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Microalgae Growth Inhibition-Based Reservoirs Water Quality Assessment to Identify Ecotoxicological Risks

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Abstract: This work intended to assess the adaptability of bioassay with *Raphidocelis subcapitata* to be used as a complement to the water quality assessment parameters of reservoirs imposed by the European Water Framework Directive (WFD). Thus, water samples of Portuguese reservoirs (Miranda, Pocinho, Aguieira, and Alqueva) were analyzed in three sampling periods (spring and autumn 2019, and spring 2020). A physical and chemical report of waters was also performed. *R. subcapitata* assay proved to be sensitive, indicating the presence of a potential perturbation that was not always associated with chemical analysis performed. In general, in the spring samplings, the water samples showed more disturbances to *R. subcapitata*, which in some situations may be associated with the higher content of nutrients and metals. Microalgae assay can be an effective complementary tool to indicate the ecotoxicological potential since they responded quickly to all sample components of water samples, in a wide-ranging variety of water conditions (different sites in several reservoirs). High similarities between the final ecotoxicological and the ecological potentials, according to the WFD parameters, were detected. The ecotoxicological approach based on our results allowed to confirm that bioassays with *R. subcapitata* are suitable and sensible to detect perturbations.

Keywords: Water Framework Directive; natural waters; heavily modified and artificial water bodies; *Raphidocelis subcapitata*; growth rate; ecotoxicological potential



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1. Introduction

The Water Framework Directive (WFD) 2000/60/EC [1] is an European directive that obliges all member states to attain the good quantitative and qualitative status of all water compartments [2]. In this directive, specific physical, chemical, biological, and hydromorphological elements aim to classify the ecological potential (EP) of heavily modified water bodies (e.g., reservoirs). The analysis of the phytoplankton community is the only biological parameter used in the characterization of reservoirs, being assessed based on: (a) chlorophyll *a* concentration, (b) the Algae Group Index (IGA), (c) biovolume of Cyanobacteria, and (d) total biovolume, for the Northern reservoirs and for the evaluation of the Southern and Main Course reservoirs only the chlorophyll *a* concentration is considered [3]. For the physical and chemical parameters, only four parameters (pH, dissolved oxygen, nitrate, and total phosphorus) have established environmental quality standards (EQS) values.

For the analysis of the chemical status of natural waters only 45 priority substances are considered [2] and the set of substances to be monitored implicates *a priori* knowledge. For economic and technical motives, it is impossible to consider and quantify all compounds present in water bodies [4–6]. Thus, water quality assessment of freshwater ecosystems requires new approaches, that allow for new monitoring approaches that do not exclusively depend on chemical scrutiny, but contrastingly contemplate biological responses first [4,5].

Numerous studies have been proposing the complementary use of the biological tools for evaluating changes in water quality and assessing biological responses [6–13]. For example, microalgae (included in the phytoplankton group) have been the focus of studies on new water quality indices and tools in biological monitoring programs for assessing water quality [8–13]. These biological groups are suitable for water quality evaluation because of their nutrient supplies, fast growth rate, and small life cycles [14]. Furthermore, in general, they respond quickly to a wide-ranging variety due to changes that are potentiated by pollution caused by agriculture, medicine, domestic, and industrial wastes.

The use of bioassays with standard test organisms, such as microalgae, has demonstrated efficacy, reliability, and sensitivity, representing an important approach in the evaluation of the ecological risk and eventual toxicity of water samples [9,12,15,16]. First efforts to quantify the consequences of pollution, in this group of organisms, were considered in the first decade of the 20th century, but only from the mid-1960s were microalgal bioassays methods validated and published [17]. *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*), *Selenastrum capricornutum*, *Scenedesmus subspicatus*, and *Chlorella vulgaris* are the species that are regularly used and suggested for freshwater ecotoxicity analysis, for which standard procedures have already been implemented [18,19] or with regulatory purposes [20–22]. Furthermore, a database is accessible about the biological responses of *R. subcapitata* to a diversity of chemicals and its suitability and sensitivity relatively to other aquatic test species [23,24]. Growth assays with *R. subcapitata* is one of the most common bioassays executed by the certain Member States for freshwater monitoring programs [10,12,16,25].

The general aim of this study was to investigate the algal bioassay sensitivity with a standard species *Raphidocelis subcapitata* (a cost-effective method), to identify the ecotoxicological potential of waters from Miranda, Pocinho, Aguieira, and Alqueva reservoirs. In addition, a physical and chemical report was performed to perceive the levels of potential pollutants present in the water, and to relate them to possible effects on the *R. subcapitata* responses. The specific and final goal of this work was to assess the relationship between ecological potential obtained by WFD parameters [general support chemical and physical parameters, specific pollutants and priority substances and phytoplankton Ecological Quality Ratio (EQR)], and ecotoxicity results. This strategy can be suitable and complementary to ecological potential demarcated by the WFD parameters to reservoirs, by implementing an approach integrating physical, chemical, and biological parameters, and ecotoxicological endpoints.

2. Materials and Methods

2.1. Study Areas

This work was performed in several Portuguese reservoirs, namely Miranda (M) and Pocinho (P) that are main course reservoirs, Aguieira (Ag), which is a northern reservoir, and Alqueva (Al) is a southern reservoir, but included in the main course typology (Figure 1).

The selection of reservoirs under study were defined based on previous studies, with pressures already documented [9,26–28], and based on previous studies by our work team, in these areas of study [12,13]. Miranda (first reservoir on the Spanish stretch) and Pocinho (first reservoir on the Portuguese stretch) belong to the hydrographic basin of the river Douro (and classified as main course reservoirs) are small and old reservoirs with diffuse anthropogenic influence [26,27]. The Aguieira reservoir belongs to the Mondego hydrographic basin (classified as a north reservoir) and was included in the WFD inter-calibration study, due to the scarcity of data and methodological divergences in the intercalibration geographic groups [28]. In its vicinity, there are food, textile, wood, and cork industries [12]. The Alqueva reservoir is located near agricultural fields, at the Guadiana River in the Alentejo region, southeast of Portugal and is one of the most recent reservoirs in the Iberian Peninsula and is the largest artificial lake in Europe [9].

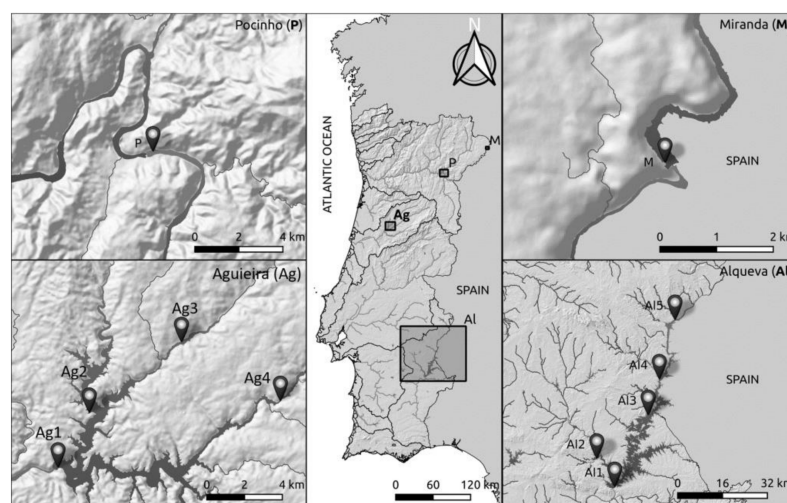


Figure 1. Map with sampling sites in the Miranda, Pocinho, Aguieira, and Alqueva reservoirs, Portugal. M—Miranda ($41^{\circ}29'24.802''$ N, $6^{\circ}15'55.925''$ W). P—Pocinho ($41^{\circ}08'10.884''$ N, $7^{\circ}06'39.074''$ W). Ag1—Aguieira dam ($40^{\circ}20'27.942''$ N, $8^{\circ}11'38.616''$ W); Ag2—Falgaroso do Maio ($40^{\circ}22'01.884''$ N, $8^{\circ}10'28.283''$ W); Ag3—Granjal ($40^{\circ}24'03.488''$ N, $8^{\circ}07'01.150''$ W); Ag4—Pinheiro de Ázere ($40^{\circ}22'22.256''$ N, $8^{\circ}03'19.055''$). Al1—Alqueva dam ($38^{\circ}12'07.957''$ N, $7^{\circ}29'19.717''$ W); Al2—Amieira ($38^{\circ}17'35.785''$ N, $7^{\circ}33'41.484''$ W); Al3—Monsaraz ($38^{\circ}25'58.085''$ N, $7^{\circ}21'03.721''$ W); Al4—Lucefécit ($38^{\circ}32'49.092''$ N, $7^{\circ}18'13.988''$ W); Al5—Juromenha ($38^{\circ}44'15.763''$ N, $7^{\circ}14'15.144''$ W).

To Miranda, Pocinho, Aguieira, and Alqueva reservoirs, the water level throughout the year varying between a maximum of 535, 134, 126, and 152 m and the minimum level of exploitation of 522, 124, 110, and 130 m, respectively, reaching peaks in autumn and in spring, according to the Sistema Nacional de Informação de Recursos Hídrico. The climate in these regions is strongly influenced by Mediterranean conditions being characterized by mild/cold winters and hot summers (mainly the southern part of Portugal) [3,9,12,13]. Miranda can also present continental influences with extreme weather fluctuations, due to its location in the Nordeste Transmontano.

2.2. Sampling and General Support Physical and Chemical Parameters Measured In Situ

Samples for water quality evaluation were collected during three sampling periods (Spring 2019—Spr19; Autumn 2019—Aut19; Spring 2020—Spr20), from 11 sites in four reservoirs (see Figure 1). The study sites were defined based on previous studies in these reservoirs [9,12,13,29]. The selection of these sampling points was also associated with other factors, namely: (i) the size of the reservoir; (ii) accessibility to sampling sites; (iii) position of previously defined monitoring stations by SNIRH—Sistema Nacional de Informação de Recursos Hídrico; (iv) assess the potential effects of different impacts from the surrounding areas.

In situ, with a multiparameter probe (Multi 3630 IDS SET F), a few meters at the margin (with accessibility on foot or in piers), several sub-superficially (<0.50 m depth) general physical and chemical parameters were evaluated: pH, dissolved oxygen (mg/L and %), and temperature ($^{\circ}$ C). At each site, 2 L of water were collected, and the entire sampling process, sample transport, and further analysis (chemical analyses and *R. subcapitata* assays) were carried out in accordance with previous work [12,13].

2.3. Laboratory Procedure

2.3.1. Physical and Chemical Characterization

In the laboratory, the concentration of several compounds was determined using different methods. Nitrites (NO_2^-) and nitrates (NO_3^-) were quantified by liquid chromatography of ions, total Kjeldahl nitrogen (N_{total}) determinations were quantified by the Kjeldahl nitrogen method after mineralization with selenium, according previous

studies [12,13,30]. Total phosphorus (P_{total}), and other metallic elements were also quantified (iron, manganese, arsenic, cadmium, copper, mercury, nickel, lead, and zinc) by the application of inductively coupled plasma mass spectrometry (ICP-MS) [31]. Polycyclic aromatic hydrocarbons (PAHS) and pesticides were not considered in our study, because, according to previous studies [12,13], the values of these compounds (quantified in autumn of 2018 in the same sampling sites) were inferior to the detection limits of the analytical technique, in addition to, that no significant changes in the areas adjacent to the reservoirs were documented during the present study.

2.3.2. Ecological Quality Ratio (EQR) for Phytoplankton

The methodology followed in the phytoplankton classification was already previously described [3,32]. For assessing the Ecological Quality Ratio (EQR) of phytoplankton, four biological parameters were proposed, by WFD, based on composition and abundance—Algae Group Index (IGA) and Cyanobacteria biovolume (mm^3/L); and—chlorophyll *a* concentration ($\mu\text{g}/\text{L}$) and total biovolume (mm^3/L), according to [3,32,33], to determine the final ecological potential (EP) of these water bodies. Only the real values were considered for the interpretation of the results.

Miranda and Pocinho reservoirs, despite belonging to the typology “main course”, the EQS used in biological quality (EQR) classification was carried out based on what is defined to southern reservoirs, according to [3] (Table 1). For the main course and southern typologies (e.g., Miranda, Pocinho, and Alqueva), the ecological potential, considering the biological elements proposed in the WFD, only two classes are defined: moderate or less, and good or more (Table 1). Aguieira is classified as a northern type reservoir, and the ecological potential based on biological elements proposed in the WFD is classified into four classes: Good, Moderate, Poor or Bad (Table 1).

2.3.3. Water Treatments

To carry out the microalgae assays, the collected water samples, for each sampling site of each reservoir, were divided into three treatments, according to previous studies [12,13]: NF, F1 and F2, which correspond to Non-Filtered water, filtered through a Whatman GF/C filter of 47 mm diameter with $1.2\ \mu\text{m}$ porosity, and filtered through a sterile filter system with a porosity of $0.22\ \mu\text{m}$, respectively.

2.3.4. Bioassays

Culture Maintenance of *Raphidocelis subcapitata* and Growth Inhibition Assays

A Laboratory culture of the microalgae *R. subcapitata* was maintained in Woods Hole MBL medium [34] and it was renewed for a new medium (in the exponential growth phase), once a week [12,35].

Growth inhibition assays with *R. subcapitata* were performed according to the OECD guidelines [19], with some adaptations as described in [12]. The water treatments (see the Section 2.3.3) and a blank (without algal addition, to evaluate the growth of algae present in the natural water samples) were evaluated. An additional treatment (control group) was performed with MBL medium. Per treatment, three replicates were performed, with an initial concentration of microalgae of the 5×10^4 cells/mL [12]. The assays were carried out in a climatic chamber (Incubator TC 445 S, Lovibond® Water Testing) at $24 \pm 2\ ^\circ\text{C}$ with permanent light, and twice a day, all treatments were resuspended, to avoid algae sedimentation, allowing more efficient mixing of the algae with the treatment. After 72 h exposure period, the final absorbance was measured at $\lambda = 440\ \text{nm}$ in a UV-1600PC Spectrophotometer, in each well. The final absorbance of the corresponding blank, from each treatment, was removed, to neutralize the presence and growth of other constituents that may interfere with the readings. The concentration of cells in each well was calculated, according to Pinto et al. [12], and the bioassays results were expressed in yield. In addition, the percent inhibition in yield ($\% I_y$) was calculated for each water treatment, according to the OECD guidelines [19].

Table 1. Results of the pH, dissolved oxygen (mg/L and %) and temperature, measured in situ, and nitrates (NO_3^-), nitrites (NO_2^-), total Kjeldahl nitrogen (N_{total}), total phosphorus (P_{total}), specific pollutants and priority substances concentrations ($\mu\text{g/L}$) with values above the environmental quality standards (EQS). For Portugal northern, main course and southern reservoirs established by WFD, the ecological potential and threshold values [for physical, chemical and biological (expressed in Ecological Quality Ratio—EQR) parameters], according WFD metrics, are presented. Sampling sites: Miranda—M, Pocinho—P, Aguieira—Ag1, Ag2, Ag3, Ag4 and Alqueva—Al1, Al2, Al3, Al4 and Al5; Sampling periods: Spring of 2019 (Spr19), Autumn of 2019 (Aut19) and Spring of 2020 (Spr20). Bold values stand for values outside the established thresholds.

Reservoirs and Sampling Points		General Physical and Chemical									Specific Pollutants and Priority Substances: Metals		Ecological Potential (Physical and Chemical)	Biological (Phytoplankton EQR)		Ecological Potential (Biological)
		EQS	6–9 [3]	≥5 [3]	60–120 [3] north and main course 60–140 [3] south	-	≤25 [3]	-	-	≤0.05 [3] north and main course ≤0.07 [3] south	0.07 [2]	7.8 [3]		North [3] [1.0–0.60]—Good or more [0.6–0.4]—Moderate [0.4–0.2]—Poor [0.2–0]—Bad		
Sampling periods	pH	O ₂ (mg/L)	O ₂ (%)	Temp (°C)	NO ₃ [−] (mg/L)	NO ₂ [−] (mg/L)	N _{total} (mg/L)	P _{total} (mg/L)	Hg (μg/L)	Zn (μg/L)	South and Main Course [3] ≥0.17—Good or more <0.17—Moderate or less					
Miranda (main course)	M	Spring 19	8.84	14.07	149.7	15.6	6.4	0.09	<0.5	0.03	<0.01	2.8	GOOD	0.11	MODERATE OR LESS	
		Autumn 19	7.88	4.40	50.0	18.3	2.3	0.43	<0.5	0.01	<0.01	1.1	MODERATE	0.85	GOOD OR MORE	
		Spring 20	8.64	11.04	124.1	19.0	7.4	0.16	<0.5	0.13	1.02	26.6	MODERATE	0.16	MODERATE OR LESS	
Pocinho (main course)	P	Spring 19	8.82	13.95	144.0	16.5	<0.5	<0.01	<0.5	0.03	<0.01	1.4	GOOD	0.26	GOOD OR MORE	
		Autumn 19	8.03	8.21	90.3	19.2	2.3	<0.04	<0.5	0.04	<0.01	2.6	GOOD	0.61	GOOD OR MORE	
		Spring 20	9.24	15.90	185.0	22.8	3.5	0.05	0.7	0.09	1.06	80.7	MODERATE	0.12	MODERATE OR LESS	
Aguieira (north)	Ag1	Spring 19	9.20	11.90	119.4	14.4	2.8	0.04	<0.5	0.01	0.02	6.7	GOOD	0.45	MODERATE	
	Ag2		8.99	12.40	124.9	15.0	3.3	0.07	<0.5	0.01	0.02	12.8	MODERATE	0.73	GOOD OR MORE	
	Ag3		8.31	11.30	112.1	15.2	4.0	0.04	<0.5	0.09	0.02	6.7	MODERATE	0.50	MODERATE	
	Ag4		9.15	12.20	125.2	15.5	1.2	0.02	0.7	0.02	0.02	6.8	GOOD	0.35	POOR	
	Ag1	Autumn 19	6.83	4.48	47.1	17.7	1.5	<0.01	<0.5	<0.01	0.18	7.3	MODERATE	0.61	GOOD OR MORE	
	Ag2		6.68	5.29	55.9	17.9	1.2	<0.01	<0.5	<0.01	0.14	9.4	MODERATE	0.72	GOOD OR MORE	
	Ag3		6.74	8.95	91.8	16.3	2.3	0.02	2.2	0.09	0.13	10.8	MODERATE	0.41	MODERATE	
	Ag4		6.80	6.90	72.3	17.3	1.0	0.03	0.6	<0.01	0.11	20.3	MODERATE	0.50	MODERATE	
	Ag1	Spring 20	9.61	12.92	150.2	21.9	2.7	0.04	<0.5	0.02	1.85	32.2	MODERATE	0.77	GOOD OR MORE	
	Ag2		9.70	14.18	160.1	20.5	2.2	0.04	0.7	0.03	1.57	27.7	MODERATE	0.37	POOR	
	Ag3		8.99	12.39	141.0	20.7	3.3	0.05	<0.5	0.08	1.33	28.9	MODERATE	0.33	POOR	
	Ag4		9.40	13.28	156.5	22.4	0.6	0.01	<0.5	0.03	0.63	24.7	MODERATE	0.61	GOOD OR MORE	

Table 1. Cont.

Reservoirs and Sampling Points		General Physical and Chemical								Specific Pollutants and Priority Substances: Metals		Ecological Potential (Physical and Chemical)	Biological (Phytoplankton EQR)		Ecological Potential (Biological)
		EQS	6–9 [3]	≥5 [3]	60–120 [3] north and main course 60–140 [3] south	-	≤25 [3]	-	-	≤0.05 [3] north and main course ≤0.07 [3] south	0.07 [2]		7.8 [3]	North [3] [1.0–0.60]—Good or more [0.6–0.4]—Moderate [0.4–0.2]—Poor [0.2–0]—Bad	
		Sampling periods	pH	O ₂ (mg/L)	O ₂ (%)	Temp (°C)	NO ₃ [−] (mg/L)	NO ₂ [−] (mg/L)	N _{total} (mg/L)	P _{total} (mg/L)	Hg (μg/L)		Zn (μg/L)	South and Main Course [3] ≥0.17—Good or more <0.17—Moderate or less	
Alqueva (main course)	Al1	Spring 19	8.54	9.64	114.4	23.0	<0.5	0.01	0.6	0.01	<0.01	2.7	GOOD	0.85	GOOD OR MORE
	Al2		8.71	9.39	112.8	23.7	<0.5	0.02	0.6	<0.01	<0.01	1.0	GOOD	1.36	GOOD OR MORE
	Al3		8.79	9.99	118.2	23.1	0.6	0.04	0.6	0.01	<0.01	4.1	GOOD	0.83	GOOD OR MORE
	Al4		8.54	12.72	152.6	23.8	0.7	0.07	0.7	0.01	0.14	1.2	MODERATE	0.58	GOOD OR MORE
	Al5		9.05	16.93	199.5	23.0	0.9	1.70	2.1	0.09	<0.01	3.2	MODERATE	0.08	MODERATE OR LESS
	Al1	Autumn 19	8.23	8.05	83.7	16.6	<0.5	0.02	<0.5	0.07	0.04	6.1	GOOD	0.73	GOOD OR MORE
	Al2		8.33	8.04	83.3	16.9	<0.5	0.04	0.5	0.05	0.02	5.9	GOOD	0.55	GOOD OR MORE
	Al3		8.32	8.54	88.8	16.9	<0.5	0.04	0.7	0.04	0.04	5.4	GOOD	0.79	GOOD OR MORE
	Al4		8.26	7.51	77.6	16.9	0.5	0.66	1.0	0.05	0.06	8.3	MODERATE	0.78	GOOD OR MORE
	Al5		8.38	10.96	108.6	14.6	1.7	0.12	1.6	0.07	0.02	6.3	GOOD	0.12	MODERATE OR LESS
	Al1	Spring 20	8.78	8.29	114.0	32.0	<0.5	0.02	<0.5	0.04	4.78	691	MODERATE	0.87	GOOD OR MORE
	Al2		8.87	8.48	119.1	33.3	<0.5	0.02	<0.5	0.05	2.20	89.2	MODERATE	0.86	GOOD OR MORE
	Al3		8.96	8.43	115.2	31.7	0.8	0.09	0.6	0.03	2.37	51.3	MODERATE	0.72	GOOD OR MORE
	Al4		9.21	10.01	133.6	32.0	<0.5	0.09	0.8	0.06	2.37	41.2	MODERATE	0.22	GOOD OR MORE
	Al5		8.59	7.04	97.0	32.0	<0.5	0.04	1.0	0.18	2.04	51.4	MODERATE	0.10	MODERATE OR LESS

2.4. Statistical Analysis

Before statistical analysis, the test variable (Yield) was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). A one-way ANOVA were performed twice (ANOVA-1 and ANOVA-2), in each sampling season. ANOVA-1 was conducted to check the differences between water treatments and control. A Dunnett's test was applied to discriminate the treatments that were significantly different from the control group (CTRL) ($p < 0.05$). ANOVA-2 followed by Tukey's test was performed to determine the differences between the surface water treatments (NF, F1, and F2) ($p < 0.05$). The program SPSS Statistics v26 was used for all the statistical analysis performed.

3. Results and Discussion

3.1. WFD Approach Based on Physical, Chemical, and Biological Parameters

General physical and chemical parameters determined at the sampling points in the three sampling periods are presented in Table 1, for the four reservoirs, as well as the EQS for heavily modified and artificial water bodies values established for the "Good Ecological Potential" (GEP).

A GEP for all sampling sites of all reservoirs, in the three sampling periods was obtained, since the pH values recorded are within the range of EQS. Dissolved oxygen revealed values above within limits (EQS, Table 1), with exception of the Miranda and the Aguieira reservoirs (Ag1 and Ag2), in Aut19, since the oxygen values were slightly inferior to the quality limits (Table 1). However, in the laboratory, oxygen levels were higher (7.23 mg/L in Miranda, and 7.75 mg/L in the site Ag1), possibly due to transport and handling of the samples until they reach the lab, fitting with the proposed EQS limits.

Nitrate concentrations showed values below 25 mg/L (the maximum established for a GEP classification), for all water samples and reservoirs. For regulated public water systems, the maximum contaminant level for nitrites and total Kjeldahl nitrogen, adopted by the Environmental Protection Agency (EPA), was 1 mg/L and 10 mg/L (EPA, 2018). Although nitrites and total Kjeldahl nitrogen are not included in the WFD, the values were always inferior to the maximum limits recommended by the EPA (for drinking water), except for the Al5 site (1.7 mg NO_2^- /L; Spr19). For the P_{total} (Table 1), values were above the EQS, in Miranda and Pocinho, in Spr20; in Aguieira, at site Ag3, during all sampling periods; and in Alqueva, at site Al5 in Spr19 and in Spr20. These reservoirs are in areas exposed to several pressures as agricultural runoff (high usage and concentrations of fertilizers) and effluent discharges [13], and therefore this nutrient enrichment (phosphorus in this study) may be associated with these anthropic activities in the surrounding areas. However, the selection of physicochemical quality limits regarding nutrients concentrations has been interrogated due to their inconsistency dependent on geographic, environmental, or geologic features [25].

Regarding the metallic elements, only Hg and Zn exceeded the EQS (Table 1) [2,3]. Regarding Hg concentrations that presented values above 0.07 $\mu\text{g/L}$, in the site Al4 (Spr19), and all sites of the Aguieira reservoir in Aut19 and Spr20. For Zn, concentrations above 7.8 $\mu\text{g/L}$ (Table 1) were quantified in the site Ag2 (Spr19); in Ag2, Ag3, and Ag4 and Al4 (Alqueva) in Aut19, and in all sites for all reservoirs in Spr20. All other metallic elements presented values (iron, manganese, arsenic, cadmium, copper, nickel, and lead) within the limits required for the GEP classification [2,3]. Concentrations of mercury are worrying as this is a harmful contaminant, and it is included in the list of high-priority environmental pollutants within the European WFD, in the Convention for the Protection of the Marine Environment of the North-East Atlantic and the United States Environmental Protection Agency.

Physical and chemical results are in line with the recorded in previous studies [12,13,26,27]. Considering the general physical and chemical characterization and the specific pollutants and priority substances analyzed, in general, in Spr19 sampling period the most sampling points presented a GEP (Table 1; sites M, P, Ag1, Ag4, Al1, Al2, and Al3), since the parameters are within the range of environmental quality standards. In Aut19, only P and

Al3 presented a GEP. In Spr20, all sites were classified as moderate (Table 1). This allows us to infer that over the sampling periods, in general, the ecological potential worsened and, consequently, the water quality as well.

In the analysis of the biological parameter, phytoplankton was expressed in EQR, for all reservoirs (Table 1). For Miranda, both spring samplings revealed a worse classification (moderate or less) of ecological potential than the autumn sampling. Pocinho showed the worst ecological potential only in the spring 2020 sampling. Regarding Aguireira, between the first and the last sampling, the Ag2 and Ag3 sites showed a worsening of the ecological potential, and the Ag1 and Ag4 sites, an improvement. In the Alqueva, only the Al5 site had the worst classification of ecological potential, in all sampling periods. The remaining sites, in all sampling periods, presented ecological potential in the category of good or more (Table 1). The EQR represents the relation between the noticed values of biological parameters relatively to the reference values. Thus, an EQR of one represents reference conditions and zero represents severe impact. Thus, higher EQR stands to a better ecological potential. The low classifications of EQR can be associated with the high abundances of Cyanobacteria perceived in spring samples, (e.g., cyanobacterial bloom of *Microcystis*). This relationship has been previously reported in other studies, where the presence of these organisms has already been reported in the reservoirs under study [12,13]. In a great amount of water basins of the Iberian Peninsula, Cyanobacteria are the main constituents of the phytoplankton representing an ecological risk for water bodies [12,36–38]. Significant disturbances in the aquatic environment are due to the enhanced growth of phytoplankton, potentiated by some abiotic factors, and the ecological potential is altered quickly from “Moderate” to “Bad”. In fact, eutrophic waters, evaluated in Moderate/Poor ecological potential, jeopardize the fulfillment of Natura 2000 objectives. Overall, we observed that, regarding the biological parameter under study, the sites classified as having moderate or lower ecological potential are potentially related to the physical and chemical results, that turned out to be outside the EQS limits. However, the main effects of pollution on phytoplankton (EQR) may be mostly associated with the high concentration of nutrients, as suggested by D’Costa et al. [39].

3.2. *R. subcapitata* Growth Inhibition Assays

Figures 2–5 show the *R. subcapitata* growth inhibition results, for the sampling periods and the reservoirs: Miranda, Pocinho, Aguireira, and Alqueva. In all sites of all reservoirs, and all sampling periods, there was a significant decrease in yield, which indicates an inhibition of growth. This inhibition would be expected, considering the composition of the media since, in the control group, the medium is suitable to its growth, including all components favorable to its development, in a controlled manner.

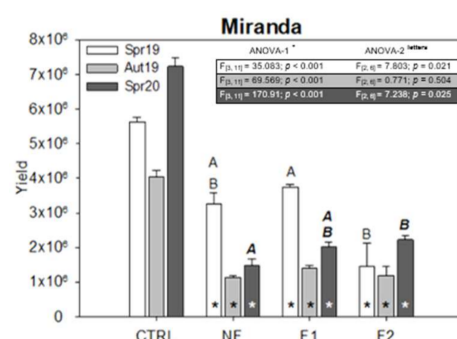


Figure 2. Results of Yield of *R. subcapitata* from the Miranda reservoir, when exposed to treatments: control (CTRL) and natural water treatments (NF—Non-filtered water; F1 and F2—filtered with 1.2 μm and 0.22 μm respectively). Data are stated as mean $\pm$ standard error (SE); $n = 3$. * Stands for significant differences detected by Dunnett test. Different letters (A, B for Spr19; and A, B for Spr20) stands for significant differences between treatments, detected by Tukey test).

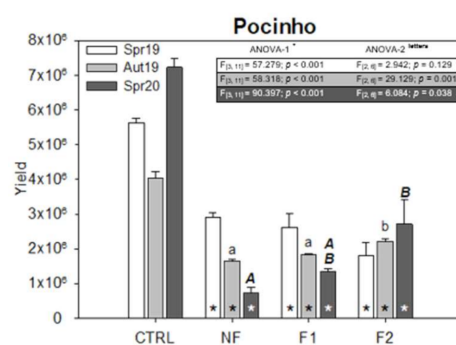


Figure 3. Results of Yield of *R. subcapitata* from the Pocinho reservoir, when exposed to treatments: control (CTRL) and natural water treatments (NF—Non-filtered water; F1 and F2—filtered with 1.2 μm and 0.22 μm respectively). Data are stated as mean $\pm$ standard error (SE); $n = 3$. * Stands for significant differences detected by Dunnett test. Different letters (a, b for Aut19 and A, B for Spr20) stands for significant differences between treatments, detected by Tukey test).

If we only consider the water treatments, in general, with the application of F1 and F2 treatments, there was a significant increase in yield (relatively to NF), in some sampling periods and sites, namely: Spr19 at Ag3, Al3 and Al5 sites; Aut19 at sites P, Ag1, Ag2, Ag4; Spr20—at the M, P, Ag2, Ag4, and Al5 sites. F2 is the filtration treatment that offerings greater growth rates, which reflects the positive effects of this treatment and the sensitivity of this test organism. Since the NF treatment represent the natural water sample, with all components, including phytoplankton and zooplankton, this increase of yield on filtration treatments (F1 and F2) may be associated with several factors, namely: (i) removal of zooplankton, (ii) phytoplankton removal, (iii) removal of all suspended particles, (iv) removal of bacteria, and (v) nutrients and dissolved compounds as metals. Zooplankton (i) can feed on algae and as such a decrease in growth rate registered in NF treatment was observed. With filtrations, this group of organisms is removed and the herbivory of zooplankton on phytoplankton does not occur. On the other hand, (ii) eutrophic ecosystems have a great variety of phytoplankton species, which can compete. In aquatic ecosystems, as reservoirs, there may be several freshwater algae that bloom with particular environmental situations, such as Cyanobacteria, and they can be very often the dominant part of the phytoplankton. Based on the results of a study with Spanish surface waters [40], the main factors pointed out by the authors for the development of Cyanobacteria, are the temperature, solar radiation, and excess of nutrients from livestock and fertilizers, including P_{total} [40–43], as reported here. In fact, our findings are supported by the work of Álvarez et al. [40] that already reported the phenomenon of competition between two green algae (*Kirchneriella* sp. and *Scenedesmus* sp.) and Cyanobacteria (*Microcystis aeruginosa*), detected in the Spanish eutrophic reservoir (Umia). Furthermore, Cyanobacteria have already been detected in Portuguese reservoirs such as Aguieira [12,37]. Thus, the removal of these species associated with potential competition phenomena allows a greater development of *R. subcapitata* exposed to water treatments subjected to prior filtration. Furthermore, these filtrations also allowed the removal of suspended particles (iii) that may also be associated with an increase in the growth rate of *R. subcapitata*, since these components can represent a significant role in the diffusion of light in water. In fact, in the presence of all components in suspension (NF treatment) a greater dispersion of radiation can occur, and consequently weakens the luminosity, which is essential for the productivity of phytoplankton, namely the development of *R. subcapitata*. Removal of bacteria (iv) associated with the F2 treatment may also have implications for algae growth rates. Algae under natural growth conditions (for example in the NF treatment with all components present in the sample), can compete for the growth-limiting nutrients (as phosphorus or nitrogen) with potentially present bacteria. In fact, Brussaard and Riegman [44] reported that, for inorganic phosphorus, bacteria are better competitors comparatively to the algae, which helps to understand our results (higher yield in F2 treatment). In this sense, with the

removal of bacterioplankton (F2 treatment), the nutrients (phosphorus) (v) became more available for the *R. subcapitata* to satisfy nutritional P demands. In fact, higher levels of phosphorus were reported in reservoirs belonging to the hydrographic basin of the river Douro (Miranda and Pocinho), in Spr20; in Agueira, during all sampling periods at site Ag3; and in Alqueva, at site Al5 in Spr19 and in Spr20, which reflects the high utility of this water treatment, and the responsiveness and sensitivity of this test organism. On the other hand, some reports suggest also stimulatory effects of heavy metals on algae (*Chlorella* sp.) growth by preventing loss of carbon compounds [45], although generally, they harm microalgae cultures.

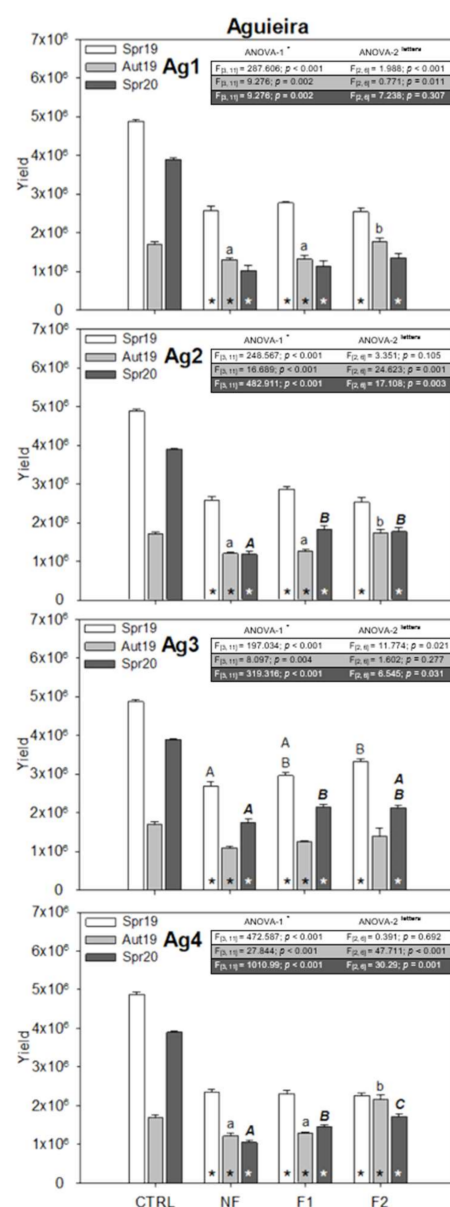


Figure 4. Results of Yield of *R. subcapitata* from the Agueira reservoir, when exposed to treatments: control (CTRL) and natural water treatments (NF—Non-filtered water; F1 and F2—filtered with 1.2 μm and 0.22 μm , respectively). Data are stated as mean $\pm$ standard error (SE); n = 3. * Stands for significant differences detected by Dunnett test. Different letters (A, B for Spr19; a, b for Aut19 and A, B, C for Spr20) stands for significant differences between treatments, detected by Tukey test).

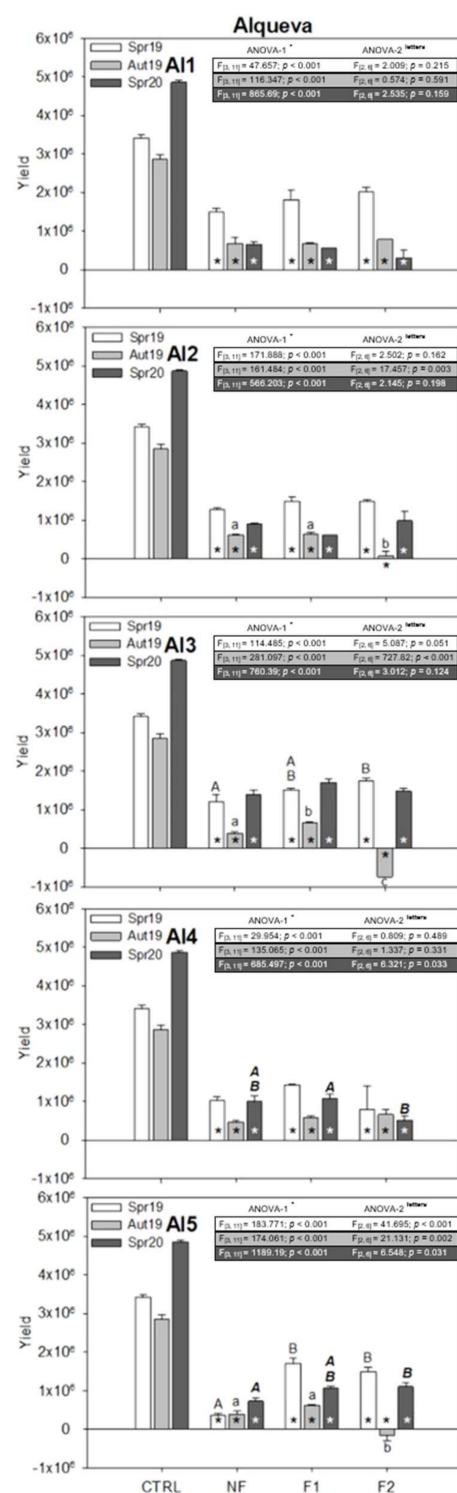


Figure 5. Results of Yield of *R. subcapitata* from the Alqueva reservoir, when exposed to treatments: control (CTRL) and natural water treatments (NF—Non-filtered water; F1 and F2—filtered with 1.2 μm and 0.22 μm , respectively). Data are stated as mean $\pm$ standard error (SE); $n = 3$. * Stands for significant differences detected by Dunnett test. Different letters (A, B for Spr19; a, b for Aut19 and A, B for Spr20) stands for significant differences between treatments, detected by Tukey test).

Contrary to what was previously reported, with the application of the filtration treatments (F1 and F2), there was a significant decrease in yield (relatively to NF), but less frequently, in some sampling periods and sites, namely: Aut19 at sites Al2, Al3, and Al5 and in the period of Spr20 at sampling sites Ag3 and Al4, although in these last two sites significant decrease only occurred in the F1 treatment. This decrease may be associated with others dissolved compounds, which despite being present in all water treatments, however, may be more bioavailable in the F2 treatment, since all biological elements (e.g., phytoplankton) of the natural sample were eventually removed by filtration. In fact, the important efficient role of algae in removing several emerging compounds has already been described [45]. *Chlamydomonas*, *Chlorella*, and *Scenedesmus* sp. are the most frequently reported and extensively studied species in these proof-of-concept studies [45], and they were observed in the samples of studied reservoirs. However, several other species (e.g., *Selenastrum*, *Coelastrum*, *Rhodomonas* sp.) identified as relevant for bioremediation [45] were observed in this work. In these studies, higher levels of mercury and zinc were reported in all sites of Agueira in all sampling periods, and in Spr20 in Miranda, Pocinho, and all sites of Alqueva reservoirs. However, only in Al4 and for the Spr20 period there was a decrease in yield with the application of treatments, which can be associated with the greater levels of metals reported. In the remainder, other unquantified compounds may have been present and have interfered with these growth rates. In this work, mostly the presence of these metals (levels above EQS) was associated with an increase in yield, in treatments with filtrations. In fact, algal growth may also be inhibited by contaminants, as metals, that affect nutrient relationships, and consequently algae growth [46]. Algae growth represents the suitable functioning of several physiological and biochemical pathways, as for example the photosynthesis or nutrient uptake [47]. The toxicity mechanisms whereby they affect its uptake and utilization vary according to nutrient, physical, and chemical conditions of the water and algal species [46]. For example, Hg even at low concentrations causes deleterious effects (perturbations in normal photosynthetic mechanism, injuries in the enzymatic activities or blockage of cell division) in microalgae [46,47], since they can compete with or substitute beneficial metals. Zn is considered an essential metal because it is required for the normal physiological performance of most living organisms; however, extreme concentrations have been involved with diverse perturbations, as mentioned in the studies of Monteiro et al. [47] and Filová et al. [48].

Another remark that we observed with these results was that, in some periods and sampling sites, the application of treatments with filtration did not benefit or harm the growth rate of *R. subcapitata*. Examples of this finding are the sites: M in the Spr19 and Aut19 periods; P in the Spr19 period; Ag1 in both spring periods, Ag2 and Ag4 in the Spr19 sampling period, Ag3 in the Aut 19 period; Al1 in all periods, Al2 in both spring periods, Al3 in the Spr20 and Al4 period and both 2019 periods. In fact, in most of these situations, these results were to be expected, given the low levels of contamination reported, except for the Spr20 period, where all sites had higher levels of the Hg and Zn, and in some sites the phosphorus concentration (Table 1). So, despite only quantifying a minority of the compounds present in these natural waters, the biological responses observed allow us to infer with some degree of evidence and certainty, that the levels of pollution were low in these reservoirs, during the study period. In general, this bioassay, under the conditions tested here, demonstrated high sensitivity and reliability. In fact, the sensitivity of *R. subcapitata* in water quality assessment of reservoirs has been previously reported in other works [9,12,25].

3.3. Ecotoxicity Results, Purposed Ranges of Ecotoxicological Potential, and Classes of Disturbances

Table 2 shows the results of the ecotoxicity tests (microalgae bioassays) performed on natural water treatments, based on the parameter: percent inhibition of yield (% Iy), compared to the control group. Overall, ecotoxicity results presented a good concordance between treatments and physical and chemical parameters and contaminants content, for the same location and sampling periods. Thus, the greater percent of yield inhibition

was recorded when the better the quality of the sample was observed, and therefore less disturbance. Therefore, based on the biological responses under study (percent inhibition of yield), compared to the control, five ecotoxicity classes were proposed (Table 2) in order to achieve an approach of the ecotoxicological potential for each sampling site and sampling period. Based on the criteria to define the equivalent quality potential, to those presented in the WFD, an estimation of the ecotoxicological potential has been suggested. Classes of ecotoxicity have been assigned from non-perturbed ($% Iy = \geq -10$) to highly perturbed ($% Iy = < -90$), according to the ecotoxicity degree of the percent inhibition of yield. So, for each class and to facilitate the analysis of global results, different colors were assigned to each class, as shown in Table 2. The definition of this range of ecotoxicity classes had as main influences the percentage of effect of 10%, 50%, and 90% (values with high significance in ecotoxicology), in which the effect considered was the inhibition of yield. Consequently, this range was adjusted, whereby equivalent variations were defined with five ecotoxicity classes, as suggested in the work by Roig et al. [25]. In the previous work, the authors designed an approach to evaluate the ecotoxicological status of rivers (Ebro River watershed, NE Spain), in which the ecotoxicity of pore water has been evaluated in several test organisms, including *P. subcapitata* (current name, *R. subcapitata*). Roig et al. [25] also proposed five classes of ecotoxicity, based on different endpoints, since they evaluated several aquatic organisms. For *R. subcapitata*, this range was defined according to the EC_{50s} values and were expressed as % dilution, for pore water assays, from non-toxic (>100) and highly toxic (<10).

For the 33 analyzes performed (11 sites $\times$ 3 sampling periods), with the application of filtration treatments (F1 and F2), 19 results did not show evident changes for the parameter under analysis (percent inhibition of yield), 8 results showed improvement, and 6 showed undesirable changes (Table 2). Regarding the sites vs sampling periods, which did not show beneficial or harmful changes with the application of filtration treatments (M in Aut19 and Spr20; P in Spr20; Ag1 in all periods; A3 in Spr20; Ag4 in Spr19 and Spr20; A11 in Aut19; A12 in all sampling periods; A13 and A14 in Spr20 and A15 in all periods), all revealed the moderately perturbed toxicity class (orange), except for Ag1, which presented the marginally perturbed toxicity class (yellow) (Table 2). These results reflect some degree of contamination, which was not fully confirmed in this work with the chemical analysis carried out, however, most of these situations may be associated with higher levels of phosphorus and metals in some sites and sampling periods, as previously described (Table 1). For locations: M, P, A13, A14 in Spr19; A13 and A14 in Spr20, the application of filtration treatments induced a worsening in terms of ecotoxicity class. However, these may be associated with higher levels of mercury, which is quite toxic to algae [46,47], or other contaminants presented in water, (not quantified). On the other hand, in the sampling period Spr19 at sites Ag3, A11; in the Aut19 period at locations P, Ag2, Ag3, Ag4; and in the Spr20 period at the Ag2 and A11 sites, there was an improvement in perturbation class extremities, with the use of filtration treatments. Therefore, in these cases the removal of all suspended particles, phytoplankton, zooplankton, and bacterioplankton communities could potentially explain this water quality improvement.

The set of results of our work, allowed to ascertain that an ecotoxicological approach, provided by appropriate bioassays with natural waters, can detect variations in water composition, with efficiency and accurate sensitivity.

Table 2. Classes of disturbances, purposed ranges of ecotoxicological potential and ecotoxicity results for *R. subcapitata* assays after exposure to natural water treatments treatments (NF—Non-filtered water; F1 and F2—filtered with 1.2 µm and 0.22 µm respectively). Values represent the percent inhibition in yield (% *I_y*), comparatively to the control. Sampling points: Miranda—M, Pocinho—P, Aguieira—Ag1, Ag2, Ag3, Ag4 and Alqueva—Al1, Al2, Al3, Al4, and Al5; Sampling periods: Spring of 2019 (Spr19), Autumn of 2019 (Aut19) and Spring of 2020 (Spr20).

Classes of Ecotoxicity				Non Perturbed						Slightly Perturbed						Marginally Perturbed						Moderately Perturbed						Highly Perturbed					
percent inhibition in yield (% <i>I_y</i>)				≥ −10						≥ −10 to −30						≥ −30 to −60						≥ −60 to −90						< −90					
M				P			Ag1			Ag2			Ag3			Ag4			Al1			Al2			Al3			Al4			Al5		
	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20	Spr19	Aut19	Spr20			
NF	−42.2	−72.0	−79.5	−48.4	−59.2	−89.9	−47.3	−47.0	−45.0	−51.8	−23.1	−29.3	−35.2	−28.3	−73.6	−69.6	−55.0	−73.1	−55.9	−62.9	−64.5	−69.7	−89.3	−76.1	−78.2	−86.4	−83.5	−86.4	−86.3	−81.5	−71.2	−79.3	−85.0
F1	−33.4	−65.2	−71.9	−53.7	−54.6	−81.3	−43.2	−41.3	−39.2	−52.6	−22.4	−26.0	−26.0	−23.4	−71.1	−53.1	−45.0	−63.0	−47.0	−56.4	−55.8	−58.2	−50.1	−76.3	−77.9	−76.7	−79.8	−78.2	−88.3	−87.5	−65.1	−77.8	−78.1
F2	−74.3	−70.3	−69.2	−67.8	−45.1	−62.5	−47.8	−48.2	−31.9	−53.6	3.8	1.8	−17.2	27.3	−65.5	−54.7	−45.4	−56.1	−41.1	−56.6	−49.1	−76.7	−56.3	−72.2	−97.5	−125.8	−76.7	−105.7	−93.6	−79.8	−69.5	−89.3	−77.3

3.4. Physicochemical and Ecological Potential vs. Ecotoxicological Approach

Despite the tests with organisms cannot substitute the biological methodology (biologic indices by WFD) for the ecological potential assessment of reservoirs, in general, it has been perceived that the results of bioassays with *R. subcapitata* show some degree of agreement with biological EQR (Figure 6). However, the Pocinho and Alqueva were the reservoirs that showed the least consistency between the ecotoxicological potential and the ecological potential according to the WFD parameters, mainly taking into account the biological EQR. For example, if we only consider the biological elements under study (phytoplankton) (bioassays vs. biological EQR), some discrepancies may arise in the interpretation of the results. Despite *R. subcapitata* as a phytoplankton element, in the evaluation of the biological EQR in main course and southern reservoirs, only the chlorophyll a content is considered (biomass endpoint, without composition, and abundance evaluation), which is a very ambiguous analysis parameter. Thus, due to the different sensitivity between WFD parameters individually considered, and the occurrence of confounding features that may possibly perform a significant interference in the water ecotoxicity evaluation, the final ecological potential according to WFD parameters has been calculated. In fact, this approach is more in line with the results of the ecotoxicological potential, since considering the contamination classes of ecotoxicity and respective colors, they present greater similarities (Figure 6). When we pay more attention in the sites where the ecological potential evaluation was poor or moderate, a positive similarity between ecological and ecotoxicological potential was largely demonstrated (e.g., M, Ag1, Ag4, Al2, and Al5). So further research is necessary to refine and adjust the discrimination of the mild levels of contamination. We believe that an accurate bioassay is an equivalent and reliable tool of organism functional responses, and they might have added influence on the judgement making procedure, then measures based on chemicals compounds concentrations or other physical and chemical parameters, as suggested in previous studies [12,13,25]. In addition, faster assessment results are obtained, without the need for specialization in taxonomy and identification. The above suggestion determines that the biological responses of aquatic organisms, as *R. subcapitata* commonly occur quickly and can attend as a prompt warning methodology to evaluate the potential toxicological responses, as already mentioned in previous studies [10,12,25]. In addition, ecotoxicological tests also allow the determination of phytoplankton's capacity to subsist and produce biomass under potential worrying situations.

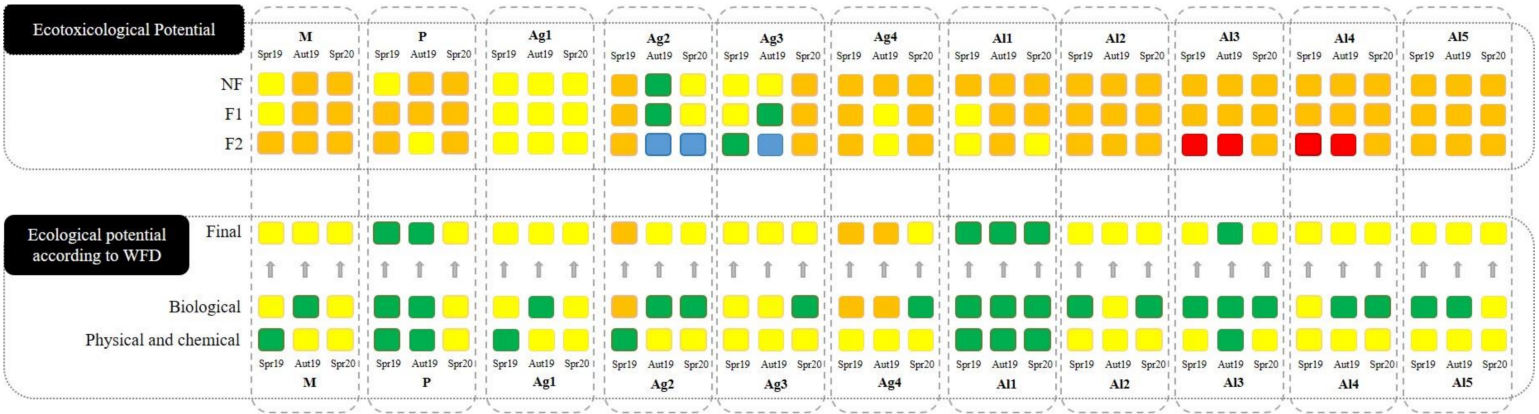


Figure 6. Ecotoxicological potential of the sampling sites and periods, according to natural water treatments (NF, F1 and F2) ecotoxicity results (defined in Table 2) and ecological potential according WFD parameters (Table 1).

4. Conclusions

Algae constitute a basic level in aquatic trophic chains and serve as food for several aquatic organisms, it is essential to recognize and define the threats that can be associated with changes in their natural environment, namely variations in their production and vital capacities. Studies designed to evaluate the associations that occur between living organisms and their environment, as natural waters of reservoirs, provide important information about the status of the freshwater ecosystem and the organism capacity or incapability to cope, when stress factors are present. The originality of this work also comprises in articulating the relations between the biological responses and potential factors that cause negative effects (stress), in which algae are continuously exposed in the surrounding freshwater ecosystems.

The results of this study support those cost-effective and rapid and short-term assays with standard species, performed with natural waters, can be advantageous to complement the determination of the ecological potential of reservoirs. In the studied reservoirs, results of bioassays with *R. subcapitata* obtained good complementarity and sensitiveness. We also verified that seston components represented some degree of stress for algae. For future analogous studies, we also suggest the evaluation of the water treatments like those performed in this work, mainly the F2, to evaluate seston quality. Moreover, high compatibilities between ecotoxicological potential and the ecological potential, established following the WFD parameters, were found especially when considering biological, physical, and chemical parameters evaluated. This approach has demonstrated, in most sites and sampling periods, a good concordance with the ecological potential. These results inspire future work in the applicability of cost-effective ecotoxicity tools for the assessment of the ecological potential of reservoirs and their assimilation in current monitoring programs.

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