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# Selecting the Most Sustainable Phosphorus Adsorbent for Lake Restoration: Effects on the Photosynthetic Activity of *Chlorella* sp.

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**Abstract:** To promote the conservation of aquatic ecosystems, it is essential to delve into restoration techniques for selecting the most sustainable option for combating eutrophication. Hence, we study the effects of novel phosphorus (P) adsorbents (magnetic carbonyl iron particles, HQ, and two non-magnetic P adsorbents: CFH-12® and Phoslock®) on the growth and photosynthetic activity of *Chlorella* sp. More specifically, the intrinsic photochemical efficiency of PSII ($\Phi_{PSII}$) and the nonphotochemical quenching (NPQ) were measured in *Chlorella* sp. after different contact times with different concentrations of these adsorbents. Our initial hypothesis was that non-magnetic P adsorbents have more effects on the organisms than magnetic ones. However, our results did not show strong evidence of inhibitory effects caused by HQ nor CFH-12® (no significant effect size on $\Phi_{PSII}$), while Phoslock® showed inhibitory effects on the photosynthetic activity of *Chlorella* sp. for any of its concentrations (NPQ = 0). Lastly, we compared the effect of the studied P adsorbents in a real application scenery (Honda wetland, Spain). For this study case, it is likely that CFH-12® and HQ doses would not cause any negative effects on photosynthetic efficiency while Phoslock®, by limiting light availability, will drastically reduce it.

**Keywords:** phosphorus; *Chlorella* sp.; toxicity; photosynthetic activity; eutrophication; lake restoration; sustainability assessment



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## 1. Introduction

Inland waters cover less than 2% of the Earth's surface, but support 12% of known species, and more than half of all fish species, with high levels of endemism. This biodiversity is being worldwide threatened by a multitude of stress agents. In fact, monitored freshwater species populations have declined by an average of 84%, migratory fish by 76%, aquatic megafauna by 88%, and mega-fishes by 94%. The main threats to the biodiversity of inland waters are habitat loss, climate change, invasive species, and eutrophication [1–5].

Worldwide, eutrophication has negative effects on both ecological and economic dimensions [2,6]. Anthropogenic activities such as agriculture have a key role in the eutrophication of aquatic ecosystems since this activity translocates high concentrations of phosphorus (P) from agricultural lands to aquatic ecosystems by runoff [7,8]. Considering that P is the main limiting nutrient for primary production in freshwater ecosystems, it requires a reduction in its concentration in the water column in order to restore them. To achieve this goal, three different but frequently complementary techniques are recommended: (i) the reduction in external P loads; (ii) the increase in P retention; and (iii) the increase in P export [1]. Although the reduction in the P external load is considered the essential step for achieving a successful restoration project, the reduction in the internal P load has notable impacts on lake water quality [9,10]. To obtain it, several P-sorbing materials have been proposed as these adsorbents take the P from the medium being this

P is no longer available for primary producers [11]. The P adsorbents most used for lake restoration are iron (Fe) and aluminum (Al) oxides [12–14]. However, the use of these compounds has shown several disadvantages such as (i) the reduction in P adsorption efficiency with aging and the increase in toxicity at high values in the case of Al [15,16]; (ii) Fe is sensible to redox conditions [17]; (iii) both Fe and Al compounds produce flocs when the pH of the lake water decreases [18]; and (iv) Al flocs reduce the sediment stability hindering macrophytes colonization [19,20].

In order to counteract the disadvantages showed by Al and Fe salts, novel P adsorbents have been proposed for use in inland water restoration projects. One of these P adsorbents is Phoslock®, a lanthanum (La) modified bentonite, which has been widely used for combating eutrophication [11,21]. Phoslock® has shown a high P removal efficiency in a wide pH and even under anoxic conditions [21,22], although it is not cost-effective [23] and it has shown chemical interferences with other substances [24,25]. On the other hand, CFH-12®, a dried Fe oxide has been proposed to be used in lake restoration since it has a high P removal efficiency (7.6–8.1 Fe: P molar ratio), it does not change water pH and it is not sensible to redox conditions [26].

Nevertheless, none of these mentioned adsorbents nor the P adsorbed by them can be recovered from the lake after their application. Accordingly, other novel P adsorbents, magnetic particles (MPs), have been proposed to be used in lake restoration since they allow recovering P-loaded MPs after their application [27–29]. These MPs are characterized by a high efficiency for removing P from the water column [30] and once P is adsorbed, P-loaded MPs can be recovered from the ecosystem by using magnetic separation techniques. Later on, P can be desorbed by using basic solutions and reused as a fertilizer [31]. Therefore, the use of MPs in lake restoration can support two important worldwide problems affecting the biogeochemical P cycle: the eutrophication of the aquatic ecosystems [7,32,33] and the exhaustion of P reserves used for making fertilizers [34–36]. In addition, the use of MPs is a cost-effective method for restoring eutrophicated lakes in comparison with other methods since they can be reused up to four times by achieving a high P removal efficiency [37]. Another advantage of using MPs is that they can be supplied by companies (i.e., BASF) or synthesized in the laboratory optimizing some selected properties [29–31]. Among MPs, carbonyl iron (Fe) particles (HQ) have been proposed as a promising tool for combating eutrophication since they combine both high maximum P adsorption capacity and low economic cost (e.g., [27–29]).

Before applying P-sorbing materials in a real whole-lake project, it is essential to carry out a complete toxicological assessment of these compounds in order to choose the most sustainable. Phytoplankton, as primary producers, plays a key role in aquatic ecosystems and accordingly, any potential toxic effects would affect the entire aquatic food chain. To date, there are some studies that evaluate the effect of adding P adsorbents on the phytoplankton community. It is likely that the most complete study (due to the diversity of adsorbents used) is that of Álvarez-Manzaneda [38], where short-term standardized laboratory tests were carried out for evaluating acute toxicological effects of magnetic (carbonyl Fe particles, HQ, and magnetite) and non-magnetic (CFH-12® and Phoslock®) P adsorbents on *Raphidocelis subcapitata*. Doubtless, the most studied P adsorbent is Phoslock®, with a wide variety of works that evaluate its effect on the phytoplankton community (e.g., [39–41]). On the contrary, the effects of MPs have been much less studied ([38,42], among others).

Despite this diversity of mentioned works, there is a lack of information about the sublethal effects of these novel adsorbents. In particular, photosynthetic activity can be useful in risk assessment due to their sensitivity to environmental changes [43–45]. In this study, the chlorophycean algae *Chlorella* sp. was chosen as an organism test since microalgae have shown to be excellent aquatic models, easy to culture, and sensitive to pollutants and *Chlorella* sp. has a short growth cycle, which makes it suitable for ecotoxicity studies [46]. Our working hypothesis is that the addition of P adsorbents can affect photosynthetic efficiency by affecting light and nutrient availability. Additionally, we hypothesize that non-

magnetic adsorbents, that cannot be removed from the medium, have higher toxic effects than MPs. More in detail, in this paper we study the effects of a magnetic (carbonyl iron particles, HQ) and two non-magnetic P adsorbents (CFH-12® and Phoslock®) on the growth and photosynthetic activity of *Chlorella* sp. The results obtained in these ecotoxicological tests can undertake risk assessments of these adsorbents in aquatic ecosystems and assist the environmental decision-making.

## 2. Materials and Methods

### *2.1. Test Species and Culture Medium*

*Chlorella* sp. (365 μm$^3$; diameter: 8.8 μm) was selected as the test species since this unicellular green alga is cosmopolitan and can be easily cultured in the laboratory [47]. It was cultured from a collection of the University of Granada by using 2000 mL volume with Bold's Basal Medium (BBM; [48]). The stock was maintained with a photoperiod of 12:12 h, at a temperature of 21 ± 1 °C, and with an agitation of 200 rpm to avoid the sedimentation of algae cells. This culture was maintained in exponential growth for five generations before the experiment, in order to ensure its proper growth and quality, as recommended by the International Organization for Standardization (ISO, [49]).

### *2.2. General Characterization of Adsorbents*

All adsorbents used in the present study have been commercially supplied. Table 1 summarizes the main characteristics of the studied P adsorbents. As can be observed, they have different chemical compositions, maximum P adsorption capacity, and size. More in detail, HQ (BASF, Ludwigshafen, Germany) are spherical, with a relative polydispersion and a ferromagnetic behavior with negligible remnant magnetization. HQ has been thoroughly characterized by previous studies [27,28]. CFH-12® (Kemira, Helsinki, Finland) is a dried amorphous solid composed of non-magnetic Fe oxides with a high and stable adsorption of P [50]. Phoslock® (CSIRO, Canberra, Australia) is a granular material; it is a Lanthanum-modified clay that has been used as a P adsorbent due to its high P adsorption capacity even in anoxic conditions or after sediment resuspension events [51,52]. Of all these distinctive features, it is key to highlight the different particle sizes among the studied P adsorbents since it is well known that it affects not only the maximum P adsorption capacity (increasing as the particle size is reduced; e.g., [27]) but also affects the magnetization (reducing as the particle size is reduced; e.g., [27]), sedimentation (reducing as the particle size is reduced; Stokes's law) and, the toxicity (increasing as the particle size is reduced; e.g., [53]).

**Table 1.** Main physico-chemical characteristics of the P adsorbents. Composition is referred to as total [1] and atomic surface [2] (%).

| Adsorbent | Composition | Maximum P Adsorption Capacity (mg g$^{-1}$) | Size | References |
|---|---|---|---|---|
| HQ | Fe (98); C (0.9); N (0.9); O (0.5) [1] (manufacturer) | 18.83 | 805 ± 10 nm | [27] |
| CFH-12® | O (59); Fe (28); C (9); S (2); Mg (0.5) and Ca (0.3) [2] | 15.1 | 0.85–2 mm (93%); <0.85 nm (6%) | [26,30,52] |
| Phoslock® | O (66); C (6); Si (19); Al (5); La (1); Na (1); Mg (1); Ca (1) and Fe (1) [2] | 13.6 | 2–4 × 1–3 mm (manufacturer) | [30,52] |

### *2.3. Toxicological Tests with Chlorella sp.*

The concentrations of P adsorbents were selected according to previous results obtained by Álvarez-Manzaneda et al. [38] in algal growth inhibition tests carried out with *Raphidocelis subcapitata*. For the magnetic adsorbent (HQ), concentrations were 0.05, 0.1, 0.5, 1, and 1.5 g L$^{-1}$ while for non-magnetic, concentrations were 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®. Each concentration and control treatment comprised four replicates.

Once the algal stock culture was maintained in exponential growth for five generations, algae were inoculated in each suspension. For the case of the magnetic adsorbent (HQ), after 24 h of contact time and just before the experiment, the adsorbent was removed by using a magnetic device specially designed by applying Comsol Multiphysics software® version 5.3. (COMSOL Inc., Burlington, MA, USA). It consists of a handle and a rake (Ø = 5 cm) with a platform that contains an array of 9 cylindrical neodymium magnets individually inserted into its base. The removal process was carried out by immersing the magnet twice for 3 s in each vessel. Later, all test vessels (30 mL) were located for 48 h in an isolated room at 21 ± 1 °C and a photoperiod of 12:12 h.

#### 2.3.1. Effects of Adsorbent Addition on Physico-Chemical Variables

At the beginning and the end of the experiment, pH and dissolved oxygen saturation (DO; %) were measured by using a multi-parameter probe (HI 9829, Hanna instruments, Eibar, Spain) while turbidity was measured with a turbidimeter (LW-TN3024, XS, Carpi, Italy) at the end of the experiment. Dissolved inorganic P (DIP; [54]) and total dissolved Fe (Tot-$Fe_{dis}$; [55]) concentrations were also quantified at the end of the experiment after filtration (Whatman GF/F) and by using a spectrophotometer (Hitachi F-2000, Dynamica Ltd., Livingston, UK and Biochrom-Libra S50, Biochrom Ltd., Cambridge, UK; respectively). Tot-$Fe_{dis}$ was measured in all the adsorbent treatments except in Phoslock® since Fe does not compose it.

#### 2.3.2. Effects of the Adsorbents on Algal Growth

At the end of the experiment (15 h), 1 mL from each test vessel was sampled and preserved with Lugol's solution (1%, final concentration) for quantitative analysis. Cell concentration (cell $mL^{-1}$) of *Chlorella* sp. was estimated by using Neubauer's counting chamber for all treatments of each adsorbent.

#### 2.3.3. Photosynthetic Activity of *Chlorella* sp.

For measuring in vivo chlorophyll a (Chl a) fluorescence of *Chlorella* sp., 3 mL were collected from each test vessel at different times: 30 min and 1.5; 3; 5; 7.5; 10; 12.5; 13.5, and 15 h. Fluorescence was later measured by using a portable pulse-modulation fluorometer (Water-ED PAM, Walz, Germany). Each measure was repeated six times for 10 s, immediately after sampling, so each sample was measured for 1 min. The PAM method was used as it has been successfully used in toxicity experiments with *Chlorella vulgaris* [56].

The intrinsic photochemical efficiency of PSII (Photosystem II, $\Phi_{PSII}$) in the light was determined according to this formula [57]:

$$\Phi_{PSII} = \frac{\Delta F}{F'm} = \frac{F'm - F't}{F'm} \quad (1)$$

where

F′m: instantaneous maximum fluorescence induced by a saturating light pulse (~5300 µmol photons $m^{-2}$ $s^{-1}$ in 0.8 s).
F′t: current steady-state fluorescence of light-adapted cells induced by an actinic light ~419 W $m^{-2}$ in light-adapted cells.

On the other hand, the nonphotochemical quenching (NPQ) was used as a proxy of the excess energy dissipation as heat. It is the most important short-term photoprotective mechanism activated by saturating radiation intensities. NPQ was determined directly from the PAM fluorometer as follows:

$$NPQ = \frac{Fm - F'm}{F'm} \quad (2)$$

where

Fm: maximal fluorescence of the dark-adapted sample.
NPQ was calculated for each sample from the Fm value stored by the software.

Finally, the $\Phi_{PSII}$ and NPQ areas were calculated for both diel cycles (in quadruplicate for each treatment), according to González-Olalla et al. [58] as:

$$A = \int_{b}^{a} f(x)dx \quad (3)$$

where

a = initial measurement time
b = final measurement time
f(x) = curve describing $\Phi_{PSII}$ or NPQ over time for each treatment.

OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA) was used for calculating the area under the curve that represents the balance between photoinhibition and repair of PSII throughout the diel cycle.

The effect size of adsorbents for each treatment on $\Phi_{PSII}$ integral and NPQ areas was calculated as follows:

$$\text{Effect size of adsorbent}(\%) = \frac{X_C - X_T}{X_C} \times 100 \quad (4)$$

where

X = Variable response considered in samples under control (C) and treatments (T).

Negative values are indicative of a stimulatory effect and positive values indicate an inhibitory effect of stress factors.

### *2.4. Statistical Analyses*

After testing the requirements of normality (Shapiro–Wilk test), and homoscedasticity (Levene test) of the residuals, significant differences in final pH values, final DO saturation values, algal concentration, DIP, and Tot-$Fe_{dis}$ concentrations, effect size on $\Phi_{PSII}$ integral and NPQ areas and turbidity values were tested by using one-way ANOVA, followed by Tukey's HSD post hoc test (95% confidence level). In the case that data did not satisfy homoscedasticity assumptions (Levene test, $p < 0.05$) such as pH in CFH-12®, DO in HQ, turbidity in HQ and CFH-12®, as well as Tot-$Fe_{dis}$ concentration in HQ experiment, significant differences among treatments were tested by using Kruskal–Wallis test. Regarding the analyses for $\Phi_{PSII}$ and NPQ diel cycles, since the data met the normality and homoscedasticity assumptions, for testing the existence of significant differences over time among treatments repeated measures ANOVA (RM-ANOVA) was used and Tukey HSD was used as post hoc test. These analyses were carried out by using the R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) program.

## 3. Results

### *3.1. Effect of Adsorbents Addition on Physico-Chemical Variables*

Tables 2 and 3 show the mean values of the physico-chemical variables measured at the initial and at the final time of the experiment and Table 4 summarizes the results of the statistical analyses for those variables. Concerning pH values, significant effects of treatment (adsorbent concentration) were observed for all adsorbents except for CFH-12® (Kruskal–Wallis test; $p = 0.28$). However, the trends were not the same between the adsorbents. While for HQ and Phoslock® an increase in pH has been observed as the adsorbent concentration increases, that trend was not clear in CFH-12®. For the case of DO saturation values, a significant decrease at the end of the experiment was observed for all adsorbents except for Phoslock®, where all treatments had a 100%. In relation to the effect on turbidity of adding P adsorbents, the same pattern for all adsorbents was observed. As

expected, a significant increase in turbidity was observed as the adsorbent concentration increased, recording the highest turbidity values for Phoslock© treatments.

**Table 2.** Mean values and standard deviations of the physico-chemical parameters were measured at the beginning and the end of the experiment for the different adsorbents and treatments.

| | | pH | | DO (%) | |
|---|---|---|---|---|---|
| **Treatment (g $L^{-1}$)** | | **Initial** | **Final** | **Initial** | **Final** |
| HQ | Control | 7.47 ± 0.02 | 7.52 ± 0.01 | 100.00 ± 0.00 | 96.00 ± 1.04 |
| | 0.05 | 7.49 ± 0.06 | 7.55 ± 0.04 | 100.00 ± 0.00 | 94.75 ± 1.05 |
| | 0.1 | 7.63 ± 0.05 | 7.70 ± 0.05 | 100.00 ± 0.00 | 93.60 ± 0.34 |
| | 0.5 | 8.26 ± 0.07 | 8.33 ± 0.09 | 98.30 ± 0.92 | 93.70 ± 0.64 |
| | 1 | 8.30 ± 0.11 | 8.30 ± 0.04 | 92.08 ± 1.98 | 91.63 ± 0.23 |
| | 1.5 | 8.44 ± 0.04 | 8.28 ± 0.04 | 88.90 ± 1.41 | 91.63 ± 0.40 |
| CFH-12® | Control | 7.42 ± 0.04 | 7.27 ± 0.29 | 93.75 ± 5.54 | 91.33 ± 0.21 |
| | 0.02 | 7.41 ± 0.03 | 7.44 ± 0.01 | 86.88 ± 0.57 | 91.23 ± 0.37 |
| | 0.04 | 7.39 ± 0.02 | 7.44 ± 0.04 | 87.50 ± 0.85 | 90.38 ± 0.26 |
| | 0.1 | 7.44 ± 0.05 | 7.43 ± 0.02 | 85.40 ± 6.66 | 90.38 ± 0.57 |
| | 0.24 | 7.44 ± 0.05 | 7.43 ± 0.01 | 86.80 ± 5.10 | 88.13 ± 1.18 |
| | 0.6 | 7.36 ± 0.03 | 7.40 ± 0.02 | 80.07 ± 7.14 | 87.27 ± 0.12 |
| Phoslock® | Control | 7.83 ± 0.10 | 7.70 ± 0.16 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| | 0.1 | 7.85 ± 0.04 | 7.82 ± 0.07 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| | 0.5 | 7.92 ± 0.01 | 7.92 ± 0.06 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| | 1 | 7.94 ± 0.04 | 7.95 ± 0.10 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| | 1.5 | 7.95 ± 0.02 | 7.98 ± 0.09 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| | 2 | 7.93 ± 0.05 | 8.16 ± 0.10 | 100.00 ± 0.00 | 100.00 ± 0.00 |

**Table 3.** Mean values and standard deviations of the physico-chemical parameters were measured at the end of the experiment for the different adsorbents and treatments. n.d. means non-detectable as values were below the limit of detection (0.01 mg Fe $L^{-1}$).

| **Treatment (g $L^{-1}$)** | | **Turbidity (NTU)** | **DIP (mg $L^{-1}$)** | **Tot-$Fe_{dis}$ (mg $L^{-1}$)** |
|---|---|---|---|---|
| HQ | Control | 28.23 ± 2.00 | 145.77 ± 8.49 | n.d. |
| | 0.05 | 57.34 ± 7.15 | 142.81 ± 8.28 | n.d. |
| | 0.1 | 69.50 ± 6.45 | 137.64 ± 7.83 | 0.01 ± 0.02 |
| | 0.5 | 254.00 ± 15.71 | 100.12 ± 4.00 | 18.67 ± 0.60 |
| | 1 | 223 ± 15.72 | 88.96 ± 1.54 | 19.81 ± 1.50 |
| | 1.5 | 157.75 ± 24.10 | 72.86 ± 4.38 | 15.97 ± 3.48 |
| CFH-12® | Control | 32.80 ± 15.66 | 63.57 ± 1.53 | n.d. |
| | 0.02 | 42.37 ± 0.85 | 67.22 ± 0.99 | n.d. |
| | 0.04 | 46.74 ± 3.44 | 65.58 ± 3.73 | n.d. |
| | 0.1 | 64.50 ± 3.11 | 64.72 ± 1.34 | n.d. |
| | 0.24 | 98.75 ± 4.27 | 62.50 ± 4.71 | n.d. |
| | 0.6 | 261.00 ± 32.91 | 58.95 ± 2.27 | n.d. |
| Phoslock® | Control | 121.25 ± 10.60 | 71.65 ± 1.72 | - |
| | 0.1 | 106.50 ± 12.45 | 56.63 ± 0.56 | - |
| | 0.5 | 137.75 ± 29.33 | 54.08 ± 0.90 | - |
| | 1 | 204.75 ± 8.77 | 50.68 ± 1.59 | - |
| | 1.5 | 264.50 ± 9.11 | 44.68 ± 5.86 | - |
| | 2 | 366.50 ± 8.96 | 42.84 ± 2.55 | - |

**Table 4.** Results of the statistical analyses of physico-chemical parameters and algal concentration for the different adsorbents. df = degrees of freedom; * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$). Statistical values generated come from the [a]: Kruskal-Wallis test and [b]: one-way ANOVA.

| | HQ | | | CFH-12® | | | Phoslock® | | |
|---|---|---|---|---|---|---|---|---|---|
| | **df** | **F** | ***p*** | **df** | **F** | ***p*** | **df** | **F** | ***p*** |
| pH [a,b] | 5 | 292.07 | *** | 5 | 0.59 | ns | 5 | 8.40 | *** |
| DO [a,b] | 5 | 101.83 | *** | 5 | 2.90 | * | - | - | - |
| Turbidity [a,b] | 5 | 12.36 | *** | 5 | 11.87 | *** | 5 | 178.01 | *** |
| DIP [b] | 5 | 182.99 | *** | 5 | 1.39 | ns | 5 | 6.69 | ** |
| Tot-Fedis [a,b] | 5 | 10.76 | ** | - | - | - | - | - | - |
| Algal concentration [b] | 5 | 0.68 | ns | 5 | 3.87 | ** | 5 | 10.53 | *** |

In the case of DIP, it is important to note that, as it was foreseeable, significant differences among treatments were found for magnetic adsorbents and Phoslock® evidencing a DIP reduction as adsorbent concentrations increase. By contrast, CFH-12® caused a DIP reduction but no significant differences were achieved (Table 4). Finally, Tot-$Fe_{dis}$ concentrations higher than the detection limit were just measured for HQ, and only for concentrations of this adsorbent greater than 0.1 g $L^{-1}$.

### *3.2. Effect of Adsorbents Addition on Algal Growth*

Algal growth was measured at the end of the experiment (Figure 1). Significant differences in algal concentration among treatments were found for all adsorbents except for HQ (Table 4). More in detail, for CFH-12® significant differences among control and 0.1 and 0.24 g $L^{-1}$ were found (Tukey HSD post hoc; $p < 0.005$ and $p < 0.05$, respectively). In the case of Phoslock®, significantly higher values were found for 2 g $L^{-1}$ compared to the control and to 0.1 g $L^{-1}$ of this adsorbent (Tukey HSD post hoc; $p < 0.05$).

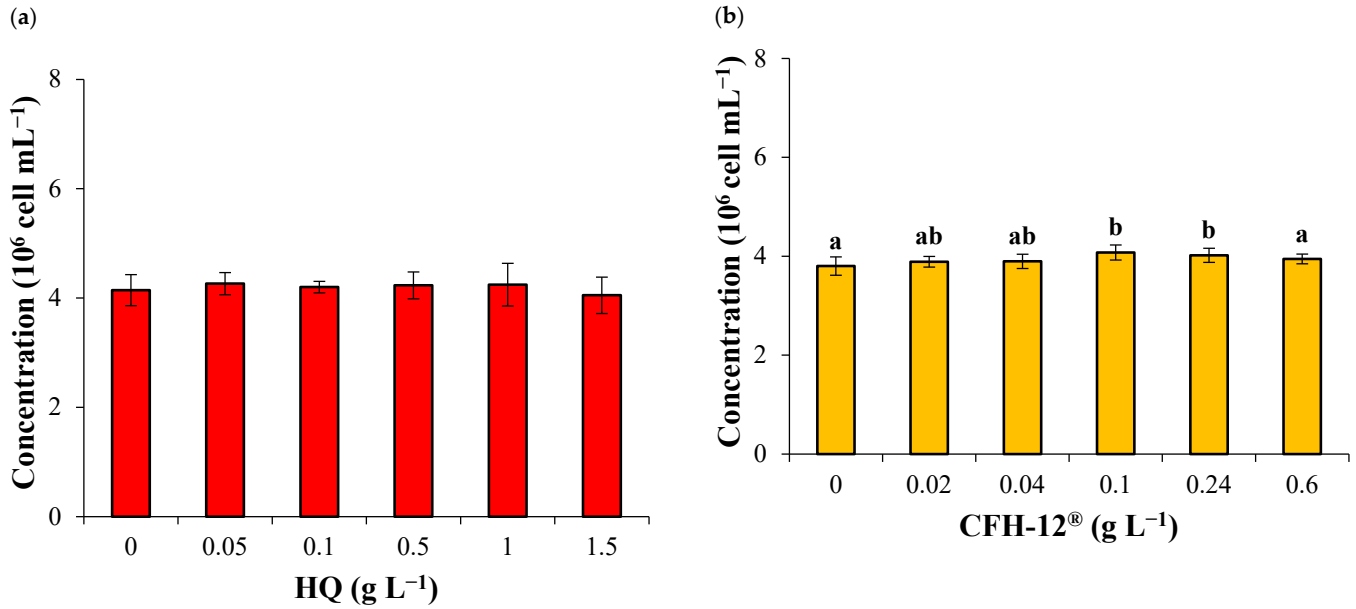


**Figure 1.** *Cont.*

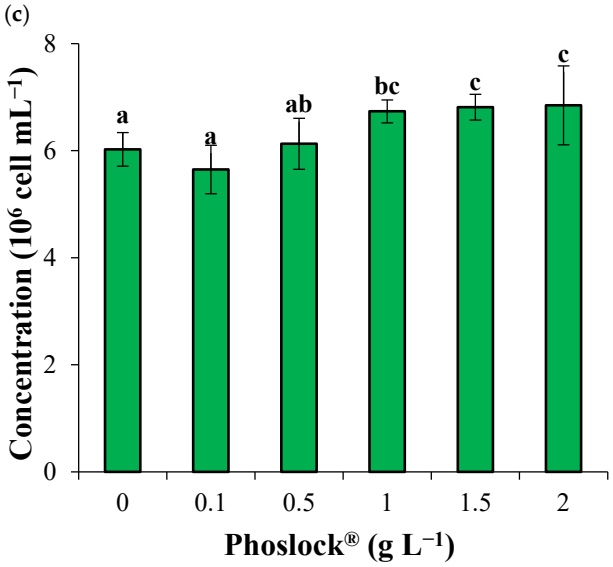


**Figure 1.** Algal concentration in the different treatments of the four adsorbents tested. (**a**) HQ; (**b**) CFH-12® and (**c**) Phoslock®. Data are expressed as mean values ± SD. Significant differences ($p < 0.05$) based on post hoc multiple comparisons are indicated by different letters.

### *3.3. Effects of the Adsorbents on the Photosynthetic Activity of Chlorella sp.*

As expected, a similar pattern in diel cycles of $\Phi_{PSII}$ was found for all adsorbents and treatments (Figure 2). It consisted of a U-shaped curve evidencing the highest values at the beginning of exposure and after the radiation stress was removed. If we compare the effects of the different concentrations of each adsorbent on the photosynthetic efficiency, we can observe that in the case of HQ, there are significantly higher values of $\Phi_{PSII}$ in the control compared to the highest concentrations only when there were no radiation conditions (Figure 2a). For CFH-12® (Figure 2b), no significant differences were detected between treatments, but significant differences were observed over time just under light conditions (Tukey HSD post hoc; $p < 0.05$). Finally, for Phoslock®, only significant differences between the control and the highest concentration were obtained at 11:00, 20:30, and 23:00 h (Figure 2c; Tukey HSD post hoc; $p < 0.05$).

In addition, in order to identify in which treatments the effect compared to the control is more remarkable, we calculated the effect size of the different treatments of each adsorbent on the $\Phi_{PSII}$ area (Figure 3). For HQ, although no significant differences among treatments were observed (one-way ANOVA; $p > 0.05$), an inhibitory effect (reflected by positive values) on $\Phi_{PSII}$ was observed for the three highest concentrations (Figure 3a). For CFH-12®, no significant differences among treatments were found (Figure 3b). Finally, all Phoslock® concentrations resulted in an inhibitory effect on $\Phi_{PSII}$ (Figure 3c), being this inhibition significantly higher at the highest concentration compared to the lowest concentration (Tukey HSD post hoc; $p < 0.05$).

On the other hand, NPQ diel cycles exhibited a clear response to the adsorbents exposition (Figure 4). Our results show significant differences over time for HQ (Figure 4a) and additionally, the two highest concentrations exhibited significant differences with all the other concentrations from 9.30 h onwards (Tukey HSD post hoc; $p < 0.05$). For CFH-12® (Figure 4b), significant differences were just found over time during light conditions (Tukey HSD post hoc; $p < 0.05$) but no significant differences were found among treatments (RM-ANOVA; $p > 0.05$). Finally, for all Phoslock® treatments, the value of NPQ was zero throughout the experiment and so, no figure is included.

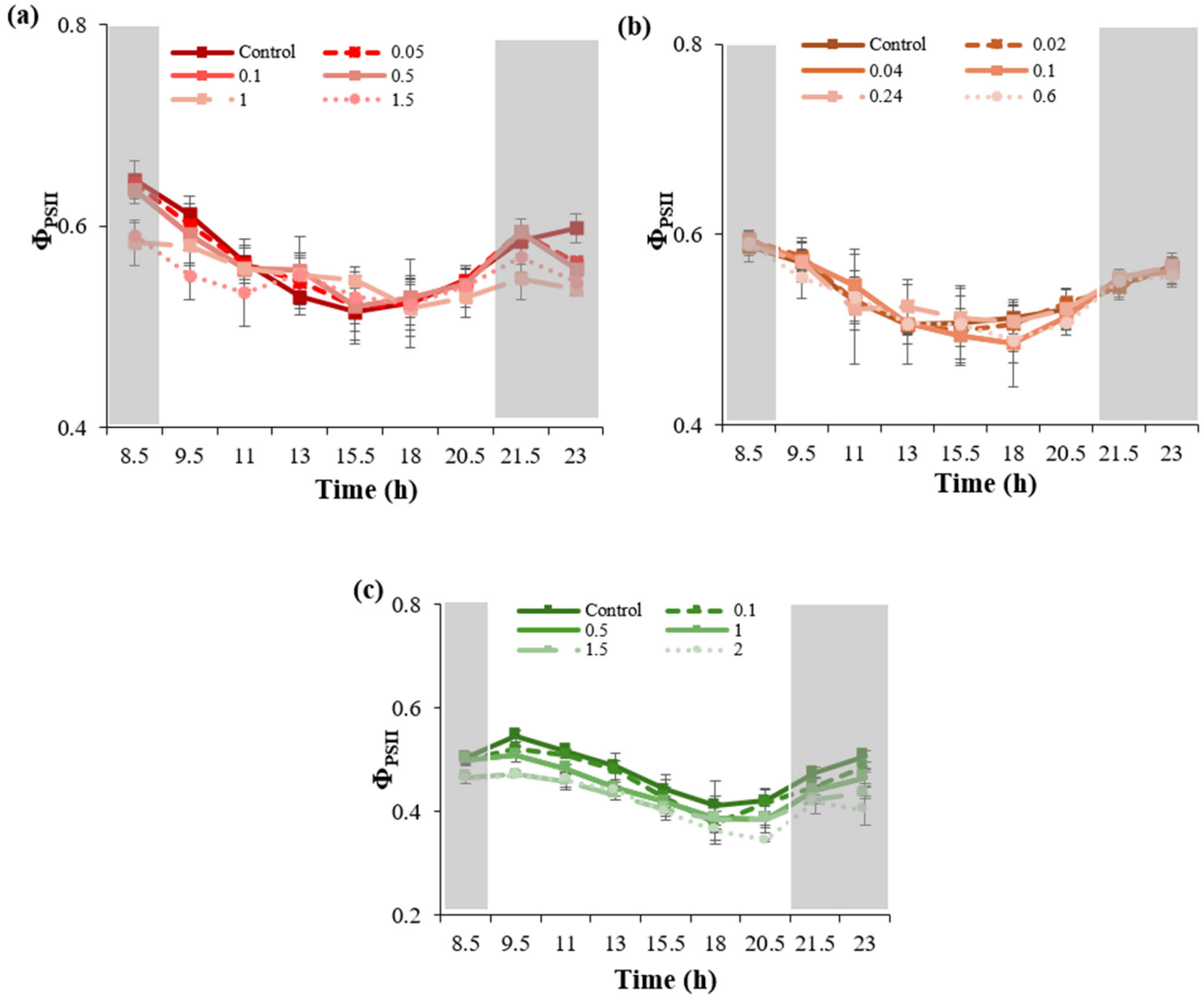


**Figure 2.** Effective quantum yield (ΦPSII) of *Chlorella* sp. exposed to different concentrations of (**a**) HQ; (**b**) CFH-12® and (**c**) Phoslock®. Data are expressed as mean values ± SD. Shaded areas represent dark exposure periods.

Due to the biological meaning of the NPQ variable coping with adsorbent stress, a positive value of the effect size means low stress. From Figure 5 it can be seen that a similar pattern exists for HQ, with only positive effects (which means less stress on the algae) at lower adsorbent concentrations (<0.5 g $L^{-1}$). However, for CFH-12® a positive effect has been observed for all concentrations except for 0.04 g $L^{-1}$.

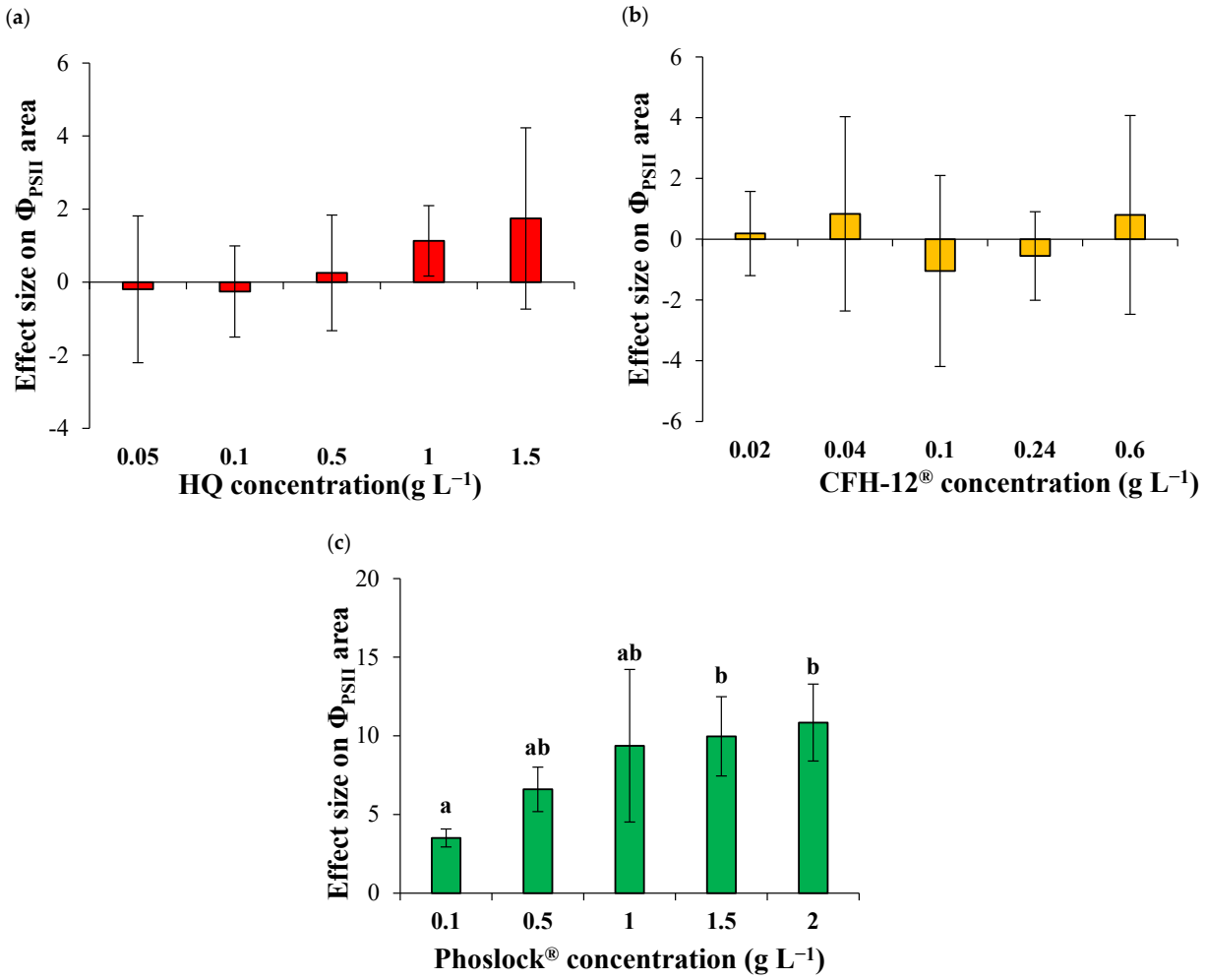


**Figure 3.** Effect size of (**a**) HQ; (**b**) CFH-12® and (**c**) Phoslock® treatments on $\Phi_{PSII}$ area. Data are expressed as mean values ± SD. Significant differences ($p < 0.05$) based on post hoc multiple comparisons are indicated by different letters.

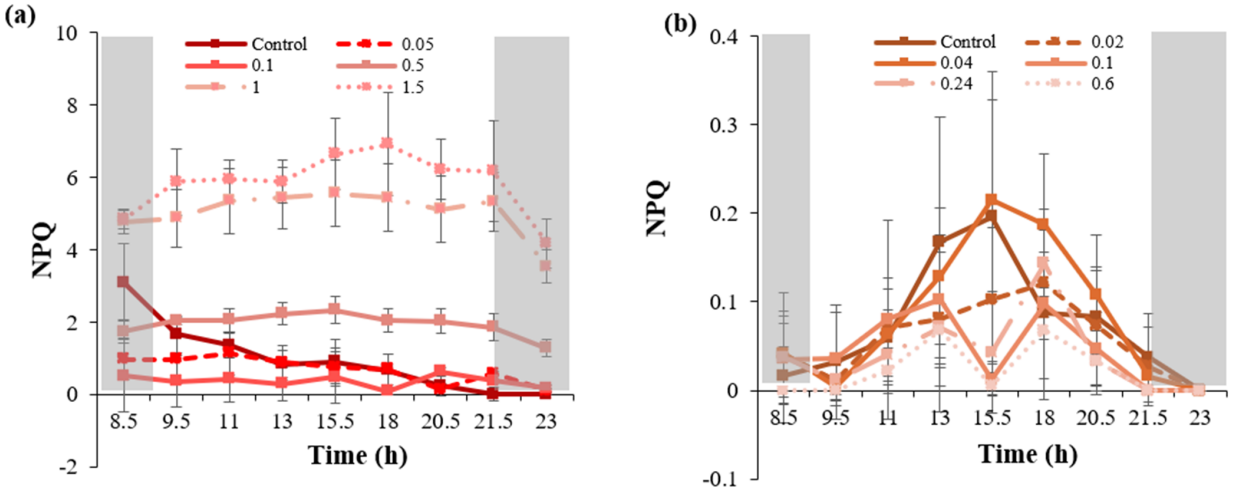


**Figure 4.** Non-photochemical quantum yield (NPQ) of *Chlorella* sp. exposed to different concentrations of (**a**) HQ and (**b**) CFH-12®. Data are expressed as mean values ± SD. Shaded areas represent dark exposure periods.

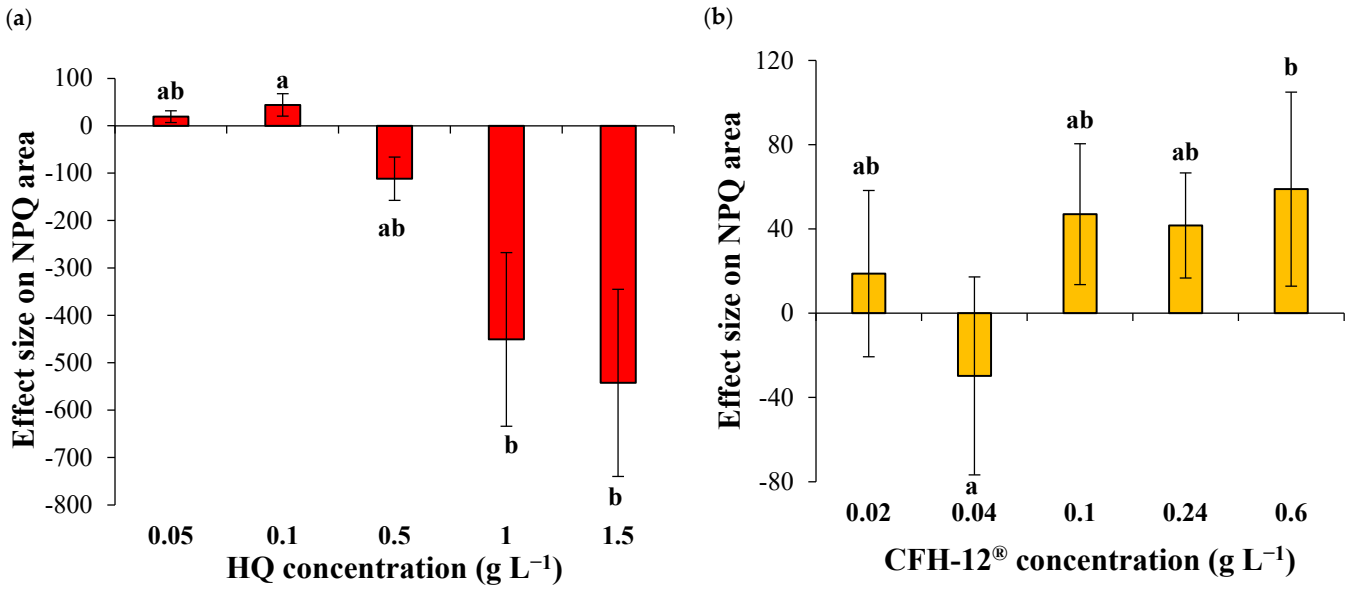


**Figure 5.** Effect size of (**a**) HQ and (**b**) CFH-12® treatments on NPQ area. Data are expressed as mean values ± SD. Significant differences ($p < 0.05$) based on post hoc multiple comparisons are indicated by different letters.

## 4. Discussion

Although various methods are available for restoring eutrophic aquatic ecosystems, none has received universal acceptance as the most effective approach (e.g., [30]). When selecting the P adsorbent to be applied in the chemical inactivation technique (for reducing P internal load), it is essential to take into account not only the maximum capacity to adsorb P or its stability regardless of the physical-chemical conditions but also its harmless nature for aquatic organisms at the dose to be applied in a real scenario. Although there is a wide variety of studies focussed on studying the sublethal effects of P adsorbents (e.g., [24,39,42,59], it is necessary to delve even deeper into these effects.

Overall, our results indicate that there is no strong evidence to infer that neither HQ nor CFH-12® caused an effect on the photosynthetic activity of *Chlorella* sp., since significant effects have only been observed in very specific situations and for adsorbent concentrations so high that are very unlikely to be used in real conditions. Regarding Phoslock®, our results suggest the existence of inhibitory effects on the photosynthetic activity of *Chlorella* sp. for any of the concentrations tested. Therefore, we should reject our initial hypothesis that non-magnetic P adsorbents (as they are not removed from the medium) have more effects on the organisms than magnetic ones since there are substantial differences between the non-magnetic P adsorbents used in this study (CFH-12® and Phoslock®). As the primary producer, the main physico-chemical and chemical drivers of *Chlorella* sp. photosynthetic activity are light and nutrient availability. In relation to light conditions, all P adsorbents drastically caused a significant reduction in light availability. For HQ results, significant differences between the control and the two highest concentrations (1 and 1.5 g $L^{-1}$) in the photosynthetic efficiency were observed only during the dark period. In addition, the significant effects of the two highest concentrations during the light period on NPQ may be due to the synergistic effects of HQ addition and light incidence that increased the stress on *Chlorella* sp. at those concentrations. The high values in NPQ even during dark conditions and the lowest values in $\Phi_{PSII}$ during the dark period may indicate an enhancement of the sensitivity of *Chlorella* sp. due to HQ addition. In fact, there are several studies that show additive or synergistic interactions between different compounds such as metals and light conditions [60]. More specifically, an increase in the effects of metals on the photosynthesis of different algal species when combined with light irradiation has been observed [61,62]. At this point, and despite the inherent limitations for these comparisons

(BBM vs. natural lake water), it is interesting to note that Funes et al. [30] recorded that, although turbidity measurements were not included, the addition of 1.4 g HQ $L^{-1}$ did not cause significant changes in the concentration of total suspended solids with respect to the controls, in experiments carried out with water from a eutrophic natural aquatic ecosystem. Therefore, if even in the very unfavorable conditions that are included in the present study (linked to the high turbidity caused by HQ), negative effects have not been observed on the photosynthetic efficiency of *Chlorella* sp., it is expected that in a real application scenario, this P adsorbent does not cause any negative effect on photosynthetic efficiency. For CFH-12® and Phoslock®, it is especially interesting that during light exposure, and despite the notable increase in water turbidity, photosynthetic efficiency was not significantly affected after the addition of CFH-12®; however, an opposite pattern for Phoslock® was observed where a significant decrease in photosynthetic efficiency was found but just for the highest concentration at certain moments. Similarly, the effect size showed the existence of an inhibition of photosynthetic efficiency in all treatments. That inhibition in the PSII may be a consequence of the extremely high turbidity values caused by Phoslock® addition that would block the radiation causing a "shading effect". In fact, turbidity values for this adsorbent were the highest among all P adsorbents and very similar to those obtained by van Oosterhout and Lürling [39]. This result is consistent with zero values reported for NPQ in the present study for all Phoslock® treatments since NPQ is expected to be zero or close to zero in darkness [63,64].

On the other hand, it is striking that Phoslock®, which has the bigger particle size, is the most harmful adsorbent. Even though some authors have shown that differences in toxicity may be linked to particle sizes by promoting the aggregation/sedimentation of the particles [65], in the present study the reduction in photosynthetic activity is mainly related to the generation of a color that prevents the penetration of solar radiation. Anyway, the particle sizes of the adsorbents used must be considered in order to distinguish among possible long-term effects although, as we previously mentioned, the smallest adsorbent: HQ was removed from the medium after 24 h of contact so long-term effects are not expected for this adsorbent. Contrary to our results, Kang et al. [41] did not observe any effect of Phoslock® (1 g $L^{-1}$ as the highest concentration) on the photosystem II efficiency of *Microcystis aeruginosa,* although these authors noted a decline in the microcystins/Chl a ratio related to a reduction in light availability at higher Phoslock® doses.

Regarding changes in nutrient availability after P adsorbents addition, all P adsorbents, except CFH-12®, caused a significant reduction in DIP concentration. Despite this reduction, no nutrient deficiency was found in any of the treatments, as DIP concentrations always exceeded the minimum concentrations needed for phytoplankton growth (3 µg P-DIP $L^{-1}$; Reynolds [66,67]). At this point, it is relevant to consider that, depending on aqueous chemical composition, P adsorbent addition may significantly affect not only P but also Fe availability which could cause indirect effects on aquatic organisms. In the present study, significantly higher Tot-$Fe_{dis}$ concentrations, compared to the control, have only been found when HQ was added at concentrations greater than 0.5 g $L^{-1}$. Our results confirm those obtained by Álvarez-Manzaneda and de Vicente [42] who measured a drastic increase in Tot-$Fe_{dis}$ concentrations when HQ concentrations were higher than 0.5 g $L^{-1}$. Similarly to those values recorded by Álvarez-Manzaneda and de Vicente [42], Tot-$Fe_{dis}$ concentrations are much higher than others reported by Funes et al. [29] when adding HQ under anoxic and oxic conditions, to natural waters. Therefore, and as Álvarez-Manzaneda and de Vicente [42] suggested, our results confirm that HQ is better dissolved in BBM (algal growth medium) than in natural waters. Anyway, these high Tot-$Fe_{dis}$ concentrations did not have any significant effect on either algal concentration or photosynthetic efficiency. However, the addition of CFH-12® which is a non-magnetic Fe oxide, did not cause any Fe release to the medium. Similarly, Fuchs et al. [26] performed a laboratory experiment using undisturbed sediment cores from three Danish lakes, under anoxic conditions and found that CFH-12® addition did not significantly affect Fe efflux relative to the untreated cores.

Apart from light and nutrient availability, other factors affecting microalgae growth may include pH or aeration of the system [68]. All treatments and controls were kept well oxygenated throughout the experiment (DO > 80%), while significant changes in pH are relevant to be mentioned. HQ and Phoslock® addition conducted a significant pH increase while no effect on pH was observed after CFH-12® addition. Previous studies have also detected the same tendency after HQ addition but no significant effects on *Chlorella* sp. growth associated with pH changes were observed [42]. Similarly, Álvarez-Manzaneda et al. [38] carried out an experiment with *Raphidocelis subcapitata* in contact with HQ and these authors found that pH values remained within the validity criteria specified by the guideline [49]. Nevertheless, additional research is required as some authors have observed negative effects on the photosynthetic activity of different algal species when pH increases [69]. However, these effects were found when pH was higher than 9 [70], a much larger value than that recorded in the present study. Regarding Phoslock®, van Oosterhout and Lürling [39] recorded a pH reduction after 2 h of contact time with 3.2 g $L^{-1}$. By contrast, other studies have found no significant changes in pH after Phoslock® addition [22,51]. Despite pH changes recorded in the present study, pH ranged from 7.3 to 8.5, which are similar values to those used in standardized experiments [42,71] and also in the range of the values reported by Rachlin and Grosso [72] as harmless for *Chlorella vulgaris*, so negative effects of the pH on *Chlorella* sp. are not expected.

In this work, no significant effect has been observed on the algal concentration when adding HQ and CFH-12® while a significant increase in algal concentration was found for Phoslock® concentrations higher than 1 g $L^{-1}$. For the case of HQ, it is striking that for the same algae (*Chlorella* sp.) and for the same P adsorbent, Álvarez-Manzaneda and de Vicente [42] observed algal growth inhibition (please note that they both are different endpoints) higher than 50% for HQ concentrations above 0.15 g $L^{-1}$ ($EC_{50}$ was 0.15 g $L^{-1}$). Actually, those authors measured an average inhibition of 83% for the highest HQ concentration (2 g $L^{-1}$) while no changes in algal concentration have been found in the present study for 1.5 g HQ $L^{-1}$. One of the possible explanations for the observed differences could be the different turbidity values recorded in both studies as turbidity values were much lower in the present study than those measured by Álvarez-Manzaneda and de Vicente [42] who measured up to 1000 NTU at 1.5 g $L^{-1}$. Additionally, it is important to note that, even though the algae used in both experiments was the same, the experimental performance was very different regarding the duration of the experiment and the initial algal concentration in the test vessels. The shorter duration of this experiment (14.5 h) in comparison with that carried out by Álvarez-Manzaneda and de Vicente [42] which extended for 72 h, may be the main reason for the different results obtained for both experiments since toxicity depends not only on dose but also on exposure time [73]. In fact, several authors pointed out that effective and lethal concentrations decrease when exposure time increases [74,75].

All in all, our results are more consistent with those observed for *Raphidocelis subcapitata* by Álvarez-Manzaneda et al. [38] who found $EC_{50}$ of 1.50 g HQ $L^{-1}$. Again, it is important to take into account the difficulty of comparing the results obtained in different studies due to, among others, interspecies differences in sensitivity [76] and also to methodological aspects such as contact time and different medium composition as described by Chen et al. [77] and Millington et al. [78]. Concerning CFH-12®, there are very few studies carried out to evaluate the toxicity on phytoplankton (Álvarez-Manzaneda et al. [38]). While those authors obtained for *R. subcapitata*, an $EC_{50}$ of 0.42 g $L^{-1}$ after 72 h of contact time, in the present study no significant effect on algal concentration was found between 0.6 g $L^{-1}$ and controls after 14.5 h. In the case of Phoslock®, we have observed an unexpected and significant increase in algae concentration for concentrations higher than 1 g $L^{-1}$. Contrarily, van Oosterhout and Lürling [39] noted negative effects of Phoslock® on the growth rate of *Scenedesmus obliquus* and *Microcystis aeruginosa* in concentrations higher than 0.5 g $L^{-1}$ while Álvarez-Manzaneda et al. [38] did not observe any inhibition patterns in an experiment carried out with with *R. subcapitata.*

Therefore, if we compare the results of the effects of the studied P adsorbents on photosynthetic efficiency and on algal concentration, strong discrepancies emerge. Similar results were found by Ouyang et al. [79] who observed different responses in growth and photosynthesis of *C. vulgaris* after their contact with zinc and cadmium. By contrast, other studies evidenced a link between the inhibitory effect of different toxicants on algal growth and the suppression of photosynthesis [80,81].

Lastly, we aim to compare the effect of the studied P adsorbents in a real application scenery such as the well-studied hypereutrophic Honda wetland of the Albufera de Adra (Almería, Spain; e.g., [29,82]). Based on the mass of sedimentary mobile P in the lake and the maximum P adsorption capacity of each adsorbent, and even considering an overdose linked to the possible chemical interferences in P adsorption mechanisms (e.g., [29]), it is likely that CFH-12® and HQ doses would not cause any negative effects on photosynthetic efficiency while Phoslock®, by limiting light availability, will drastically reduce it.

## 5. Conclusions

Considering that eutrophication is a worldwide and growing environmental problem, it is imperative to prioritize efforts to study the best method to face eutrophication before its use in a whole-lake application. In this context, this study was focused on the assessment of the toxicological effects of different P adsorbents (one magnetic and two non-magnetic adsorbents) on the growth and photosynthetic activity of *Chlorella* sp. Basically, our results indicate that there is no strong evidence to infer that neither HQ nor CFH-12® caused an effect on the photosynthetic activity of *Chlorella* sp., since significant effects have only been observed in very specific situations and for adsorbent concentrations so high that are very unlikely to be used in real conditions. If we take into account the abiotic factors that could affect photosynthetic efficiency, the absence of effect in the presence of these two adsorbents was unexpected taking into account the high turbidity recorded after their addition, although in no case there was P limitation of the primary production. Regarding Phoslock®, our results suggest the existence of inhibitory effects on the photosynthetic activity of *Chlorella* sp. for any of the concentrations tested. Therefore, we should reject our initial hypothesis that non-magnetic P adsorbents (as they are not removed from the medium) have more effects on the organisms than magnetic ones since there are substantial differences between the non-magnetic P adsorbents used in this study (CFH-12® and Phoslck®). When comparing the effects of P adsorbents on algal concentration and on photosynthetic activity based on previous studies reported in the literature, it is important to take into account the inherent difficulties due to, among others, interspecies differences in sensitivity and also to methodological aspects such as contact time and different medium composition. While the results suggest that based on the mass of sedimentary mobile P in the lake and the maximum P adsorption capacity of each adsorbent, under controlled conditions, CFH-12® and HQ doses are unlikely to negatively impact photosynthetic efficiency, the potential real-life application of these adsorbents requires further study. Environmental conditions in a real ecosystem introduce additional variables, such as chemical interactions, changes in physico-chemical variables, and long-term effects, which are difficult to simulate in a laboratory experiment. Moreover, while Phoslock® reduced light availability and subsequently impacted the photosynthetic efficiency of *Chlorella* sp., the extent of these effects in a natural ecosystem remains uncertain and should be further examined. Therefore, caution is warranted when extrapolating these findings to real application sceneries, as further research in more complex ecological contexts is necessary to validate these conclusions.

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