacar la ausencia de diferencias significativas entre los tratamientos con fertilizante comercial y con P recuperado. De manera similar, la biomasa de las partes aéreas y de las raíces,

así como la concentración de P en las partes aéreas fue significativamente más baja en los controles que en los tratamientos, mientras que no se encontraron diferencias significativas entre los tratamientos (fertilizante comercial *vs* P recuperado). Estos resultados sugieren que el P desorbido desde las MPs puede usarse como fertilizante líquido. Por tanto, las MPs podrían ser porpuestas para contrarrestar la alteración antrópica del ciclo biogeoquímico del P.

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