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Screening for tolerance to natural phenols of different algal species: Toward the phycoremediation of olive mill wastewater

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ABSTRACT

Olive Mill Wastewater (OMWW) is a by-product of olive oil production and it is rich in nutrients (e.g. P, N and K) and phenolic compounds. These latter are aromatic compounds, and their concentrations can reach up to 11 g L^{-1} in OMWW. A complete remediation of OMWW is required since phenols are known to cause toxicity once released in the environment: particularly, their effect on microorganisms is species-specific and primarily depends on the chemical structure of the compound. Microalgae have already been tested to remediate OMWW, data are promising but how different phenols affect algal growth is still poorly known. In this work, ten microalgal species belonging to different phylogenetic groups and natural habitats, were grown in the presence of three phenolic compounds found in OMWW (tyrosol, coumaric acid, caffeic acid). Algal growth and removal of phenolic compounds were assessed. Tyrosol was the only compound allowing growth of each tested microalga similarly to what observed in control media. Growth of microalgae and removal of phenolic compounds were not always related, and a multi-step phenolic removal mechanism was suggested. Species such as *Nannochloropsis salina* and *Porphyridium purpureum* rapidly died after the addition of coumaric acid or caffeic acid but a high removal percentage (60–100 %) of the phenols was still observed and it was likely due to their absorbance onto the cell surface. On average, freshwater species showed a higher growth performance compared to the one of marine species; in particular, *Tetradesmus obliquus* and *Anabaena* sp. showed the best results. This work elucidates a species-specific effect for each phenolic compound on algal growth and it also highlights that growth and removal are not related phenomena.

1. Introduction

Freshwater resources are limited on Earth and their availability strongly depends on the region considered [1]. Water scarcity together with the Global Climate Change (GCC) and the increased water demand due to a continued human population growth, will be a global challenge [2,3]. Even nowadays, some human communities are already undergoing water shortage [4]. Moreover, especially in developing countries, a wrong discharge of wastewaters can decrease quality and safeness of the available potable water and also hamper ecosystem health, which indirectly influences human health [5]. To guarantee drinkable water and its usage to every human community as well as to reduce stress and pollution of ecosystems, a better management of current freshwater sources and an improvement in wastewater treatment techniques would be desirable [5].

Following the circular economy concept, a possible strategy for wastewater treatment is to use wastewaters as growth media to cultivate microalgae, thus removing polluting substances and generating a safe and re-usable treated water. Microalgae due to their metabolic flexibility [6] can adapt to many different growth conditions, therefore they are suitable organisms for wastewater treatment applications recently named phycoremediation [7]. For instance, an interesting feature of

Abbreviations: OMWW, Olive Mill Waste Water; μ_{max}, maximum growth rate; Nt_e, maximum algal density; μ_{rm}, maximum removal rate; GR ratio, growth rate ratio; Nt_e ratio, maximum algal density ratio.

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some microalgae is the use of organic carbon as source of energy and C-skeletons while fixing inorganic carbon through photosynthesis, as a result of a mixotrophic metabolism. Thereafter, these organisms thrive assimilating the micro- and macro-nutrients present in wastewaters. Phycoremediation is a way to reduce costs of microalgal biomass production and the produced biomass adds value to the remediation process itself.

The added value of such microalgae-based strategy consists in many different molecules of human interest which can be extracted from the algal biomass, like triacylglycerols (TAG) for biofuels production, polyunsaturated fatty acids (PUFAs) and pigments (e.g. β-carotene and phycobiliproteins) for nutraceutical applications [8].

In the Mediterranean area where about 97 % of global olive oil production is found, one of the most abundant agro-industrial waste is represented by Olive Mill WasteWaters (OMWWs), a by-product of the olive oil extraction the [9]. OMWWs are rich in suspended solids (SS), have high chemical and biological oxygen demands (COD and BOD), low pH value and they are also rich in lignin, tannins and other phenolic compounds [9,10]. As a whole, these factors contribute to the environmental impact of OMWWs [11] and the role of phenolic compounds is still unclear.

These compounds are characterized by the presence of one or more phenolic groups, generating simple and complex molecules, with a variety of known and unknown functions [12]. For instance, phenolic compounds can act both as antioxidants, but also as pro-oxidants, in order to regulate cell proliferation or to induce apoptosis [12,13]. The phenolic compounds found in OMWWs can reach a concentration of roughly 11 g L^{-1} [10]; tyrosol (211 mg L^{-1}), p-coumaric acid (117 mg L^{-1}) and caffeic acid (140 mg L^{-1}) are among the most abundant [14]. Composition of phenol compounds is highly variable (including amounts of ferulic acid, trans-cinnamic acid, vanillic acid) and depends on several factors, including the cultivar of the olive tree, the maturity of the fruits at harvest and their conservation, the extraction process, the climatic conditions and the geographical area of origin [15–17].

When phenolic compounds are discharged in the environment, they can cause adverse physiological effects on the biota, depending on the phenolic structure, its concentration, and the biota living in the area [9,10,18]; hence this complexity limits our ability to understand the real magnitude of the environmental impact of phenolic compounds and our ability to look for the proper effective treatment.

Microalgae have already been used to remediate OMWWs, but little is known regarding phenolic effects on these organisms; what is known is that phenolic compounds interact with the algal cell in several ways, but common steps are usually followed: 1) biosorption on the cell wall, 2) transport inside the algal cell, 3) bioaccumulation, 4) conversion and/or degradation. Biosorption on microalgae biomass is a passive mechanism where phenolic compounds interact with functional groups present on the cell wall surface [19], thus they are withheld on it. This process does not require an "active" role of the cell and even dead algal biomass can retain compounds; however the process is more efficient with alive biomass since it can be followed by bioaccumulation and biodegradation processes [19]. Another mechanism which does not include the direct contact of the alga cell with phenolic compounds in the media is the releasing of oxidative enzymes, such as laccase, in the surrounding environment as observed in the green alga *Tetraselmis suecica* by Otto and Schlosser [20].

As shown by Das and co-authors [21], microalgae can degrade organic carbon molecules, such as phenolic compounds through their mixotrophic metabolism. In this way, phenolic compounds can be used as a carbon source or electron donors/acceptors [22]. In their study, Das and co-authors [21] considered the biodegradation pathway of phenol by a specific strain of diatom (BD1IITG). This strain was able to absorb the phenol and degrade it through two metabolic pathways. As shown in Fig. 1 phenol is firstly converted in catechol and then it is meta- or ortho-cleaved, with the latter pathway being more prevalent [21]. For the biodegradation of more complex molecules than phenol, additional steps may be hypothesized which then converge to the pathway proposed by Das and co-authors [21]. Wu and co-authors [22] proposed an additional biodegradation mechanism, by which algae take advantage of phenolic compounds as electron donors to enhance the degradation of other organic molecules.

A first step to screen for the most suitable algal species to remediate OMWW was to study the effect of tyrosol, p-coumaric acid and caffeic acid on the growth of ten different freshwater and marine microalgal species spanning among different taxonomic groups. Removal rate of each compound was also assessed for the algal candidates.

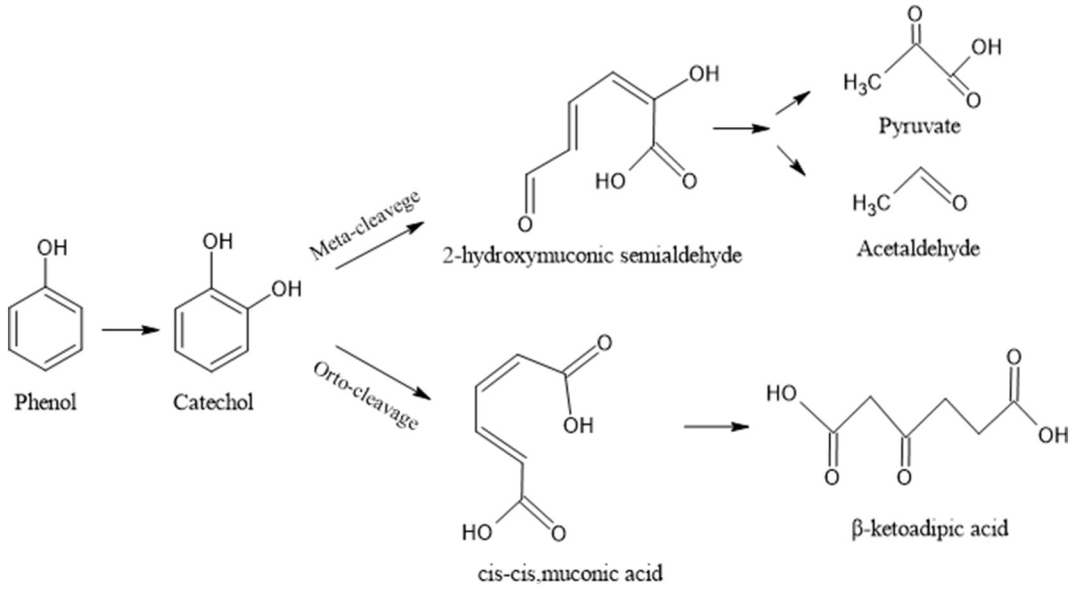

Fig. 1. Biodegradation pathway of phenol as proposed by Das and co-authors [21]. The aromatic ring is first hydroxylated and then the compound undergoes a meta or orto-clevage which lead to the production of pyruvate and acetaldehyde or β-ketoadipic acid respectively. Chemical structures were generated using Chemsketch software (Advanced Chemistry Development, Inc.)

2. Materials and methods

2.1. Microalgal strains and culture maintenance

Ten algal species from three evolutionary lineages (cyanobacteria, green and red lineages) were investigated: six species were marine, the remaining four were freshwater species. A detailed list of the species and the respective growth media is reported in Table 1.

Microalgae were maintained in 250 mL Erlenmeyer flasks containing 100 mL growth medium. The cultures were incubated at 20 °C, 100 μmol photons m^{-2} s^{-1} and 24 h light photoperiod. AMCONA growth medium was prepared according to Fanesi and co-authors [23] and used for marine species (Table 1), Zarrouk for *Arthrospira Platensis* according to Zarrouk [24], BG11 for the freshwater species (Table 1) according to Allen and Stanier [25], 3 N-BBM for *Anabaena* sp. according to Bischoff and Bold [26].

2.2. Experimental design

Growth of algae in presence of phenolic compounds was assessed for 15 days in 6 well plates at 20 °C, 100 μmol photons m^{-2} s^{-1} and 24 h light photoperiod.

Algae were transferred from the Erlenmeyer flasks in standard conditions (see Section 2.1) to wells at the concentration of 1×10^5 cells mL^{-1} for unicellular algae and 0.25 mg mL^{-1} for colonial algae (*Anabaena* sp. and *Arhtrospira platensis*). Each well contained 10 mL of culture and the experiments were performed in three biological replicas.

Among the phenolic compounds naturally present in wastewaters produced during the olive oil extraction (Deeb et al., 2012), tyrosol, coumaric acid and caffeic acid were selected and used in the experiments. Chemicals for solutions were acquired from Sigma-Aldrich Corporation: tyrosol ($C_8H_{10}O_2$), MW 138.16 g mol^{-1}, purity 98 % (HPLC); p-coumaric acid ($C_9H_8O_3$), MW 164.05 g mol^{-1}, purity ≥98 % (HPLC); caffeic acid ($C_9H_8O_4$), MW 180.16 g mol^{-1}, purity ≥98 % (HPLC). Final concentration of each phenolic compound in growth media was a fifth (1/5) of the respective concentration in an "average" OMWW [14]: tyrosol 42.12 mg L^{-1}; p-coumaric acid 23.42 mg L^{-1}; caffeic acid 28.08 mg L^{-1}. Since coumaric and caffeic acids were soluble only in pure ethanol, preliminary growth tests were run adding only the solvent volumes to the growth media. They showed algal behaviour was not affected (Appendix B).

Growth in the presence of phenolic compounds was compared to growth in standard growth media (according to Table 1) without the addition of phenols; cultures in standard growth media are hereafter named CTRs.

Except for caffeic acid, phenolic compounds did not alter the colour of the media: when caffeic acid was added, the medium acquired a brownish colour (Fig. 2).

Table 1
List of experimental microalgae grouped by taxonomy, habitat, origin and growth medium.

Species	Habitat	Origin and ID code	Growth medium
Chlorophyta			
Dunaliella salina.	Marine, hypersaline	Isolated from Margherita di Savoia saltpans (Italy)	Amcona
Tetradesmus obliquus (Turpin) Wynne 2016	Freshwater	CCAP 276/3A	BG11
Chlorella vulgaris Beyerinck 1890	Freshwater	CCAP 211/11b	BG11
Bacillariophyceae			
Phaeodactylum tricornutum Bohlin 1898	Marine	UTEX 646	Amcona
Eustigmatophyceae			
Nannochloropsis salina D.J. Hibberd 1981	Marine	CCAP 849/3	Amcona
Rhodophyta			
Porphyridium purpureum (Bory) K.M. Drew & R. Ross 1965	Marine	CCAP 1380/3	Amcona
Cyanobacteria			
Arthrospira platensis Gomont 1892	Brackish, Freshwater	Gottingen SAG 85.79	Zarrouk
Anabaena sp.	Freshwater	CCAP 1403/4a	3N-BBM
Synechococcus sp.	Marine	UTEX LB 2380	Amcona
Haptophyta			
Isochrysis galbana Parke 1949	Marine	Roscoff RCC 1353	Amcona

2.3. Algal growth

Algal growth was monitored daily for 15 days by counting cells using an automatic cell counter (Casy TT, Innovatis AG, Reutlingen, Germany) or by measuring OD (optical density) at 750 nm (UV-1900i, SHIMADZU CORP.). The optical density of *Anabaena* sp. and *A. platensis* biomass was monitored and converted into dry weight (DW) through calibration curves between OD_{750} and DW.

Maximum growth rate (μ_{max}) was determined through non-linear regression of experimental algal growth curve; β-function as reported by Yin [28] (Eq. (1)) and also reported to analyse algal growth by Lee and co-authors [29], and by Petrucciani and co-authors [30] was used. In the β-function N is the algal concentration, C_m is the maximum growth rate which is achieved at time t_m, μ_{max} is the maximum growth rate normalized on Nt_m (Eq. (2)), t_b is the reference time and t_e is the time at the end of the growth. The δ parameter, which can influence the curvature, is set to 1 as suggested by Yin [28] to simplify the equation as it follows:

$$\frac{dN}{dt} = C_m\left(\frac{t_e - t}{t_e - t_m}\right)\left(\frac{t - t_b}{t_m - t_b}\right)^{\frac{t_m - t_b}{t_e - t_m}} \quad (1)$$

$$\mu_{max} = \frac{C_m}{Nt_m} \quad (2)$$

2.4. Quantification of phenolic compounds

Every two days from the addition to growth media, the content of phenolic compounds was quantified using a modified Folin-Ciocolteau method as reported by Chandra and co-authors [27]. Quantification was carried out also on modified media without algal cells to check photodegradation of phenols. Quantification was done directly on 125 μL aliquot of modified medium where no cells were inoculated while quantification in cultures was done on the supernatant obtained after 10′ centrifugation at 3000 rpm of 1 mL culture; an aliquot of 125 μL of the supernatant was added to 125 μL of Folin reagent and the solution was mixed vigorously. After 6′ of reaction time, a volume of 2.25 mL of 4 % $NaHCO_3$ was added to the solution, mixed and left in the dark for 90′. Phenolic content was assessed spectrophotometrically through analysis of the absorbance at 760 nm (UV-1900i, SHIMADZU CORP.) and using a calibration curve realized with concentrations from 0 to 100 mg L^{-1} of gallic acid as standard phenolic compound. Results were reported percentage of the initial concentration.

To evaluate if phenolic compounds were absorbing in the same wavelength range as chlorophylls, their absorbance was spectrophotometrically measured in growth media without algae from 350 nm to 750 nm.

2.5. Phenolic compound removal rate

Maximum removal rate (μ_{rm}) was assessed through non-linear regression of phenolic compound concentration. A logit model (Eq. (3)) was used. In logit model N is the phenolic compound concentration

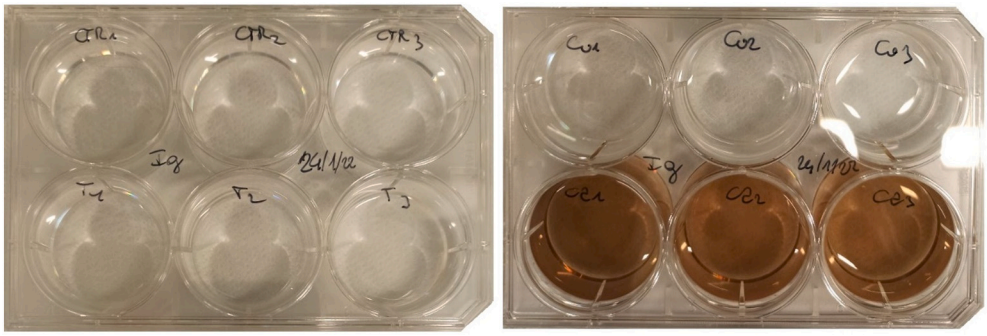

Fig. 2. Experimental set up: 6 well plates before the algal inoculum. Each well contained a maximum volume of 10 mL and phenols were added directly in the well. Each condition was performed in triplicate. On the top left there are the three control replicas (CTR_{1-3}); on the bottom left the tyrosol (T_{1-3}); on the top right the coumaric acid (Cu_{1-3}); on the bottom right the caffeic acid (Ca_{1-3}). The addition of caffeic acid modified the colour of the growing medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

removed from the medium, K is the maximum phenolic compound concentration removed from the medium, μ_{rm} is the maximum removal rate. The regression curves were shown as percentages of phenolic compound present in the medium as a function of time (Appendix A). Removal rates were compared within each species and across them.

$$\frac{dN}{dT} = \mu_{rm}\frac{K-N}{K}\,N \tag{3}$$

2.6. Growth ratio analysis

To compare responses across all species to phenolic compounds a growth rate (GR) ratio (Eq. (4)) was calculated normalizing growth rates of treated algae by the average growth rate of their relative control (CTR) (see Section 2.3 Growth rate analysis). Similarly, ratio of maximum density (Nt_e Ratio) achieved at t_e was also calculated (Eq. (5)).

Both GR and Nt_e ratios were grouped by phenolic compound and their averages were compared.

$$GR\ ratio = \frac{\mu_{max}\ Treatment}{Mean\ \mu_{max}\ CTR} \tag{4}$$

$$Nt_e\ ratio = \frac{Nt_e\ Treatment}{Mean\ Nt_e\ CTR} \tag{5}$$

2.7. Statistical analysis

All data are shown as arithmetic mean and standard deviation (±SD) of three to six ($3 \le n \le 6$) independent biological replicates. The results were analyzed using software Graphpad prism 9.0.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Tukey multiple comparison post-hoc test was used to compare data within and across algal species. GR ratios and removal rates of freshwater and marine species were compared by *t*-test. All statistical analyses were performed with a significance level of $\alpha = 0.05$. Letters were used in figures and tables to distinguish significantly different groups ($P < 0.05$).

3. Results and discussion

3.1. Algal growth

Microalgae survival and/or death in presence of phenolic compounds have been widely discussed especially in the case of pollutants produced by petro-chemical industries such as: phenol, cresol, chlorophenol and nitrophenol [31,32]. Tolerance of microalgae largely varies and it is not so rare to observe tolerance up to 150 mg L^{-1} of cresol, 200 mg L^{-1} of cresol and 270 mg L^{-1} of p-cresol for *Dunaliella salina*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* respectively [33–36]. Concerning phenols deriving from the processing of olives, most research focused on the remediation of OMMWs rather than on the cytotoxic examination of such phenolic compounds and a few authors claimed a toxic effect on algae even at low concentration (5 mg L^{-1}) [37].

According to the overview table (Table 2) where data regarding growth are reported, and to the growth curves reported in Appendix A, phenolic compounds stimulated or inhibited algal growth in terms of rate and/or density depending on the algal species and on their chemical form. The results are in line with similar studies showing specific effects depending on algae and phenols [36]; concentration even higher than 5 mg L^{-1} (from 23 up to 42 mg L^{-1}) did not induce toxicity in part of the microalgal species here tested, such as *C. vulgaris* and *T. obliquus*. Among the treatments the addition of tyrosol was the solely condition which allowed growth of all the algae.

Chlorophyta grew in each treatment and they were the only group tested whose members survived till the end of the experiment in each condition. Among them, the cell density at day 15 and the growth rate of *T. obliquus* and *C. vulgaris* grown in the presence of tyrosol or coumaric acid were comparable to the values of their respective CTR. Thus, treatment with one of these phenolic compounds did not show any effect on growth. The same species grown in the presence of caffeic acid showed slightly higher μ_{max} and final density as compared to CTR and to other treatments. As the phylogenetically related species, the third green alga *D. salina* did not show any effect due to the addition of tyrosol, both in terms of growth rate and cell density at day 15; nevertheless it showed a particular sensibility for coumaric and caffeic acids; in fact, at the end of the experiment, cell density of cultures exposed to coumaric and caffeic acids was lower than the CTR one. Growth data of *T. obliquus* and *C. vulgaris* agree with those in literature where these algae are renowned to remediate wastewaters and to tolerate heavy metals, phenols or toxic compounds [38–40].

Several researches on algal phenol tolerance highlighted the importance of a cell wall in the resistance and detoxification of phenolic compounds [41,42]: cell wall is supposed to act as filter avoiding internal damage to organelles such as plastids, mitochondria and nuclei [43]. Contrary to the literature, our data suggested that growth is not strictly related to the presence or absence of a cell wall; *D. salina*, a cell wall-lacking alga, was indeed one of the few species who survived in each condition.

Among the aforementioned species, *T. obliquus* grown in phenols differed in shape compared to the CTR shape and produced an EPS matrix (Extracellular Polymeric Substances) which promotes cell adhesion (Appendix C). It is known that *Tetradesmus* strains might undergo morphological changes under stress [44,45] and production of extracellular matrix structures [46–48]. EPS are rich in polysaccharides and proteins, they have several functions including defence against toxic compounds [49,50]; therefore, it might be supposed a role of EPS against OMWW phenols.

For the diatom *P. tricornutum*, the final cell density of cultures treated with tyrosol and coumaric acid was slightly lower than the CTR one. On the contrary, caffeic acid was harmful to the alga who died in a couple of days. Growth rates of CTR, tyrosol and coumaric acid grown cells were

Table 2
Algal growth with or without phenolic compounds (CTR). Data are reported as mean ± standard deviation. Results of statistical analysis (One-way ANOVA) are reported on the right of each value. Values followed by different letters are significantly different ($P < 0.05$). The statistical analysis was made within each species. Density of *Anabaena* sp. and *Arthrospira platensis* are reported as mg mL^{-1}. β-Function non-linear regression model was used to calculate growth rate and Nt_e. Coefficient of determination (R^2) of non-linear regression model is reported.

Taxon	Species	Growth condition	Growth rate (d^{-1})				Density at 15 day (Cells mL^{-1} or mg mL^{-1})				Nt_e (Cells mL^{-1} or mg mL^{-1})				R^2			
Chlorophyta	*Dunaliella salina*	Control	0.22	±	0.01	b	9.76E+05	±	1.56E+05	a	9.35E+05	±	9.20E+04	a	0.92	±	0.01
		Tyrosol	0.23	±	0.02	b	8.76E+05	±	2.89E+04	a	8.75E+05	±	3.33E+04	a	0.92	±	0.02
		Coumaric acid	0.23	±	0.00	b	2.10E+05	±	1.77E+04	b	2.52E+05	±	2.47E+04	b	0.95	±	0.03
		Caffeic acid	0.36	±	0.01	a	3.89E+05	±	1.64E+05	b	3.44E+05	±	4.91E+04	b	0.78	±	0.23
	Tetradesmus obliquus	Control	0.23	±	0.05	b	3.94E+06	±	2.95E+05	ab	4.86E+06	±	1.05E+06	a	0.98	±	0.01
		Tyrosol	0.23	±	0.02	b	3.49E+06	±	2.71E+05	b	4.24E+06	±	2.99E+05	a	0.99	±	0.01
		Coumaric acid	0.18	±	0.00	b	3.96E+06	±	7.50E+04	ab	4.14E+06	±	1.04E+05	a	0.98	±	0.00
		Caffeic acid	0.32	±	0.04	a	4.14E+06	±	2.74E+05	a	4.19E+06	±	3.47E+05	a	1.00	±	0.00
	Chlorella vulgaris	Control	0.22	±	0.01	b	2.59E+06	±	3.50E+05	a	3.13E+06	±	8.29E+04	a	0.98	±	0.03
		Tyrosol	0.23	±	0.02	b	2.01E+06	±	8.34E+05	a	2.81E+06	±	4.61E+05	ab	0.93	±	0.09
		Coumaric acid	0.23	±	0.00	b	2.03E+06	±	5.90E+04	a	2.44E+06	±	1.05E+05	b	0.99	±	0.01
		Caffeic acid	0.36	±	0.01	a	2.00E+06	±	1.45E+05	a	2.43E+06	±	1.66E+05	b	0.98	±	0.01
Diatom	*Phaeodactylum tricornutum*	Control	0.24	±	0.00	a	1.59E+07	±	1.03E+06	a	1.58E+07	±	7.72E+05	a	0.96	±	0.01
		Tyrosol	0.24	±	0.00	a	1.20E+07	±	3.48E+05	b	1.30E+07	±	1.69E+05	b	0.97	±	0.01
		Coumaric acid	0.25	±	0.01	a	1.19E+07	±	5.86E+05	b	1.29E+07	±	8.53E+05	b	0.96	±	0.01
		Caffeic acid	−1.17	±	0.00	b	0.00E+00	±	0.00E+00	c	0.00E+00	±	0.00E+00	c	1.00	±	0.00
Eustigmatophyte	*Nannochloropsis salina*	Control	0.22	±	0.01	b	1.54E+07	±	8.07E+05	a	1.57E+07	±	1.21E+06	a	0.99	±	0.01
		Tyrosol	0.23	±	0.01	b	1.05E+07	±	1.44E+06	b	1.02E+07	±	1.04E+06	b	0.97	±	0.01
		Coumaric acid	0.63	±	0.00	a	0.00E+00	±	0.00E+00	c	8.25E+05	±	7.66E+04	c	0.93	±	0.01
		Caffeic acid	0.68	±	0.10	a	0.00E+00	±	0.00E+00	c	3.40E+05	±	3.65E+04	c	0.90	±	0.06
Rhodophyta	*Porphyridium purpureum*	Control	0.16	±	0.02	a	3.33E+05	±	1.02E+05	a	4.71E+05	±	5.52E+04	a	0.89	±	0.03
		Tyrosol	0.13	±	0.05	a	9.16E+04	±	1.53E+04	b	1.89E+05	±	5.12E+04	b	0.85	±	0.11
		Coumaric acid	−0.35	±	0.14	b	0.00E+00	±	0.00E+00	b	0.00E+00	±	0.00E+00	c	0.66	±	0.03
		Caffeic acid	−0.68	±	0.17	c	0.00E+00	±	0.00E+00	b	0.00E+00	±	0.00E+00	c	0.73	±	0.03
Cyanobacteria	*Arthrospira platensis*	Control	0.21	±	0.00	a	3.71E+00	±	1.09E-01	a	4.33E+00	±	1.34E-01	a	0.94	±	0.01
		Tyrosol	0.18	±	0.03	a	9.07E-01	±	1.38E-01	b	9.64E-01	±	8.73E-02	b	0.86	±	0.05
		Coumaric acid	0.19	±	0.00	a	3.79E+00	±	3.93E-01	a	4.12E+00	±	3.46E-01	a	0.99	±	0.01
		Caffeic acid	0.17	±	0.01	a	1.73E+00	±	5.46E-02	c	1.77E+00	±	7.95E-02	c	0.99	±	0.01
	Anabaena sp.	Control	0.14	±	0.01	a	9.34E-01	±	6.01E-02	a	1.04E+00	±	5.44E-02	a	0.97	±	0.01
		Tyrosol	0.07	±	0.01	b	7.18E-01	±	6.81E-03	c	8.25E-01	±	2.81E-02	b	0.93	±	0.01
		Coumaric acid	0.05	±	0.00	b	7.21E-01	±	1.12E-02	c	7.49E-01	±	1.28E-02	b	0.98	±	0.01
		Caffeic acid	0.05	±	0.00	b	8.15E-01	±	2.44E-02	b	7.79E-01	±	8.24E-03	b	0.97	±	0.02
	Synechococcus sp.	Control	0.24	±	0.01	a	1.60E+07	±	2.65E+05	a	1.42E+07	±	1.49E+06	a	0.98	±	0.02
		Tyrosol	0.20	±	0.02	a	1.49E+07	±	3.44E+06	a	1.70E+07	±	2.77E+06	a	0.96	±	0.01
		Coumaric acid	0.69	±	0.09	a	0.00E+00	±	0.00E+00	b	1.76E+05	±	4.76E+03	b	0.96	±	0.07
		Caffeic acid	−1.68	±	1.24	b	0.00E+00	±	0.00E+00	b	0.00E+00	±	0.00E+00	b	0.94	±	0.04
Haptophyte	*Isochrysis galbana*	Control	0.21	±	0.01	b	1.78E+07	±	9.18E+05	a	1.80E+07	±	6.38E+05	a	0.99	±	0.00
		Tyrosol	0.22	±	0.00	b	1.50E+07	±	3.33E+05	b	1.53E+07	±	3.88E+05	b	0.99	±	0.00
		Coumaric acid	0.50	±	0.00	a	0.00E+00	±	0.00E+00	c	1.23E+06	±	2.79E+04	c	1.00	±	0.00
		Caffeic acid	−0.34	±	0.09	c	0.00E+00	±	0.00E+00	c	0.00E+00	±	0.00E+00	d	0.91	±	0.04

comparable.

The responses of the eustigmatophyta *N. salina* and the rhodophyta *P. purpureum* were similar: coumaric acid and caffeic acid proved to be toxic since algae died and no cell was detected after 4 to 7 days of exposure. Regarding the treatment with tyrosol, growth of *N. salina* was less affected than growth of *P. purpureum*: the eustigmatophyta cell density at the end of the experiment was 1.5 times lower than its CTR value while *P. purpureum* cell density was 3.5 times lower compared to its CTR one.

Within the cyanobacteria, *Anabaena* sp. showed a 9 to 23 % lower biomass in the presence of phenols than the CTR biomass after 15 days of growth. Similarly, growth rates were statistically lower than the CTR growth rate but comparable among the treatments (Table 2). *A. platensis* displayed positive growth for each treatment even if achieving different biomass values at the end of the experiment: the biomass obtained in tyrosol and caffeic acid treatments was significantly lower than the one of CTR and coumaric acid grown cultures (Table 2). On the contrary, growth rates were statistically similar among all the growth conditions (Table 2). The third cyanobacterium, *Synechococcus* sp., proved to be more susceptible than the previous phylogenetically related species: when coumaric acid was added to the culture an initial growth was recorded (μ_{max} of 0.20 ± 0.02 d^{-1}), rapidly followed by cell density decrease and death of the cells; in the treatment with caffeic acid no growth was observed (μ_{max} of −1.68 ± 1.24 d^{-1}). *Synechococcus* growth with tyrosol was comparable to CTR growth in terms of both growth rate and biomass.

Response to phenolic compounds by the haptophyte *I. galbana* was similar to that of *N. salina*: total inhibition of growth was observed for treatments with coumaric and caffeic acids while tyrosol did not prevent growth. Despite being significantly different, growth rate and cell density at the 15th day of tyrosol growth condition were only 10 % and 15 % lower than those observed at CTR condition, respectively. *I. galbana* is not covered by calcite scales (coccoliths) as other representatives of the haptophyta group [51,52] and the cell is simply encapsulated by plasma membranes making the algae easily disrupted by mechanical and chemical stress [53]. Contrary to the other cell wall-lacking alga, *D. salina*, *I. galbana* less tolerated the addition of coumaric acid and caffeic acid compared to *D. salina*, as previously reported. Based on the results it could be proposed that cell wall is more important for removal of phenolic compounds rather than for algal survival (see Section 3.2).

As reported in Table 2 and Appendix A, growth rate, Nt_e, and biomass achieved at the end of the experiment were not always positively related. For instance, a delayed toxic effect was observed in *I. galbana*, *Synechococcus* sp. and *N. salina* treated with coumaric acid (Table 2, Appendix A) where growth rate was even higher than the value in CTR cultures while the Nt_e value was lower and no cell survived at day 15. While being counterintuitive, an explanation could be found on the production of toxic intermediate compounds [54,55]: the phenolic compounds, after their biosorption and uptake inside the cell, can undergo active transformation by algal enzymes, such as oxidase, which can produce harmful electrophilic metabolites [55]. In addition, the oxidase effect on the phenolic compound may lead to an uncoupling effect on mitochondrial oxidative phosphorylation resulting in an alteration of the pH gradient and the consequent synthesis of ATP [33,56].

3.2. Removal of phenolic compounds

Removal activity of phenolic compounds agreed with data of previous studies where it appeared to be species-specific [36,43]; however it was observed that removal occurred even in the absence of algal growth. Except for *D. salina*, the removal percentage averaging the three treatments within each species was over 45 % and reached more than 90 % in *N. salina* (Table 3). According to literature, removal and further degradation of phenols by algae followed a first step of passive physiochemical biosorption onto the cell wall and a subsequent absorption into the cytoplasm, then an eventual degradation [43]. Indeed, removal of phenolic compounds from the medium was observed in the presence of all the species except for *D. salina* (Table 3); the lack of a cell wall [57] could be the reason why no removal was observed since the biosorption step is crucial for the uptake of phenolic compounds and other elements such as heavy and toxic metals [43,58].

When phenol removal occurred in presence of algal growth, most of phenolic compound removal was observed during the exponential growth phase of the culture (Appendix A). Data regarding removal percentage and rate are reported in Tables 3 and 4.

On average, *N. salina* was the best alga to perform a remediation of phenols: almost all the amount of tyrosol, coumaric acid and caffeic acid was removed from the growth media (Table 3). *P. tricornutum* proved to be very effective for the removal of tyrosol and coumaric acid (Table 3) while it removed more than half of the caffeic acid (60 ± 15 %). To the best of our knowledge, data regarding interaction between diatoms and phenols are quite meagre and *P. tricornutum* has never been previously investigated for phenol phycoremediation. Up to now, few diatoms (e.g. *Thalassiosira* sp. and diatom BD1IITG) are known to be able to remove phenols [21,59].

Regarding *P. purpureum*, the removal of phenolic compounds was highest in the case of coumaric acid with its total depletion (Table 3). *T. obliquus*, one of the four freshwater algae tested, removed almost all the content of tyrosol and coumaric acid from the media (Table 3). *T. obliquus* was more reluctant to remove caffeic acid, even if no negative effect on growth was observed (Table 2, Appendix A). The EPS formation observed in this species (see Section 3.1) (Appendix C) could also be involved in enhanced removal of phenolic compounds: similar studies on organic contaminants [49,60–63] elucidated the mechanism of algal EPS suggesting that such biofilm matrix acts as an external digestive system allowing enzymes to metabolize organic compounds outside the cell.

Table 3
Percentage removal of phenolic compounds. Data are reported as mean ± standard deviation. Results of statistical analysis (One-way ANOVA) are reported on the right of each value. Values followed by different letters are significantly different ($P < 0.05$). Statistical analysis was made within each treatment condition.

Species	Phenolic compounds removal (%)											
	Tyrosol				Coumaric acid				Caffeic acid			
Dunaliella salina	0 %	±	0 %	e	0 %	±	0 %	e	50 %	±	2 %	cde
Tetradesmus obliquus	97 %	±	3 %	a	100 %	±	0 %	a	50 %	±	8 %	cde
Chlorella vulgaris	9 %	±	2 %	de	82 %	±	7 %	a	52 %	±	10 %	cd
Phaeodactylum tricornutum	100 %	±	0 %	a	100 %	±	0 %	a	60 %	±	15 %	bcd
Nannochloropsis salina	96 %	±	5 %	a	94 %	±	9 %	a	87 %	±	0 %	a
Porphyridium purpureum	70 %	±	2 %	b	100 %	±	0 %	a	84 %	±	2 %	ab
Arthrospira platensis	22 %	±	3 %	c	92 %	±	2 %	a	40 %	±	5 %	de
Anabaena sp.	99 %	±	1 %	a	56 %	±	5 %	b	32 %	±	3 %	e
Synechococcus sp.	100 %	±	0 %	a	8 %	±	11 %	de	65 %	±	3 %	abc
Isochrysis galbana	16 %	±	8 %	cd	36 %	±	10 %	c	85 %	±	5 %	ab
Average	61 %	±	44 %	a	67 %	±	39 %	a	61 %	±	19 %	a

Table 4
Removal rate of phenolic compounds. Data are reported as mean ± standard deviation. Results of statistical analysis (One-way ANOVA) are reported on the right of each value. Values followed by different letters are significantly different ($P < 0.05$). Statistical analysis was made within each treatment condition.

Species	Phenolic compounds removal rate (d^{-1})											
	Tyrosol				Coumaric acid				Caffeic acid			
Dunaliella salina	0.00	±	0.00	f	0.00	±	0.00	e	0.46	±	0.09	c
Tetradesmus obliquus	0.38	±	0.01	d	2.66	±	0.42	a	0.45	±	0.05	c
Chlorella vulgaris	0.12	±	0.02	e	0.64	±	0.10	cd	4.89	±	3.56	abc
Phaeodactylum tricornutum	0.59	±	0.03	bc	1.01	±	0.02	bc	6.95	±	3.81	ab
Nannochloropsis salina	0.33	±	0.02	d	0.31	±	0.01	e	5.60	±	0.22	abc
Porphyridium purpureum	0.82	±	0.07	a	1.06	±	0.03	b	9.32	±	0.00	a
Arthrospira platensis	0.52	±	0.06	c	0.41	±	0.01	d	0.38	±	0.11	c
Anabaena sp.	0.64	±	0.02	b	0.30	±	0.01	e	0.31	±	0.04	c
Synechococcus sp.	0.87	±	0.00	a	0.00	±	0.00	e	1.89	±	0.23	bc
Isochrysis galbana	0.11	±	0.05	e	0.15	±	0.01	e	3.52	±	2.55	bc
Average	0.44	±	0.29	b	0.66	±	0.78	b	3.38	±	3.46	a

Another freshwater species with high remediation yield was *Anabaena* sp., in particular tyrosol was removed with a percentage of 99 % ± 1 %. Similarly to *Anabaena* sp., the other cyanobacterium *Synechococcus* sp. was able to remediate the totality of tyrosol but only 8 ± 11 % of coumaric acid from the culture media. The last cyanobacterium tested, *A. platensis*, did not achieve optimal results for tyrosol (22 ± 3 %) and caffeic acid treatments (40 ± 5 %), but the alga obtained optimal remediation of coumaric acid (92 ± 2 %). The green alga *C. vulgaris* already known to be effective in the remediation of toxic compounds and wastewaters [64,65] was primarily able to treat coumaric acid (82 ± 7 %) and caffeic acid (52 ± 10 %) but not tyrosol (9 ± 2 %). The haptophyte *I. galbana* showed the highest removal percentage for the caffeic acid growth condition together with *N. salina* and *P. purpureum* (85 ± 5 %) but low removal percentages of tyrosol and coumaric acid. Lastly, *D. salina* was almost not able to remove tyrosol and coumaric acid while it removed half of the caffeic acid, 50 ± 2 %, from the medium (Table 3).

Overall, the higher removal rate (μ_{rm}) was achieved by *P. purpureum* when treated with caffeic acid: despite the algal death and negative growth rate (Table 2) the μ_{rm} was 9.32 ± 0.00 d^{-1}. As reported in Tables 2, 3, 4 and graphically reported in Appendix A, high growth rate did not always imply high removal percentage: *P. tricornutum* and *T. obliquus* treated with caffeic acid had similar removal percentages (50 % ± 8 % and 60 % ± 15 %) but much different removal rates (0.45 ± 0.05 d^{-1} and 6.95 ± 3.81 d^{-1}).

It is worth noticing that *D. salina* and *I. galbana*, both without a rigid cell wall, did not reduce the concentration of tyrosol and coumaric acid, however reduction of caffeic acid concentration was observed. While *D. salina* removed caffeic acid in presence of cell growth (Appendix A), *I. galbana* showed a much higher removal rate than the previous alga (3.52 ± 2.55 d^{-1} in *I. galbana* and 0.46 ± 0.09 d^{-1} in *D. salina*) but without any growth. The reduction in caffeic acid concentration cannot be explained by a biosorption mechanism as suggested for other species (*P. purpureum*, *N. salina*) but other mechanisms, e.g. intermediate metabolite production, can be supposed.

Results on the phenolic compound removal are promising and expand current literature which reports high removal yield for phenolic compounds other than the natural ones here tested [36]; moreover, literature on wastewater treatment plants mostly focuses on freshwater algae exploitation while information on suitable marine algae is almost missing. At last, as reported by Lindner and Pleissner [31], few studies tried to address the underlying mechanisms adopted by algae in phenol removal by observing physiological changes.

It is challenging though to compare present results with the available data in literature since several factors such as temperature, nutrients, light and presence of metabolic intermediates may alter or modulate algae survival and removal rate [22,36].

More growth and better remediation could be obtained by acclimating algae to phenolic compounds as already reported by Cho and co-authors [33]. Acclimation to a different environment involves a modification in the proteome and metabolome that is inherited by daughter cells and increases their fitness [23,66]. As reported by Borowitzka [67], a temporary or prolonged disturbance of homeostasis due to the action of a stressor (e.g. phenolic compound), is followed by changes in algal physiology which require different times to be implemented in regulation, acclimation and adaption. If homeostasis is greatly disturbed, regulation and acclimation cannot be reached and the cell can rapidly die. Because the experiment here reported was supposed to evaluate the acute response of algal cell to the addition of phenolic compounds, no time for acclimation (at least the duplication time of 3 generations) was planned. Acclimated cells could reach higher removal and growth rates.

A second approach to improve effectiveness of the phycoremediation includes varying the number of inoculum cells especially for those algae which face death within few days. Despite Lau and co-authors [68] reported that changing the inoculum density did not change toxicity, growth rates and remediation yield of microalgae, inoculum size affects the removal of phenols when biosorption is the main removal mechanism. Indeed biosorption is a passive mechanism depending on the number of active sites on the cell wall such as carbonyl, carboxylic, amino and hydroxyl groups [69]. The higher the inoculum density the higher the number of active sites and so the removal capacity. It should also be considered that biosorption is strictly related to environmental conditions, such as pH and temperature, and to algal shape and cell wall biochemistry [70,71].

A third approach is the addition of simple organic carbon molecules (e.g. glucose) in growth media as an energy source for algal cells in order to remove the multiple functional groups attached to the aromatic ring of phenols; only then phenolic compounds become carbon sources available to cells and are completely removed from the medium [72].

3.3. Light effect on phenols

Absorbance of phenolic compounds added to growth media without algae was only observed between 350 and 390 nm. Since most of microalgae absorb light in the range of 500–800 nm [73], light availability to photosynthetic pigments was not altered by the addition of phenolic compounds.

As far as it is known [74–76], phenolic compounds are light-sensible and they can encounter a photo-degradation process if exposed to specific intensities and wavelengths. Photo-degradation of OMWW phenols has been observed when catalysts or sensitizers were added to the media [77,78], therefore an evaluation of photo-degradation of phenols under experimental conditions was carried out.

According to data reported in Fig. 3, photo-degradation of phenolic compounds was minimum or irrelevant. During the 15 days of analysis, algae were not added to the media and phenol concentrations did not decrease except for caffeic acid whose concentration decreased a few percentage points.

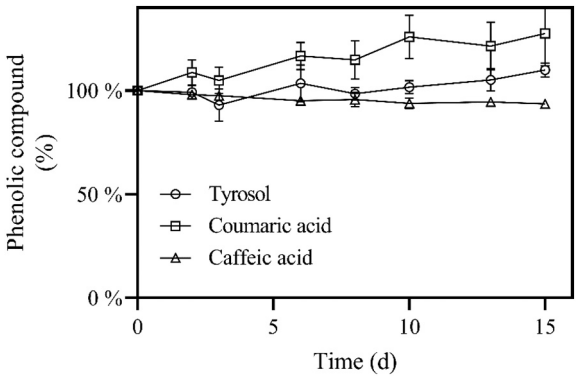

Fig. 3. Percentage variation of phenolic compounds during 15 days in absence of cells. Data are reported as mean ± standard deviation.

The observed removal of phenolic compounds was then exclusively related to the presence of algal cells within the growth media. If light did not directly influence the degradation of phenolic compounds, it indirectly contributed through the interaction with algal cells [36] whose removal pattern of phenolic compounds differs in a species-specific way (Appendix A).

3.4. Analysis of GR and Nt_e ratios

GR ratio and Nt_e ratio allowed us to evaluate the effect of phenolic compounds by comparing growth parameters, μ_{max} and Nt_e, across algal species. The lowest GR ratio was recorded for caffeic acid (Fig. 3), while the highest value was obtained by tyrosol (0.99 ± 0.15). Similarly, lowest Nt_e ratio was obtained for caffeic acid (Fig. 3) suggesting its higher toxicity compared to other phenolic compounds. Nt_e ratio of tyrosol was statistically different from the ratios in the other two conditions (p value 0.0019, p value <0.0001 respectively) with a value close to 1, meaning similar cell densities compared to those achieved in the respective CTR conditions and, thus, a lower average toxicity (Fig. 4).

These results suggest the existence of a scale of toxicity among the phenolic compounds: caffeic acid > coumaric acid > tyrosol (where caffeic acid is the more toxic and tyrosol the less toxic) (Fig. 4). The results are also in line with previous data found in literature where it is reported a rise of the toxicity of phenols with the increase of compound complexity and the number of substituents on the aromatic ring [36,79].

Analysis of GR ratio between freshwater and marine algae did not show a significative difference for tyrosol and coumaric acid treatments while a statistically lower GR ratio was observed for the marine species group when caffeic acid was added to the growth medium (Fig. 5).

Despite no difference observed in GR ratio between the first two

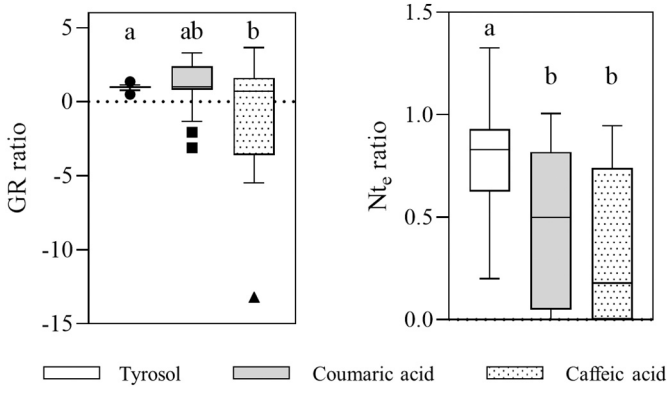

Fig. 4. Comparison of GR ratio (left) and Nte ratio (right) between the three treatments. Above each box-plot is a letter resulting from statistical analysis. Different letters are significantly different ($P < 0.05$).

treatments, Nt_e ratio (Fig. 6) and growth curves (Appendix A) showed differences: indeed, three marine species, *N. salina*, *Synechococcus* sp. and *I. galbana*, did not survive at the end of the experiment despite the initial growth (Table 2, Appendix A).

Moreover, analysis of the Nt_e ratio pointed out an average lower cell density for marine species compared to the freshwater group (Fig. 6). Again, tyrosol had almost no toxic effect on algae: maximal cell density was similar to the CTR one (Nt_e ratio of 0.99 ± 0.15 and 0.97 ± 0.16 for freshwater and marine group, respectively). The effect of coumaric acid and caffeic acid was more toxic on marine microalgae than on freshwater microalgae since lower Nt_e ratios were observed (p value <0.001, p value <0.001, respectively, Fig. 6).

It is worth noticing that the freshwater group always had positive GR ratio, Nt_e ratio and biomass after 15 days of growth (Figs. 5, 6, Table 2): hence, it is here proposed that freshwater algae tolerance against phenolic compounds was averagely higher than the tolerance of marine species. The finding is fundamental when choosing the best alga or algal group in specific applications involving OMWWs.

Regarding the comparison of μ_{rm} between freshwater and marine species (Fig. 7), values of μ_{rm} were comparable when tyrosol was added to the media while they were statistically different for treatments with coumaric acid and caffeic acid (p value 0.0435 and 0.0326 respectively). Coumaric acid μ_{rm} was higher for freshwater species compared to the one in marine species, while caffeic acid μ_{rm} was the opposite (Fig. 7). Looking at the whole picture, data suggest that growth of algae and removal of phenols are not related phenomena: growth can occur without removal and vice versa (Appendix A). Especially when the algae died immediately after exposure to phenols while phenol removal was still ongoing (e.g. *N. salina* treated with coumaric acid, Appendix A), a biosorption effect could be the explanation.

Several studies [61,80–83] evaluated the biosorption of phenols, heavy metals and toxic elements onto cell walls of living or dead microalgae, proving the existence of a passive biosorption mechanism depending on several factors (e.g. pH, solute concentration, algal biomass, algal shape). More research on the biosorption of phenols within the first 24 h of exposure (when biosorption mechanism takes place) [84] would clarify the entity of such removal process.

4. Conclusion

Response of microalgae to phenol exposure was species and phenol specific; tyrosol was the only compound tolerated by all tested algae. However, freshwater species, in particular *T. obliquus* and *Anabaena* sp., better tolerated phenolic compounds compared to marine species, suggesting that natural environments might have been one of the selective factors affecting algal tolerance [43]. Despite a higher growth performance of freshwater species, the removal rate of phenolic compounds was comparable among the two groups.

The absence of a cell wall did not hamper resistance to toxic compounds, as confirmed by data on *D. salina* whose growth was recorded in each treatment despite the lack of a cell wall; on the other hand, the cell wall might have a role in the removal of phenols by biosorption since *D. salina* growth did not cause any removal.

More studies are needed to further understand the biological response of microalgal cells to phenolic compounds in order to implement an efficient OMWW phycoremediating system. According to what reported during the Water Conference 2023 of the United Nations (UN), remediation of wastewaters shall contribute to increase the already limited freshwater resources; thus, phycoremediation of OMWWs is a promising biotechnological tool to reduce their environmental impact and to supply safe freshwater to local communities.

CRediT authorship contribution statement

Lorenzo Mollo: Conceptualization, Formal analysis, Investigation, Writing- Original draft preparation, Visualization. **Filippo Drigo**:

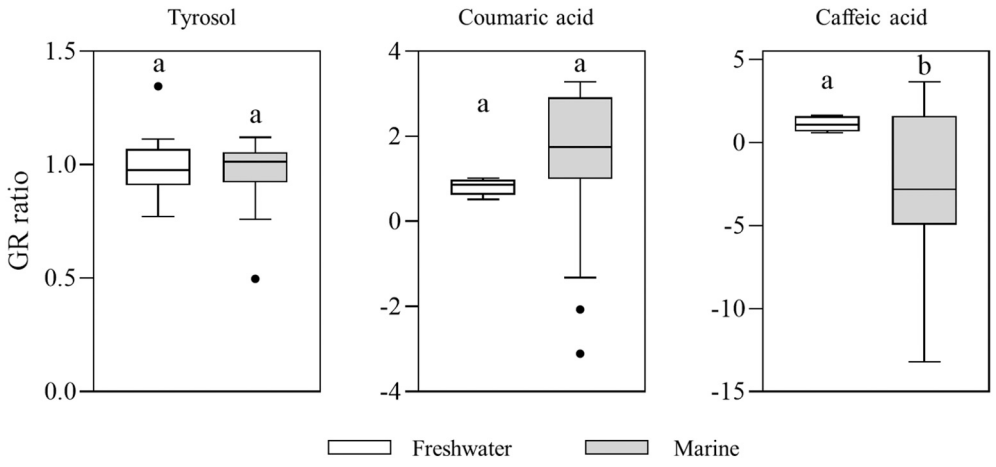

Fig. 5. GR ratio comparison among freshwater and marine species. Above each box-plot is a letter resulting from statistical analysis. Different letters are significantly different ($P < 0.05$).

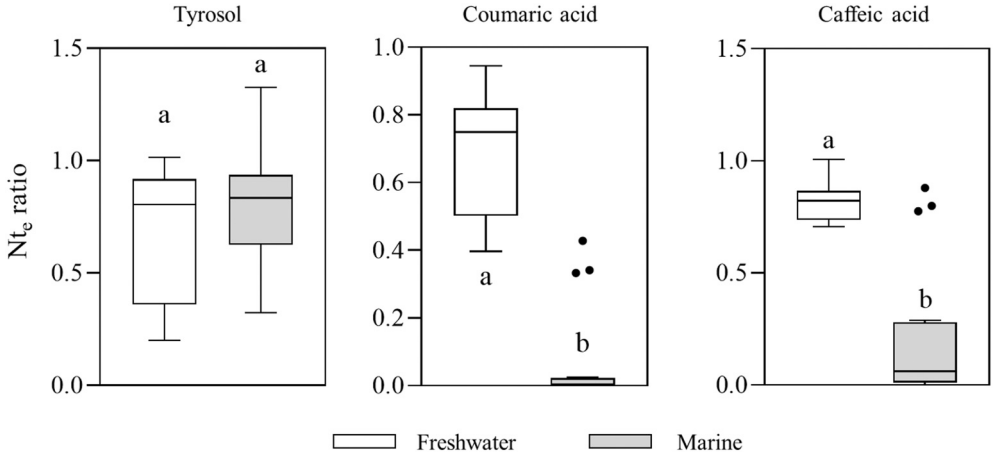

Fig. 6. Nt_e ratio comparison among freshwater and marine species. Above each box-plot is a letter resulting from statistical analysis. Different letters are significantly different ($P < 0.05$).

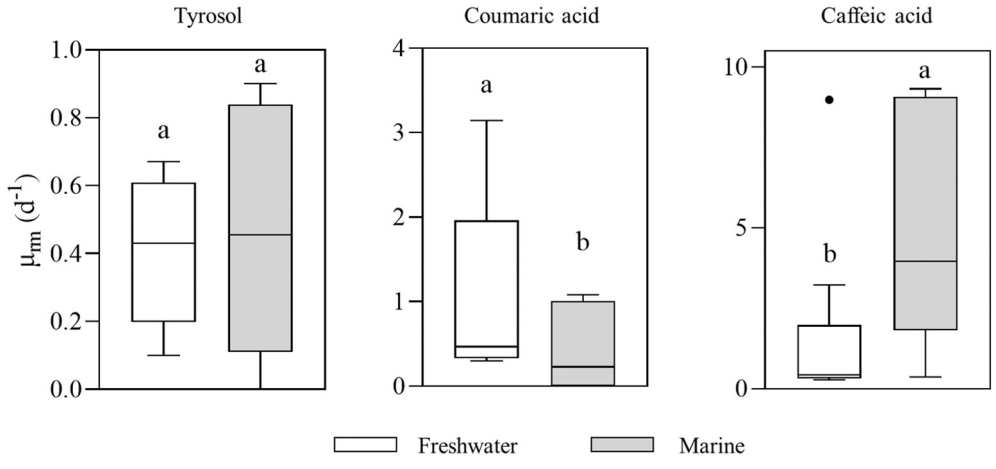

Fig. 7. Comparison of phenolic compounds removal rate between freshwater and marine species. Above each box-plot is a letter resulting from statistical analysis. Different letters are significantly different ($P < 0.05$).

Formal analysis, Investigation, Original draft preparation. **Matteo Moglie**: Conceptualization, Resources. **Alessandra Norici**: Conceptualization, Writing - Review & Editing, Supervision.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a

potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103256.

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