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Ecotoxicity screening of novel phosphorus adsorbents used for lake restoration

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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 Fe₃O₄) and two non magnetic (CFH-12® and Phoslock®). For the case of the algal growth inhibition test, the EC₅₀ was 1.5 and 0.42 g L⁻¹ for HQ and CFH-12®, respectively, and no inhibition patterns were observed neither for Fe₃O₄ 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 EC₅₀ 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 EC₅₀ 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.

Keywords: phosphorus, *Raphidocelis subcapitata*, *Daphnia magna*, toxicity, eutrophication, lake restoration

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 ($\text{Al}_2(\text{SO}_4)_3$) or polyaluminum chloride form solid $\text{Al}(\text{OH})_3$ 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 $\text{Al}(\text{OH})_3$ 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 $\text{Al}(\text{OH})_3$ 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 ($18.83 \text{ mg P g}^{-1}$) 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. $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ 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, Fe₃O₄) 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 µm³), 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 ± 1°C 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 Fe₃O₄ 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).

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. The concentrations of each adsorbent were 0.05, 0.1, 0.5, 1 and 1.5 g L⁻¹ for HQ and Fe₃O₄; 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 EC₅₀ 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 Fe₃O₄), 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 ± 2°C 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, Fe₃O₄, 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 shaken 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 ± 1°C 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 $20 \pm 1^\circ\text{C}$ 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 20°C 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 20°C 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:

$$t_{\frac{1}{2}} = \frac{\text{Ln}2}{K_d}$$

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 Fe₃O₄ and Phoslock®, EC₅₀ 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 EC₅₀ for HQ was much higher than the 0.085 g 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.

For Fe₃O₄, 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 Fe₃O₄ 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 Lüring (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 Lüring (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 & 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*

3.1.1. 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 EC₅₀, no statistically significant differences were found among the four studied adsorbents (Table 3).

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-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 EC₅₀ 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-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.

The EC₅₀ 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). For Fe₃O₄, and as expected, the EC₅₀ (2.76 g L⁻¹) was much higher than EC₅₀ and LC₅₀ reported for much smaller Fe₃O₄ 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 EC₅₀ value for immobilization of *D. magna* when exposed to Phoslock® was 1.4-2.5 times higher than the EC₅₀ 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. 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 ingested by daphnids. On the other hand, the smaller size of Fe₃O₄ (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 Fe₃O₄ 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.

3.1.2. 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).

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 Fe₃O₄. 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.

3.1.3. 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 Fe₃O₄ (0.04 µg daphnia⁻¹) while the highest was measured in tests 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 Fe₃O₄. 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 amount as 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 Fe₃O₄, 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.

In the depuration test, when comparing the four P adsorbents, lowest depuration rate constant (K_d) was found for Fe₃O₄ (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 Fe₃O₄ (0.34 h). For the case of the residual body burden at the

end of the depuration study, it was highest for HQ ($0.40 \mu\text{g Fe daphnia}^{-1}$; Figure 6) while very similar for Fe_3O_4 ($0.03 \mu\text{g Fe daphnia}^{-1}$) and CFH-12® ($0.06 \mu\text{g Fe daphnia}^{-1}$). These concentrations were higher than the measured Fe-content of non-exposed animals ($0.003 \mu\text{g Fe daphnia}^{-1}$). For the case of Phoslock®, residual body burden was similar to the background concentration ($3.5 \text{ ng La daphnia}^{-1}$). 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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List of Tables

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 ₃ O 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 (Haghserreht, 2006)	13.6 (Funes et al., 2018)

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

Major nutrients	mg L ⁻¹
NH ₄ Cl	15
MgCl ₂ ·6H ₂ O	12
CaCl ₂ ·2H ₂ O	18
MgSO ₄ ·7H ₂ O	15
KH ₂ PO ₄	1.6
NaHCO ₃	50
Trace elements	µg L ⁻¹
FeCl ₃ ·2H ₂ O	64
Na ₂ EDTA·2H ₂ O	100
H ₃ BO ₃	185
MnCl ₂ ·4H ₂ O	415
ZnCl ₂	3
CoCl ₂ ·6H ₂ O	1.5
CuCl ₂ ·2H ₂ O	0.01
Na ₂ MoO ₄ ·2H ₂ O	7

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 ₃ O 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 (-; -)*

Table 4. Comparison between predicted no-effect concentration (PNEC), predicted environmental concentration (PEC) and environmental quality standard of relevance (MAC-QS_{fw,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-QS _{fw} (mg L ⁻¹)
HQ ¹	350	1.50	15
Fe ₃ O ₄ ²	1120	2.76	27.6
CFH-12® ¹	430	0.42	4.2
Phoslock® ²	480	2.14	21.4

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 ₃ O ₄	3.50	2.04	0.34
CFH-12®	1.18	2.50	0.28
Phoslock®	5.80	3.60	0.19

List of Figures

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.

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.

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®.

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.

Figure 5. Body burdens of (a) Fe and (b) La in the *D. magna* immobilization test in indirect contact with (a) HQ, Fe₃O₄ and CFH-12® and (b) Phoslock®.

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 referred to HQ, Fe_3O_4 and CFH-12®, respectively while in (c) and (d) Phoslock® results are shown.