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Evidence for Links between Feeding Behavior of *Daphnia magna* and Water Framework Directive Elements: Case Study of Crestuma-Lever Reservoir

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Abstract: The Water Framework Directive (WFD) is the European legislation on water policy that assesses water quality according to time-consuming metrics and specific taxonomic needs. In this sense, the objective of this study was to evaluate the sensitivity of *Daphnia magna* feeding rate assays to assess/discriminate the water quality of heavily modified and artificial water bodies. Monthly, for one year, the quality of the Crestuma-Lever reservoir (in two sampling sites: Crestuma and Marina) was assessed using physical, chemical, and biological (concentration of chlorophyll-*a*) elements proposed by the WFD. Additionally, *D. magna* was exposed to the collected water samples and the feeding rates were evaluated to include an ecosystem function evaluation in water quality assessment. The WFD metrics showed that, overall, the Crestuma-Lever reservoir has a rating of Good to Moderate Ecological Potential, regardless of site. Feeding rates varied with the sampling site and months, demonstrating that feeding behavior evaluation is a sensitive tool that allows discriminate potential effects indicative of a lower water quality. This finding was recorded by the decrease in the feeding rate (Crestuma: May, Sept; Marina: Nov, Jan, May), despite the WFD classification, and once the organisms are affected by the components present in the water samples.

Keywords: water quality; ecotoxicology; bioassays; water fleas; freshwaters

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1. Introduction

The Water Framework Directive (WFD) (Directive 2000/60/EC of the European Parliament and of the Council, adopted in September 2000) is the main instrument of the European Union's Water Policy [1]. It establishes a framework for community action to improve and protect the quality of inland surface waters, transitional waters, coastal waters, and groundwater. At the same time, the WFD ensures that all water-dependent ecosystems function correctly and that all uses of water can only be accepted if they do not jeopardize the proper functioning of ecosystems [1]. According to this approach, the main objectives established in the WFD are to achieve a good ecological status for all surface waters (rivers, lakes, coastal and transitional waters) and groundwater, but also to achieve a good ecological potential for heavily modified and artificial water bodies (e.g., reservoirs), through the implementation of programs of measures specified in the Hydrographic Region Management Plans [1]. For all the aquatic ecosystems, the WFD defines a set of elements to quantify in order to assess the water quality. Namely, for reservoirs, this directive defines specific hydromorphological (hydrological regime, morphological conditions), physical, and chemical (e.g., dissolved oxygen and pH) elements to support biological elements [e.g., phytoplankton biomass-chlorophyll-*a* concentration (mg/m^3) and total biovolume (mm^3/L)] to assess the ecological potential.

To introduce a more integrative and ecological perspective in the assessment of water quality, it is essential to obtain an overview of the stresses and fluctuations that occur in aquatic ecosystems [2]. In order to evaluate the water quality in a more realistic scenario, and to complement the parameters proposed by the WFD, it is necessary to include new tools for characterizing and qualifying the status of water bodies. Currently, research is focused on real-scale scenarios using remote sensing studies that allow the evaluation of water quality as well as water quantity (e.g., [3,4]). On the other hand, some studies have emerged with the objective of proposing the use of different biological tools to assess water quality [2,5–7]. Indeed, ecotoxicological assays have been indicated as a sensitive tool to complement the assessments proposed by the WFD. This ecotoxicological approach showed that biological responses to a complex mixture (natural waters), which can contain thousands of substances, are impossible to all be quantified are sensitive to discriminate water quality [2,6]. In this sense, standardized ecotoxicological assays with aquatic organisms of different trophic levels (such as microalgae, macrophytes, and microcrustaceans) have often been proposed as good indicators and are sensitive to water pollution [5,6]. Pinto et al. [2] demonstrated that acute assays with *Raphidocelis subcapitata* can be a potential tool to assess the ecological potential of a reservoir. Rodrigues et al. [7] found that the use of the parameters proposed by the WFD combined with ecotoxicological tools, such as assays with *Daphnia magna* (e.g., feeding rate assays), allowed a more realistic assessment of the ecological potential of three Portuguese reservoirs (Miranda, Pocinho, and Alqueva).

Daphnia magna is a small zooplanktonic crustacean, used as a standard species in ecotoxicological studies [8,9], namely in bioassays required by national and European legislation for the ecotoxicological assessment of new chemical agents, urban and industrial effluents, and freshwater ecosystems [10]. The diversity of the quantity of the diet provided to *Daphnia*, and other cladocerans, has been the subject of some studies (e.g., [11]), through the effects evaluation of feeding behavior assays after exposure to different stressful situations (e.g., pharmaceuticals, pesticides, and metals) [2,12,13]. These assays offer a very sensitive and ecologically relevant diagnosis for use in water quality assessment [12] as several authors have already demonstrated that the presence of contaminants and other seston components can affect the feeding rate of these organisms [14,15]. This renders feeding behavior a potential and important parameter to be included in a water quality monitoring program [14]. At the same time, this ecotoxicological tool can also be used to assess the functioning of ecosystem services. Feeding rate is an individual response, physiologically linked with effects on growth and reproduction, representing one of the organisms' most important biological processes [6]. Several studies have already showed that this bioassay measures a responsive endpoint, feeding behavior, to different stresses and environmental conditions (e.g., [16]), providing crucial information regarding the evaluation of the ecosystem function [14].

According to this background, the present study aimed to evaluate the potential of *Daphnia magna* feeding rate assays to assess the water quality of a reservoir (Crestuma-Lever, north of Portugal), as a complement to the parameters proposed by the WFD.

2. Materials and Methods

2.1. Study Area and Sampling Procedure

The Crestuma-Lever reservoir (Figure 1) is located 10 km north of the city of Porto, downstream of the Douro River, at the eastern end of the Municipality of Vila Nova de Gaia. The Crestuma-Lever reservoir was formed in 1985, after the dam was closed [17]. The freshwater flow into the Crestuma-Lever reservoir is controlled by hydroelectric needs on both sides of the border and the need for irrigation in Spain, thus introducing annual, seasonal, daily, and hourly variations to the natural flow regime [18] (annual average integral flow 7,388,900 × 1000 m^3 [19]). This reservoir is essential for industrial and technological development, as well as for navigation, throughout the year, due to the existence of the marine Angra do the Douro. It is also used to supply drinking water in urban and rural areas [20] through the Lever Water Treatment Plant (WTP). The Crestuma-Lever reservoir is a reserve freshwater mass used to supply water to Porto, Gaia, and neighboring municipalities, covering Gondomar, Santa Maria da Feira, Castelo de Paiva, Penafiel, and Marco de Canaveses.

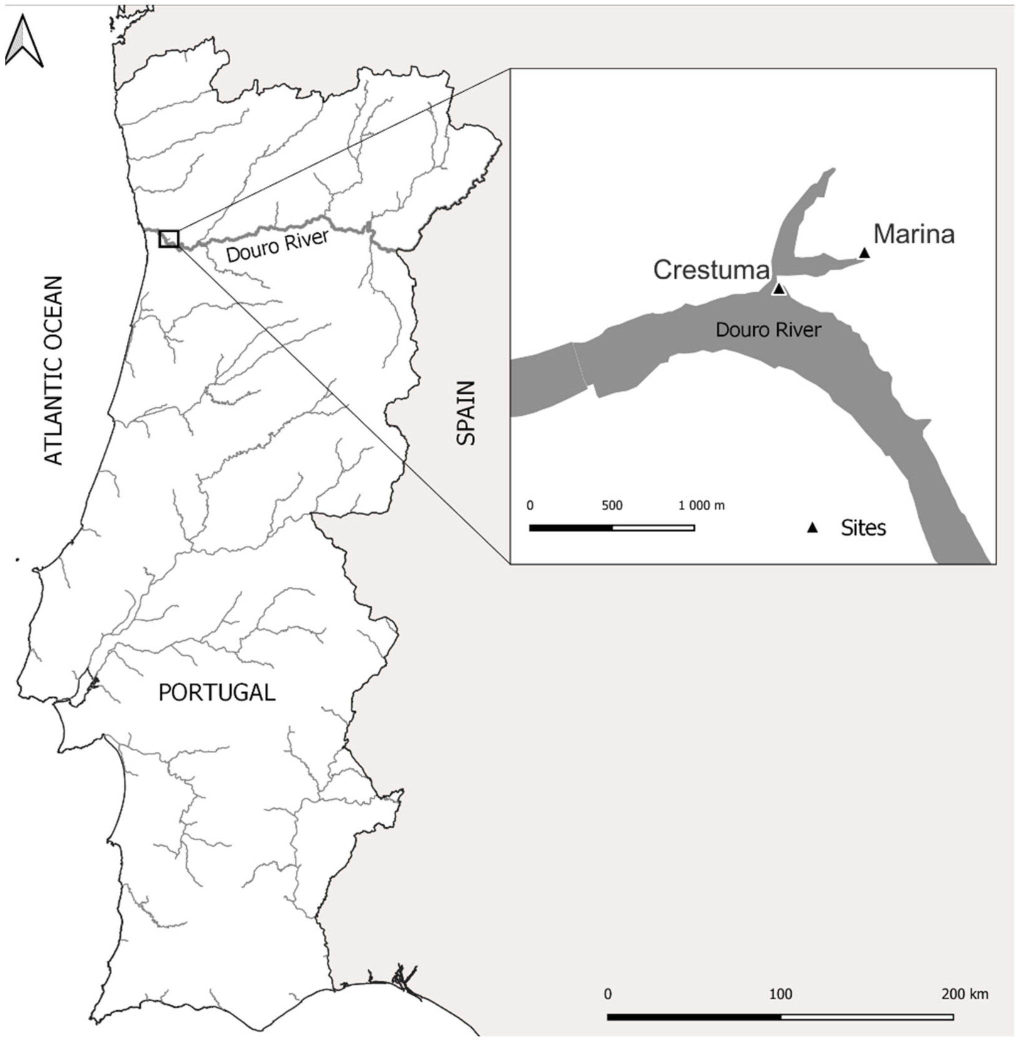


Figure 1. Map location of the study area with the sampling sites positions in the Douro River: the Crestuma-Lever reservoir: Crestuma (41°04′38.2″ N, 8°28′20″ W) and Marina (41°04′45″ N, 8°27′58″ W).

To conduct the present study, two sampling sites were chosen in the Crestuma-Lever reservoir: firstly, in Crestuma, in-front of the Lever WTP (samples were collected in a floating pier); secondly, in Marina, on the right-side of the Douro River, the marine Angra do Douro (sample were collected from the margin) (Figure 1). In each sampling site, monthly and in the morning period, over a year (October 2012 to September 2013) 6 L of water samples were collected in plastic bottles and transported to the laboratory, in the dark at 4 °C, for further analysis and bioassays. Additionally, in situ, several physical and chemical parameters were measured [transparency (Secchi disk), pH, oxygen (mg/L and %), conductivity (µS/cm), total dissolved solids (m^{-1}), and temperature (°C)], using a multiparameter probe (HI 9828 multiparameter meter with GPS). In the laboratory, the physical and chemical characterization of the water samples was conducted on the sampling day (*see* Section 2.2). The feeding rate assays were carried out with *Daphnia magna* and began within a maximum period of 24 h after the water samples were collected (*see* Section 2.3).

2.2. WFD Approach—Physical and Chemical, and Biological Elements Analysis

In the laboratory, the water samples were processed according to the parameters proposed by the WFD for heavily modified water bodies. The quantification of nutrients [nitrites (mg NO_2/L), nitrates (mg NO_3/L), ammoniacal nitrogen (mg NH_4/L), and total phosphorus (mg P_{total}/L)] were measured in the water samples, using a bench spectrophotometer (model C200 from Hanna Instruments). The turbidity level, as well as the five day biochemical oxygen demand (BOD_5), were also quantified according to standard guidelines [21]. According to the WFD, the physical and chemical parameters are classified as Good or More, and Moderate ecological potential, based on the corresponding environmental quality standards (EQS) [22].

In 2012/13, one of the biological indicators that the WFD proposed for phytoplankton biomass is the concentration of chlorophyll-*a* (mg/m^3). According to this, the concentration of chlorophyll-*a* (mg/m^3) in water samples was measured according to Lorenzen [23]. Based on the WFD guidelines, for the determination of the ecological potential (EP), the results of chlorophyll-*a* were expressed based on the corresponding environmental quality standards (EQS). Taking into account the sampling periods of the present study (2012–2013), the classification of the ecological potential of the Crestuma-Lever reservoir (considered at that time as a northern type of reservoir) was carried out using only the chlorophyll content as an indicator and considering the 1st Planning Cycle (2010–2015) [21].

The final ecological potential of each sampling period and site was determined regarding the previous classification of physical and chemical parameters and biological indicators and expressed according to the Water Framework Directive scale: high, good, moderate, poor, bad [1].

2.3. Daphnia Magna—Feeding Rate Assays

The feeding rate assays were carried out monthly with the 2 water samples collected (Crestuma and Marina) and were conducted according to [12]. In each assay, individuals of *D. magna* with 4 or 5 days old and born between the 3rd to 5th broods were exposed for 24 h to 120 mL of each water sample (Crestuma and Marina). For each water sample, a feeding assay with 5 replicates, with 5 neonates in each replicate, was performed, where *R. subcapitata* (until the final concentration of 3×10^5 cells/mL) was added.

A control (Ctl) with ASTM "hard water" culture medium [24] and a blank with the water samples (without organisms) was constructed to remove the variability of possible algal growth during the assay period. Before the addition of the neonates to each condition, the absorbance was measured at 440 nm (Abs 0) with a spectrophotometer. The assays were conducted in a climatic chamber (Model F10000 EDTU) with a controlled temperature (20 $\pm$ 2 °C) and in total darkness to prevent algal growth. After 24 h of the assay, the organisms were removed from the assay conditions, and the absorbance (Abs 24) was measured again (also in Ctl and blanks). The absorbance values (Abs) were transformed into cells/mL using Equation (1), and the results were expressed in feeding rate, according to Equation (2) [25]:

$$\text{cells/mL} = -168857 + \text{Abs} \times 10^7 \quad (1)$$

$$\text{FR} = \left(\frac{\text{V} \times (\text{C } 0 - \text{C } 24)}{t}\right)/\text{n} \quad (2)$$

where FR = feeding rate (cells/organisms/h); V = volume of medium in the test vessel (mL); C 0 = initial cell concentration (numbers/mL); C 24 = the final cell concentration (numbers/mL); *t* = duration of the experiment (h); n = number of organisms per vessel.

The results of the feeding rate were analyzed using a one-way analysis of variance (one-way ANOVA), separately, for each sampling month. Whenever differences in the one-way ANOVA ($p < 0.05$) were recorded, a Dunnett test was performed to extract the differences between the FR result (Crestuma and Marina) and the control (Ctl).

3. Results and Discussion

3.1. Physical and Chemical, and Biological Elements

The general physical and chemical parameters quantified in the Crestuma and Marina water samples are presented in Tables 1 and 2, respectively. In general, both sites showed the same ecological potential regarding the results of the physical and chemical parameters, differing only in July, where Marina presented a moderate ecological potential (MEP) due to the low O_2 value (43.6%—Table 2). Moreover, Crestuma and Marina presented a MEP in October, March, June, and August, due to the low values of oxygen recorded (Table 2) and the high concentration of total phosphorus (only in October), which were not within the environmental quality standards (EQS) imposed by the WFD. Both sites presented good ecological potential (GEP) in the remaining sampling periods. Regarding the conductivity values, these remained below 300 µS/cm, while the temperature varied according to the seasons, ranging between 9.60 and 25.0 °C in Crestuma and between 12.0 and 28.2 °C in Marina. The pH values recorded in the present study were always within the reference limit (6.00–9.00) and close to neutrality; these data are in agreement with the historical water pH values for the Crestuma-Lever reservoir [26]. Transparency measurements through the Secchi disk carried out at the Marina sampling site indicate high transparency, however, the water column rarely exceeded 50 cm (Table 2), allowing us to observe the bottom in all sampling months. For the Crestuma site, the transparency only reached a maximum of 3.00 m, which indicates high turbidity due to suspended materials in the water column (high amount of seston, Table 1).

Table 1. Crestuma results of the general physical and chemical parameters measured *in situ*, and biochemical oxygen demand (BOD_5), nitrates (NO_3^-), nitrites (NO_2^-), ammoniacal nitrogen (NH_4), and total phosphorus (P_{total}). The biological element phytoplankton Chlorophyll-a (Chl-a) concentration is also presented. The Ecological Potential was classified according to [22]. BDL—Below Detection Limit. Bold values stand for outside the established environmental quality standards (EQS) established by WFD for northern Portuguese reservoirs [27].

Physical and Chemical Parameters	EQS	Crestuma—Sampling Period											
		Oct12	Nov12	Dec12	Jan13	Feb13	Mar13	Apr13	May13	Jun13	Jul13	Aug13	Sept13
Temperature (°C)		21.7	17.2	13.1	12.2	10.0	9.60	12.0	14.8	18.5	22.5	25.0	24.0
pH	6.00–9.00	7.70	7.80	7.90	7.70	8.20	8.60	7.20	9.00	8.20	8.80	7.70	8.80
Conductivity(µS/cm)		270	250	200	187	145	196	153	191	188	255	293	300
Dissolved O_2 (mg/L)	≥5.00	6.30	7.30	7.60	8.20	10.6	**3.70**	8.50	10.0	9.50	10.7	**1.70**	7.30
Dissolved O_2 (%)	60.0–120	**59.0**	64.0	66.0	72.2	93.0	**32.5**	99.3	90.9	**27.0**	120	**14.5**	87.0
TDS (mg/L)		130	230	290	55.0	60.0	98.0	81.0	95.0	118	127	146	147
Turbidity (m^{-1})		0.001	0.003	0.006	0.007	0.063	0.007	0.009	0.009	0.004	0.001	0.001	0.001
Secchi disk (m)		2.30	2.00	2.20	2.30	2.00	2.50	3.00	1.30	2.00	2.20	2.20	2.10
BOD_5 (mg/L)		2.20	2.10	1.90	2.40	1.50	2.00	2.30	1.20	1.80	1.30	1.30	0.800
NO_2^- (mg/L)		BDL	0.040	BDL	BDL	0.670	0.200	0.300	0.030	BDL	BDL	BDL	0.330
NO_3^- (mg/L)	≤25.0	BDL	BDL	BDL	BDL	5.32	6.94	1.18	4.43	0.050	0.100	0.100	BDL
NH_4 (mg/L)		0.050	0.040	0.030	BDL	BDL	0.060	0.010	BDL	BDL	BDL	0.080	0.050
P_{total} (mg/L)	≤0.050	0.070	0.010	BDL	BDL	BDL	BDL	BDL	0.030	BDL	BDL	0.020	0.030
Ecological Potential (Physicochemical)		Moderate	Good or More	Good or More	Good or More	Good or More	Moderate	Good or More	Good or More	Moderate	Good or More	Moderate	Good or More
Chl *a* (mg/m^3)	9.50	9.26	1.07	**10.3**	3.74	0.800	1.19	2.14	7.48	1.18	0.800	0.330	0.530
Ecological Potential (Biological)		Good or More	Good or More	Moderate or Less	Good or More	Good or More	Good or More	Good or More	Good or More	Good or More	Good or More	Good or More	Good or More
Final Ecological Potential		Moderate	Good	Moderate	Good	Good	Moderate	Good	Good	Moderate	Good	Moderate	Good

Table 2. Marina results of the general physical and chemical parameters measured in situ, and biochemical oxygen demand (BOD_5), nitrates (NO_3^-), nitrites (NO_2^-), ammoniacal nitrogen (NH_4), and total phosphorus (P_{total}). The biological element phytoplankton [Chlorophyll-a (Chl-a) concentration. The Ecological Potential was classified according to [22]. BDL—Below Detection Limit. Bold values stand for outside the established environmental quality standards (EQS) established by WFD for northern Portuguese reservoirs [27].

Physical and Chemical Parameters	EQS	Marina—Sampling Period											
		Oct12	Nov12	Dec12	Jan13	Feb13	Mar13	Apr13	May13	Jun13	Jul13	Aug13	Sept13
Temperature (°C)		21.6	15.8	13.5	13.6	12.0	13.6	15.2	15.9	17.7	28.2	27.2	25.8
pH	6.00–9.00	7.30	7.90	7.30	7.00	7.90	8.30	7.80	8.40	8.7.0	8.10	7.80	7.70
Conductivity(µS/cm)		300	280	220	157	105	101	132	179	162	237	289	258
Dissolved O_2 (mg/L)	≥5.00	6.80	7.40	7.10	9.30	9.00	**4.90**	7.70	10.4	8.70	5.00	**1.80**	6.50
Dissolved O_2 (%)	60.0–120	**59.0**	65.0	62.0	81.7	83.0	**38.5**	83.4	103	**27.5**	**43.6**	**15.7**	78.3
TDS (mg/L)		150	26.0	21.0	78.0	10.0	50.0	43.0	90.0	96.0	109	143	128
Turbidity (m^{-1})		0.004	0.009	0.012	0.009	0.097	0.060	0.002	0.002	0.009	0.005	0.005	0.019
Secchi disk (m)		<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500	<0.500
BOD_5 (mg/L)		2.70	2.20	2.6.0	2.10	0.400	0.900	2.00	2.00	2.10	1.20	1.20	0.600
NO_2^- (mg/L)		BDL	BLD	BLD	BLD	2.41	0.030	BLD	BLD	BLD	BLD	BLD	0.330
NO_3^- (mg/L)	≤25.0	1.51	0.110	0.530	0.500	0.890	1.52	BLD	4.43	0.130	0.160	0.170	BLD
NH_4 (mg/L)		0.080	0.050	0.040	BLD	BLD	0.040	0.010	0.010	BLD	BLD	0.060	0.360
P_{total} (mg/L)	≤0.050	0.050	BLD	BLD	BLD	BLD	BLD	BLD	BLD	0.010	0.010	0.030	0.020
Ecological Potential (Physicochemical)		Moderate	Good or More	Good or More	Good or More	Good or More	Moderate	Good or More	Good or More	Moderate	Moderate	Moderate	Good or More
Chl *a* (mg/m^3)	9.50	**25.6**	**20.8**	**16.0**	**22.1**	**18.2**	8.54	**15.0**	**15.7**	**22.4**	5.34	8.54	**14.2**
Ecological Potential (Biological)		Good or More	Moderate or Less	Moderate or Less	Moderate or Less	Moderate or Less	Moderate or Less	Good or More	Moderate or Less	Moderate or Less	Moderate or Less	Good or More	Good or More
Final Ecological Potential		Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate

The results of the nutrient concentrations (Tables 1 and 2) were below the detection limit (BDL) for almost all of the sampling periods and at both sites. The nitrites values remained relatively low throughout most of the year, which, according to Nisbet and Verneaux [28], means that the water has active auto-debugging (capacity of self-purification of surface waters, through the degradation of organic polluting compounds by the action of microorganisms). Although the nitrate values never exceeded the reference values established by the WFD (≤25.0 mg/L), the nitrate peaks were recorded (in both sampling sites) in the spring months (February to May). This may occur due to the use of fertilizers on an agricultural holding in the area adjacent to or upstream of the reservoir. Through soil leaching processes, these fertilizers and nutrients move into the aquatic ecosystem [29], and in places with shallow depths, as is the case of Marina, they end up accumulating. Moreover, the boat circulation, the increase in water residence time, and the increase in temperature in the spring and summer months may promote an increase in nitrogenous compounds. The substantial increase in the ammonia concentration observed in Marina in the last month evaluated (September) may be related to a large fire that occurred in the surrounding area, and due to the low depth recorded in the Marina sampling site. Ammonia is a direct product of combustion, and as other authors have already described, the concentration of leached ammonia increases in the surrounding areas, namely in aquatic ecosystems, following a fire [30].

Considering the results presented above, the ecological potential for the two sites was determined and, overall, the reservoirs were classified with a moderate to good ecological potential. However, this classification was determined according to the WFD metrics in force at the study time; that is, within those of the 1st Planning Cycle [19], only four physical and chemical parameters (pH, oxygen, total phosphorus, and nitrates) have defined values for environmental quality standards.

Regarding the analysis of the biological elements, phytoplankton was evaluated by taking into consideration the concentration of chlorophyll-*a* (Tables 1 and 2). Chlorophyll-*a* is a photosynthetic pigment present in many photoautotrophic organisms. Its concentration can be used as an indicator of phytoplankton biomass and is, currently, one of the metrics included in the WFD in the context of assessing the status and ecological potential of water bodies [27]. The Crestuma site was classified with a better biological EP, where every month a Good Ecological Potential (GEP) was achieved, with the exception of December, when only a MEP was obtained ([Chl *a*] = 10.3 mg/m^3, above of the EQS = 9.50 mg/m^3). On the other hand, for the Marina sampling site, only three months (March, July, and August) presented a GEP. The lowest values of chlorophyll-*a* concentration (EQS < 9.50 mg/m^3, Table 2) verified in this sampling site can justify this result, in addition to the site characteristics (shallow area) which increase the interactions between sediments and water (e.g., resuspension of nutrients and contaminants in the water column) [24]. Moreover, and according to the limits proposed by the national criteria for assessing the trophic state of reservoirs [31], and considering the concentration of chlorophyll-*a* obtained, Crestuma was classified as oligo- (<2.5 mg/m^3) a mesotrophic (2.5–10 mg/m^3), while Marina was mostly eutrophic (>10 mg/m^3) (Tables 1 and 2). Marina is an area with a longer water retention time and with a high potential to accumulate compounds (e.g., nutrients, leachates), which was reflected in the analyzed biological parameter and, consequently, in the eutrophic classification.

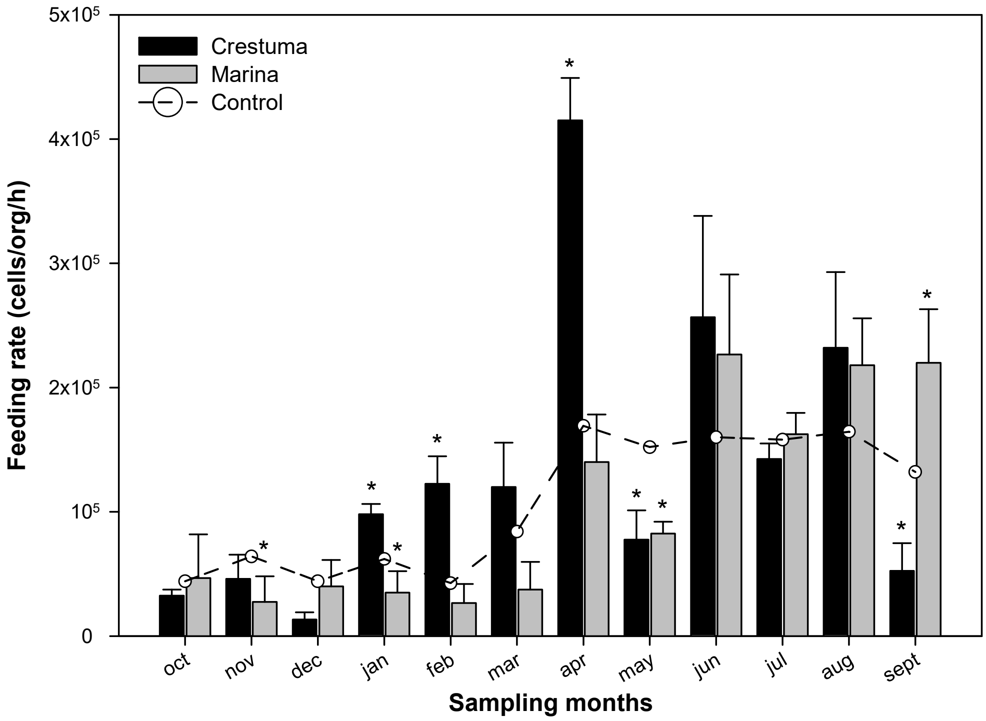
From the analysis of the results of the physical, chemical, and biological elements, the Crestuma site only showed a good final ecological potential in seven (mainly due to the physical and chemical variables) of the 12 months sampled (Table 1), according to the limits established by the WFD. Marina presented a moderate final ecological potential every month (mainly due to the biological elements), possibly as it is an area with less water recirculation within Angra do Douro Marina (Table 2, Figure 1). These two sites are in the final section of the Douro River and the water quality of this reservoir (where the two sites are included) results from the accumulation of influences suffered throughout the hydrographic basin (e.g., diffuse load from agricultural and forestry origin) [17]. From the point of view of the WFD assessment methodology, a water body must have homogeneous characteristics, thus guaranteeing a GEP. The analysis carried out in this study allowed us to verify that the diffuse load of nutrients that flows into the Crestuma-Lever reservoir, from human activities such as agriculture and forestry, influences the water quality of the Crestuma-Lever reservoir [32].

3.2. Feeding Rate Assays

The results of the *D. magna* feeding rate assays with water samples from Crestuma and Marina are shown in Figure 2 and Table 3. Overall, in the aquatic ecosystems, *Daphnia* shows seasonality, reaching a high-density peak during early spring and late winter. This density increase occurs due to the high light intensity, which combined with the availability of nutrients, allows the growth of phytoplankton and, consequently, of herbivory (by part of the zooplanktonic species), as shown by Castro and Gonçalves [33]. This link between the abiotic parameters and biological response could corroborate our results regarding feeding behavior.

Table 3. Summary of one-way analysis of variance (ANOVA) applied to *D. magna* feeding rate results. Bold values stand for significant differences between treatments.

Feeding Rate	Oct12	Nov12	Dec12	Jan13	Feb13	Mar13	Apr13	May13	Jun13	Jul13	Aug13	Sept13
d.f.	2, 9	2, 11	2, 10	2, 11	2, 8	2, 9	2, 10	2, 10	2, 8	2, 10	2, 9	2, 11
F	0.528	4.173	3.242	26.69	24.38	5.929	45.66	7.976	0.755	2.44	1.438	19.95
p	0.607	0.045	0.082	<0.001	<0.001	0.023	<0.001	0.008	0.501	0.137	0.287	<0.001



oct	nov	dec	jan	feb	mar	apr	may	jun	jul	aug	sept
9.26	1.07	10.3	3.74	0.80	1.19	2.14	7.48	1.18	0.80	0.33	0.53
25.6	20.8	16.0	22.1	18.2	8.54	15.0	15.7	22.4	5.34	8.54	14.2

Figure 2. Results of *D. magna* feeding rate after exposures to water samples from Crestuma and Marina collected throughout the sampling period. Data are expressed as mean ± standard error; * stands for significant differences when compared to control in each sampling period (Dunnett test, $p < 0.05$). Table shows the results of chlorophyll *a* concentration recorded in each sampling site and sampling period.

Overall, the Marina water samples induced lower feeding rate values compared to the Crestuma water samples. Regarding the Crestuma results, a significant increase (compared to the control) in the *D. magna* feeding rate was recorded when exposed to the water samples collected in January, February, and April (winter/spring months characterized by a GEP—Table 1), and March (month characterized by a MEP—Table 1). On the other hand, a significant decrease in the feeding rate was observed in the water samples collected in May and September (in Crestuma), despite the GEP classification in these months (Table 1 and Figure 2). Indeed, the biomass phytoplankton indicator quantified [chlorophyll-*a*] does not allow us to know the quality and diversity that occur, which may not be palatable for the higher trophic levels. Queirós et al. [13] evaluated the feeding rates of *Daphnia* spp. after exposure to water samples from the Crestuma reservoir and concluded that, at the end of summer, the seston does not have a good nutritional quality (e.g., due to a higher amount of cyanobacteria) for *D. magna*. These results are in agreement with the results presented here, namely, for the September samples. In addition, in the spring sampling, Queirós et al. [13] did not observe differences in the feeding rates, suggesting that the phytoplankton present in the water samples are of good quality for *D. magna*.

In the Marina samples, a significant decrease in the *D. magna* feeding rate was observed in November, January, and May (months characterized by a MEP—Table 2). As previously mentioned, based on [chlorophyll-*a*], Marina was classified as a eutrophic ecosystem. Eutrophic water bodies promote the development of Cyanobacteria (e.g., *Anabaena* and *Microcystis*), which are unpalatable for *D. magna*, and may produce toxins that can interfere with the metabolism of these organisms [2]. Other studies conducted in Portuguese reservoirs detected that these heavily modified and artificial water bodies may contain organic pollutants [34], and/or pollutants such as zinc and mercury, which inhibit the feeding rate of *D. magna* [6]. Regarding the exception recorded in September, where a significant increase in FR was observed, the month was characterized by a GEP. Despite the low ecological potential regarding the phytoplankton indicator [chlorophyll-*a*], the feeding rates were high, and perhaps in this month the phytoplankton community was more palatable and was used as an additional food source for *D. magna*. *D. magna* is a non-selective filter organism and can use colloidal particles, flagellates, and detritus [35], as a source of nutrients [11,36], and has the ability to select the phytoplankton that are more nutritive and palatable. On the other hand, if phytoplankton is not palatable and has no nutritional quality, *Daphnia* reduces the food intake, resorting to survival strategies (e.g., reducing the number of neonates) [37,38]. In addition, *D. magna*, when subject to different food concentrations, can adjust the filtration rates and choose between appropriate and unsuitable foods [39]. Moreover, below a certain food concentration (the incipient limiting level), the feeding rate is proportional to the food concentration, and the filtering rate (amount of water filtered per unit time) is maximal [36,39]. Above this level, the feeding rate is constant because the filtering rate decreases with the increasing food concentration in the water [36,39]. In the present study, the feeding behavior may also be affected due to the lixiviation products after the intensive fire occurred in September 2013 in the surrounding area of Marina. In this month, a substantial increase in the ammonia concentration was observed, which may have also affected the *D. magna* feeding performance. Indeed, Queirós et al. [13] have already shown that ashes leaching can cause alterations in the nutrient and phytoplankton dynamics in lentic ecosystems.

Regarding *Daphnia* sensitivity, several authors have already demonstrated that variations in *Daphnia* spp. feeding rates could be a response to the exposure of different stresses (e.g., salinity, dissolved contaminants, and seston composition in general, etc.) [12,25,40]. Rodrigues et al. [6] proved that feeding rate assays with *D. longispina* and *D. magna* were sensitive to evaluating the water quality of Portuguese reservoirs. Moreover, the authors also recorded that feeding inhibition and biochemical disturbances (promoting the antioxidant status and lipid peroxidation) were observed when *D. magna* was exposed to natural water from reservoirs. Currently, the assessment of water quality established by the WFD does not consider different time scales, responses at the level of the individual organism, or the functioning of the ecosystem [2]. Thus, in recent years, several studies have focused on different approaches to complement the WFD evaluation, namely using bioassays, such as the feeding rate assays with *D. magna* [2,6,7]. These allow an assessment of ecosystem functioning and are a measure of energy acquisition by the organism as feeding activity is linked to fitness components such as growth rate, fecundity, and survival [41]. *Daphnia* spp. provides an important link between the different trophic levels of freshwater communities, as it constitutes a key intermediate link in the food chain (primary consumer) [42]. Thus, an alteration in the population dynamics of *Daphnia* spp., due to the reduction in the feeding rate, is ecologically relevant as it can predict indirect effects on the structure and functions of different aquatic communities [43].

Therefore, from an ecological and environmental point of view, changes in feeding behavior can have repercussions on *Daphnia* populations and, consequently, implications on the ecosystem level. Reproduction, individual growth, and survival rates may be directly affected by abiotic (e.g., temperature, oxygen) and biotic conditions (e.g., food quality and quantity, host density, presence and density of competitors, and toxins), as well as by feeding behavior [36]. In good/normal food conditions, *Daphnia* produces a clutch of parthenogenetic eggs, which leads to rapid population growth [36]. However, under poor feeding conditions (poor food quantity and quality), *Daphnia* is not able to obtain sufficient energy for its development and reproduction [37,39]. In this case, the fecundity is reduced (number of neonates produced per offspring), in favor of allocating energy to expand its lifespan, and in some cases, sexual reproduction can be promoted [36,38,44]. Alterations in feeding performance can also have immediate repercussions for the community (its influence on prey quantity and composition) [45]. Ecologically, *Daphnia* has the ability to control the phytoplankton, as its primary consumer. A significant reduction in *Daphnia* (large Cladocera are classified as a highly efficient feeding *taxa*) in the natural ecosystems, the transparency of the water, and its chemical and microbial quality tends to deteriorate (eutrophication) [46]. In turn, *Daphnia* populations are controlled by pelagic fish, as these are a very important food item for juvenile fish. Consequently, a reduction in the *Daphnia* population also compromises the performance and survival of planktivorous fish, as well as the energy and biomass transfer in the aquatic trophic web.

3.3. Physical and Chemical, and Ecological Potential vs. Feeding Behavior Approach

The present study showed the possibility of relating the physical, chemical, and biological elements, and the final ecological potential achieved by the WFD with the *D. magna* feeding rates (Figure 3), to assess the water quality of the Crestuma-Lever reservoir. Considering the combined results of the physical and chemical (PC), biological elements (BIO), and feeding rate assays (FR), the ecological potential (EP) classification showed distinction in the sampling sites and months (Figure 3).

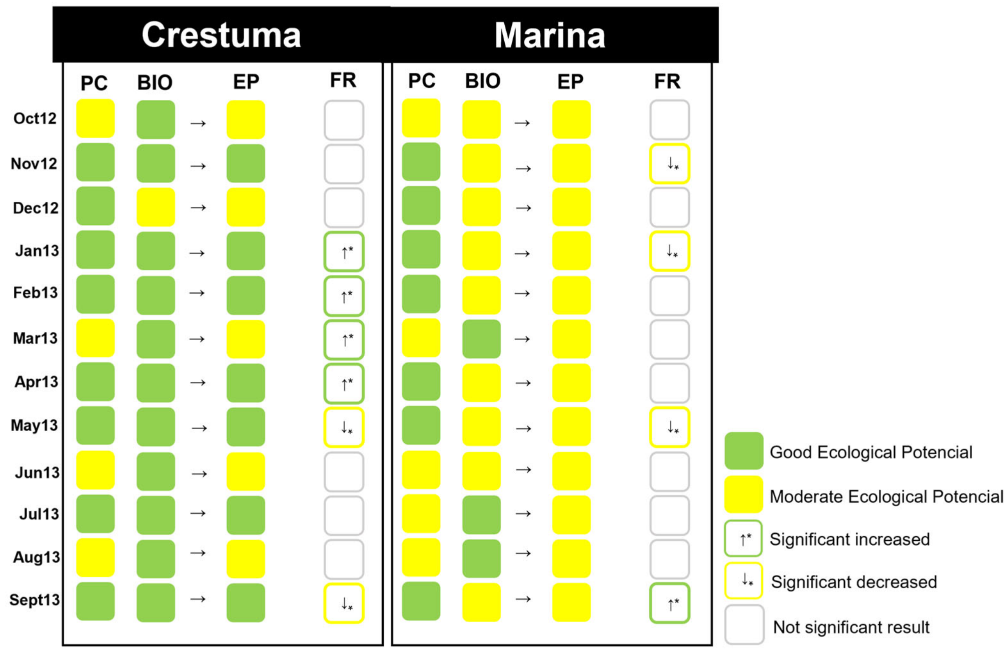


Figure 3. Physical and chemical (PC), biological (BIO) elements, and final ecological potential (FP from Tables 1 and 2) classification for Crestuma and Marina water samples, from each sampling period. *Daphnia magna* feeding rate (FR from Figure 2) results were also presented as additional water evaluation data. Green—Good Ecological Potential (GEP); Yellow—Moderate Ecological Potential (MEP). FR color evaluations were performed according to the classes of disturbances described in Rodrigues et al., [34].

Regarding the WFD approach for Crestuma, seven months showed good ecological potential (GEP), and five had moderate ecological potential (MEP) (Table 1, Figure 3). Regarding the feeding rates results, two additional months (May and September, FR in Figure 3) were identified with low quality, as a significant decrease in the feeding rate of *D. magna* was recorded, despite the GEP classification according to the WFD approach. This finding demonstrates the sensibility of this ecotoxicological tool to discriminate water quality, as the analyzed biological response (FR) is integrative of all compounds and components present in the samples. Therefore, in the overall results, we recorded that the components present in the sample (e.g., seston) or the physical and chemical properties of the samples (quantified or not), or compounds dissolved (e.g., contaminants) did influence the feeding behavior during the period studied.

Regarding the Marina water samples, all of the sampling periods showed a moderate ecological potential (Table 2, Figure 3). Similar to the Crestuma feeding rate results, in Marina, a further three months (November, January, and May, FR in Figure 3) were discriminated, presenting a low water quality. A significant decrease in the feeding rate was observed, despite the MEP classification according to the WFD metrics. This finding revealed an increase in the sensitivity of the FR to discriminate the water quality in these months, evidencing a relationship between the biological water sample components and feeding behavior. Therefore, in the overall results, despite the parameters included in the WFD (PC + BIO) showing some degree of disturbance, this was not enough to affect the feeding rates of *D. magna*.

Recent studies have demonstrated that food performance can be associated with the seston composition, potentially more associated with the quantity and quality of phytoplankton, which represent the main sources of stress for zooplankton in reservoir waters [2,6,34]. Moreover, the same authors propose the evaluation of filtered and unfiltered water treatments, with different meshes, to evaluate the seston quality. In the present study, the physical and chemical determinations performed in water samples at Portuguese reservoirs demonstrated that the heavily modified and artificial water bodies had a reasonable to good quality, as showed by previous studies [2,6,34].

4. Conclusions

The results presented in this study demonstrated that ecosystem functioning evaluation (namely through feeding rate assays) should be used as a tool to complement the methodology proposed by the WFD in water quality reservoir assessment. These results encourage further work on the applicability of cost-effective and sensitive ecotoxicity tests for water quality evaluation, as shown by the feeding rate assays (a simple, fast and sensitive tool). Thus, to better understand the effect of water samples from reservoirs on *D. magna* feeding rates (or other organisms with essential functions in the ecosystem), other parameters (e.g., the composition of the phytoplanktonic community, quantification of the specific pollutants, toxins evaluation, and priority substances, evaluation of filtered and unfiltered water) should be evaluated in future works. In this way, only an integrated approach, including ecological and ecotoxicological tools, allows for the complete assessment, management, and monitoring of the different water bodies.

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