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Urban Wastewater Phytoremediation by Autochthonous Microalgae in Winter Season: Indoor and Outdoor Trials

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Abstract: Microalgae are gaining increasing attention for wastewater (WW) depuration thanks to their ability to remove pollutants from WW. As environmental conditions change widely geographically and throughout the year, also reaching growth-limiting temperatures during the cold season, sites and seasons specific tests are needed to assess the actual implementation of microalgae phytoremediation. In this paper, two site-specific prototype-scale experiments were performed to test the ability of an autochthonous microalga to depurate urban WW efficiently during winter. Two setups were tested: one indoor and one outdoor. We evaluated dry biomass production, photosynthetic stress, and nitrogen (N) and phosphorus (P) removals from WW. In addition, *Escherichia coli* concentration was monitored on the effluent. Despite the limited growth in both conditions, N was largely removed from the medium, with the highest result recorded outdoors (almost 70%). No P removal was recorded, although P content in biomass increased both indoors and outdoors, meaning that multiple processes could occur at the same time. Moreover, a huge decrease in *E. coli* content was recorded in both conditions, suggesting potential for effluent disinfection.

Keywords: microalgae; wastewater; phytoremediation; urban wastewater; *Escherichia coli*

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1. Introduction

Water scarcity and water pollution are two of the major problems that humanity is going to face shortly [1]. Water is consumed mostly by agriculture, industry, and housing, which in turn release wastewater (WW) into the environment [2,3]. These effluents are generally rich in organic matter, nitrogen (N), and phosphorus (P), and their release into the environment can bring to loss in river quality, sediment contamination, and even eutrophication. Even if depuration is performed, WW treatment plants (WWTPs) are still considered a point source of pollution [4–7]. The most commonly used depuration process is called the activated sludge process (ASP), in which microorganisms degrade organic matter in an aerated bioreactor. In ASP, N is usually removed from WWs thanks to biological activities and alternating aerobic and anaerobic conditions. During the aerobic phase, ammonia is converted into nitrite and nitrate, which are then converted into molecular N_2 during the anaerobic phase. Molecular N_2 then leaves the medium, resulting in net nitrogen removal. Considering how important bioreactor aeration is for ASP, we

should not be surprised that it can account for up to 70% of the total energy costs of the WWTP, also representing a major source of greenhouse gases [8]. As an alternative to the ASP, the anaerobic ammonium oxidation (Anammox) has been proposed [9]. In this process, ammonia and nitrite are converted into molecular nitrogen and nitrate simultaneously with denitrification under anaerobic conditions, thus reducing aeration costs. However, its applicability in full-scale WWTP is difficult because Anammox bacteria are easily susceptible to environmental conditions, such as low temperatures [9]. Moreover, sludge produced by both ASP and Anammox has a high water content (up to 98%), so other processes, e.g., thickening and de-watering, are needed [10,11]. P biological removal is also possible, but far more complex, and chemical methods are usually preferred [12]. Chemical methods usually require the addition of magnesium salts that react under an alkaline environment, which can lead to cost increases, higher maintenance, and difficulty in recovery [13]. On the other hand, P biological removal is only possible using specific polyphosphate accumulating organisms (PAOs) that accumulate P in aerobic conditions and release P in anaerobic conditions. By alternating these two phases, it should be possible to have a net P removal from the WW [14]. These processes usually produce nutrient-rich effluents that must be depurated [10,11,15,16]. Since some models suggest that municipal WW production will increase due to climate change, further research and improvement are needed [17].

As a green alternative or implementation to ASP, microalgae-based processes are gaining increasing attention for the N and P removal from WW [18] since microalgae can remove nutrients from the medium while producing nutrient-rich biomass [19–21]. Moreover, microalgae are photosynthesizing organisms that subtract CO_2 from the environment to perform photosynthesis, releasing O_2 that can be used by nitrifying bacteria to oxidize ammonia and organic matter [22]. Therefore, microalgae implementation in the ASP can both increment nutrient depletion and reduce CO_2 emission while reducing aeration costs [23–27]. Moreover, microalgae can accumulate P, producing biomass with a high content of this nutrient that can be used, for example, as an agricultural fertilizer [28,29]. Moreover, the algal biomass resulting from WW depuration can also be used for biofuel production [30–32]. Considering that P removal from the effluent happens without the addition of other chemicals and that the algal biomass can be reused for other biotechnological applications, microalgae implementation in the WWTPs may help reduce maintenance costs [12,33,34]. It has also been shown that microalgae can remove pathogenic microorganisms, such as *Escherichia coli*, from WW contributing to effluent disinfection [35,36].

Different microalgal strains can have different responses to the environmental and culturing conditions, so it is crucial to choose the correct microalga or microalgal consortium, with some studies reporting better and more promising results using native strains from the WW [37–40]. Moreover, to properly implement microalgae inside ASP in a cost-effective way, the cultivation system and procedures must be chosen carefully to balance phytoremediation efficiency and energy consumption [18,30,41–43]. Open-pond systems, for example, have the advantage of low maintenance costs but they can occupy more land surface compared to other systems [18,30,44]. The same reactor can also be run in batch or continuous mode, increasing the possible combinations [42,45,46]. Lastly, to achieve better phytoremediation using microalgae, abiotic factors, e.g., light and temperature, should be monitored [37,47–49]. These factors can affect both microalgae growth and nutrient depletion and synergistic effects have been recorded, meaning that fine-tuning of environmental parameters is needed [50–53]. Unfortunately, these parameters are difficult to control when performing outdoor tests, meaning that seasonal variations in photoperiod and light quality, along with daily and seasonal temperature changes, are major challenges for large-scale applications. During winter, low temperatures can slow down microalgae metabolism,

reducing biomass production and nutrient recovery [37,49,54,55]. For example, Larsdotter and colleagues, who monitored a greenhouse phytoremediation plant for a whole year, reported a net P increase in the effluent during winter and linked it to decaying biomass and low algal production [56]. Unfortunately, limited literature has been produced regarding prototype or large-scale applications of microalgae in ASP during the winter season, and environmental conditions drastically change due to geography, so site-specific tests should be performed [57–59]. Table 1 offers examples reported in the literature of different phytoremediation trials conducted in the winter season, highlighting the differences in microalgae species, cultivation systems and procedures, and geographical positions.

Table 1. Prototype or large-scale experiments regarding applications of microalgae in ASP during the winter season. Algal species, cultivation systems, and procedures, along with time period and location are listed.

Algal Species	Cultivation System	Time Period and Location	Ref.
Scenedesmus obliquus	High-rate algal pond and an Airlift tubular photobioreactor both operated in continuous.	From October 2011 to March 2012 in South Spain.	[60]
Chlorella sp., *Scenedesmus* sp. and a pennate diatom	Open raceway pond operated in continuous.	From June 2013 to September 2013 in Perth, Australia.	[61]
Co-culture of *Chlorella* sp., *Stigeoclonium* sp., *Nitzschia* sp. and *Navicula* sp.	Tubular horizontal semi-closed photobioreactor connected to two open tanks. Operated in semi-continuous.	From May 2017 to May 2018 in Barcelona, Spain.	[62]
Scenedesmus sp. And *Chlorella* sp.	Raceway pond operated in semi-continuous cultivation.	From December 2019 to December 2020 in East Australia.	[63]
Scenedesmus sp.	Raceway ponds operated in semi-continuous.	Monitored for a whole year. Almería, Spain.	[64]
Consortium dominated by *Monoraphidium* sp.	Four tanks connected in parallel and operated in continuous.	Monitored for a whole year. Greenhouse in Sweden.	[56]
Chlorella pyrenoidosa	Rectangular photobioreactor operated in batch.	Winter in Shandong Province, China.	[65]
Chlorella sp. and *Scenedesmus* sp.	Raceway pond operated in batch.	Winter in Qatar.	[66]

In this paper, we performed two prototype-scale phytoremediation tests, one outdoor and one indoor, as a follow-up study of that in [67]. Both tests were performed in open pond-like systems operated in batches during the winter season using the effluent of the sludge thickening process to navigate the possibility of the integration of a microalgae-based process in an ASP-based WWTP even during adverse environmental conditions.

2. Materials and Methods

2.1. Microalgae Cultivation

The microalgal strain employed in this study was isolated from secondarily treated municipal WW and described as *Chlorella*-like as reported in Baldisserotto et al. [67]. The microalgae were provided by Alga&Zyme Factory s.r.l. (Ferrara, Italy) and were maintained in bag cylindrical photobioreactors (22 °C, 90 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR, 16–8-h light–dark photoperiod) in BG11 medium with N and P content modified to match the ammonium and the total P concentrations of the WW from which the algae were isolated: 380 mg/L for NH_4^+-N and 110 mg/L for PO_4^{3-}-P. The final recipe was NH_4Cl 1.45 g/L, K_2HPO_4 0.62 g/L, $MgSO_4 \times 7H_2O$ 0.075 g/L, $CaCl_2 \times 2H_2O$ 0.036 g/L, Na_2CO_3 0.02 g/L, EDTA-Na_2 0.001 g/L, citric acid 0.006 g/L, ammonium ferric citrate 0.006 g/L, and BG-11 Trace Metals

Solution (H_3BO_3 2.86 g/L, $MnCl_2 \times 4H_2O$ 1.81 g/L, $ZnSO_4 \times 7H_2O$ 0.222 g/L, $NaMoO_4 \times 2H_2O$ 0.39 g/L, $CuSO_4 \times 5H_2O$ 0.079 g/L, $Co(NO_3)_2 \times 6H_2O$ 0.0494 g/L) 1 mL/L. The algae used for the inoculation were at a stationary growth phase with an OD_{750} = 1.604. Absorbance at 750 nm was measured with a Pharmacia Ultrospec 2000 UV-Vis spectrophotometer (1-nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA).

The effluent of the thickening step of sludge treatment was collected in November 2021 from Hera SpA (Holding Energia Risorse Ambiente) WWTP located in Ferrara, Italy (44°51′49″ N, 11°37′47″ E). The fresh WW was composed of a solid residue precipitating to the bottom of the tank and an opaque supernatant (OD_{750} = 0.14). The WW was not mixed; only the supernatant was used as a culture medium for this study. The composition of the WW supernatant, determined using standard certified methods for water quality analyses, is shown in Table 2.

Table 2. Characterization of the WW and analytical methods employed for the analyses. * indicates values below detection limit.

Parameter	Unit	Method	Value
Total nitrogen	mg N L^{-1}	UNI EN 12260: 2004 [68]	25.2
Ammonium	NH_4^+-N L^{-1}	APAT CNR IRSA 4030 A1 Man 29 2003 [69]	24.4
Nitrate	NO_3^--N L^{-1}	APAT CNR IRSA 4020 Man 29 2003 [70]	1.4
Nitrite	NO_2^--N L^{-1}	APAT CNR IRSA 4050 Man 29 2003 [71]	0.16
Total phosphorus	mg L^{-1} P	UNI EN ISO 15587-2: 2002 ISO 17294-2: 2016 [72,73]	22.1
COD	O_2 mg L^{-1}	ISO 15705 par 10.2: 2002 [74]	80
BOD_5	O_2 mg L^{-1}	APHA Standard Methods for the Examination of Water and Wastewater 23rd 2017 5210 [75]	19
Total suspended solids	mg L^{-1}	APAT CNR IRSA 2090 B Man 29 2003 132 114 [76]	48
Al	mg L^{-1}	ISO 15587-2: 2002 + UNI EN ISO 17294-2: 2016 [72,73]	1.33
Cr	mg L^{-1}	ISO 15587-2: 2002 + UNI EN ISO 17294-2: 2016	<0.02 *
Cr (VI)	mg L^{-1}	APAT CNR IRSA 3150 C Man 29 2003 [77]	<0.02 *
Cu	mg L^{-1}	ISO 1187-2_2002 + UNI EN ISO 17294-2: 2016 [73,78]	0.021
Hg	mg L^{-1}	ISO 1187-2_2002 + UNI EN ISO 17294-2: 2016	<0.001 *
Pb	mg L^{-1}	ISO 1187-2_2002 + UNI EN ISO 17294-2: 2016	<0.005 *
Ni	mg L^{-1}	ISO 1187-2_2002 + UNI EN ISO 17294-2: 2016	<0.01 *
Zn	mg L^{-1}	ISO 15587-2: 2002 + UNI EN ISO 17294-2: 2016	0.06
Escherichia coli	CFU/100 mL	UNI EN ISO 9308-1:2017 [79]	61,100

To test the ability of this microalga to remove nutrients and grow during winter, two different setups were designed (see below). Both experiments took place in December 2021 and lasted 20 days. Samples were collected on days 0, 1, 2, 3, 6, 7, 10, 14, 17, and 20 of both experiments.

2.2. Indoor Setup

A glass tank (55 × 55 × 10 cm) placed underneath a south-facing window was used for the indoor experiment. The tank received only natural light during the day, as no other illumination was used (sunrise at 7:30 UTC + 1, sunset at 16:30 UTC + 1, average PAR during daytime: 20 $\mu mol_{photons}$ m^{-2} s^{-1}). The average temperature in the room was 19 ± 2 °C. A 4 L amount of *Chlorella*-like culture, cultivated as above, and 16 L of WW were mixed

to obtain a final volume of 20 L and a target OD_{750} of 0.3. In the following days, the culture was mixed manually before collecting the samples.

2.3. Outdoor Setup

A stainless-steel tank located at the Hera WWTP (Ferrara, Italy) was used for the outdoor experiment. The tank was positioned 1 m above ground level and was equipped with an adjustable cover to prevent contamination from the environment. Natural light was supplemented with white LED lamps (final mean PAR during daytime: 30 $\mu mol_{photons}$ m^{-2} s^{-1}, 16:8 h L:D). Data concerning average hourly air temperature 2 m above ground (°C), along with average, minimum and maximum daily temperature 2 m above ground (°C) for the control unit located in Malborghetto (Ferrara, Italy, 44°85′89.2″ N 11°65′62.5″ E) were downloaded from "dext3r", a web app for the extraction of weather data from ARPAE-Emilia Romagna database (Agenzia Regionale per la Prevenzione, l'Ambiente e l'Energia of Emilia Romagna region, Italy). The control unit was chosen considering both geographical proximity to the WWTP and the completeness of the data recorded (https://simc.arpae.it/dext3r/, accessed on 5 June 2023) [80]. Trends during the 20-day experiment of the average, maximum, minimum, and midday daily temperature are depicted in Figure 1. An 85 L amount of *Chlorella*-like algae suspension, cultured as above, and 450 L of WW were mixed to obtain a final volume of 535 L and an OD_{750} of 0.3. In the following days, the culture was mixed manually before collecting the samples.

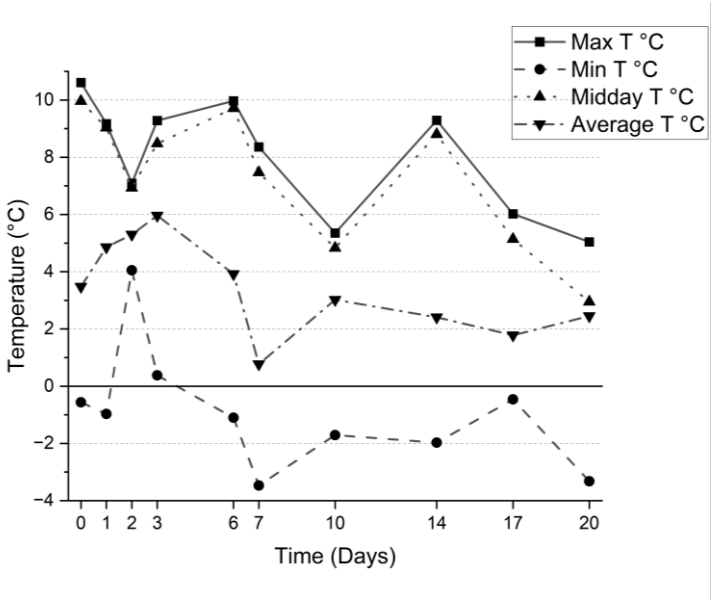


Figure 1. Maximum (Max T °C, squares), minimum (Min T °C, circles), midday (midday °C, triangles), and average (average T °C, inverted triangles) temperatures recorded in December 2021 by a control unit located in Malborghetto (Ferrara, Italy). Data were downloaded from ARPAE dext3r database.

2.4. Growth Evaluations

Dry biomass was measured at each experimental time by filtering known aliquots of samples through pre-dried and pre-weighted glass fiber filters (Whatman GF/C; 1.2 µm pore size). Filters were then rinsed with 20 mL of distilled water, dried for 72 h at 60 °C, and weighed to evaluate the biomass yield of the cultures [32].

2.5. PSII Maximum Quantum Yield and Photosynthetic Pigments Analysis

The maximum PSII quantum yield was calculated as F_V/F_M ratio using a pulse amplitude-modulated fluorometer (Junior PAM, company, Heinz Waltz GmbH, Effeltrich,

Germany). Samples were prepared following [81]. After 15 min of dark incubation, the basal fluorescence (F_0) was determined; a saturating light impulse (0.5 s) was used to measure the maximum fluorescence (F_M). Variable fluorescence (F_V) was calculated as $F_V = F_M - F_0$ [82].

For the extraction of photosynthetic pigments, cell samples were harvested by centrifugation (18,000× *g*, 10 min) and absolute methanol was used according to Baldisserotto et al. 2016 [81]. To avoid photo-degradation, extracts were manipulated under dim light. Absorbances were read at 666 nm, 653 nm, 470 nm, and 750 nm with the spectrophotometer described above, and chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) concentrations were quantified as reported by Wellburn [83].

2.6. Morphological Aspects: Light and Electron Microscopy

The microalgae were observed with an Axiophot (Zeiss, Oberkochen, Germany) photomicroscope under conventional and UV light. For fluorescence examination, an HBO 100 W pressure mercury vapor lamp (filter set, BP 436/10 FT 460, LP470) was used. Alcian Blue staining was used to highlight the presence of acidic mucopolysaccharides, following [84] with some modification. A 20 µL amount of Alcian Blue (1% in 3% acetic acid) was added to an algal pellet obtained after the centrifugation of 0.5 mL of culture. The sample preparation continued with a 30 min incubation at room temperature, followed by two rinses with distilled water. Nile Red staining (NR; 9-diethylamina-5Hbenzo[α]phenoxazine-5-one, 0.5 mg dissolved in 100 mL acetone) (Sigma-Aldrich, Gallarate, Milan, Italy) was used to highlight the presence of intracellular lipid droplet, following [85].

On the last day of both experiments, cells were harvested through centrifugation, fixated with glutaraldehyde (3% *v*/*v* in phosphate buffer 0.1 M, pH 7.2; 3 h, 4 °C), and post-fixated with OsO_4 (2% *v*/*v* in the same buffer; 1 h, RT) [67]. For TEM observations, cells were then dehydrated in acetone series and embedded in Durcupan ACM (Fluka, Sigma-Aldrich). Ultrathin sections were stained with lead citrate and uranyl acetate. Cells were then observed with a Zeiss EM910 (Electron Microscopy Center, University of Ferrara, Ferrara, Italy). For SEM observations, cells were dehydrated in graded alcohol series and dried through evaporation. A Q150RS (Quorum, Lewes, UK) sputter was used to coat the samples with gold. Cells were then observed with a Zeiss Evo 40 (Electron Microscopy Center, University of Ferrara, Ferrara, Italy) in high vacuum conditions.

2.7. Phytoremediation Analysis

Three aliquots of culture medium were collected from both cultures through centrifugation (4500× *g*, 10 min) for the quantification of nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), and phosphate ($P\text{-}PO_4^{3-}$) using a flow-injection autoanalyzer (Flowsys, Systea SpA, Company, Roma, Italy). The detection limit for the nitrate and phosphate analyses was 0.02 mg L^{-1}, while for the ammonium was 0.1 mg L^{-1}. The same method was used to evaluate the percentage of N and P in the microalgal biomass. For this purpose, an appropriate amount of biomass was collected on days 0, 6, 14, and 20 of both experiments through centrifugation (4500× *g*, 10 min) and dried through evaporation. Then, at least 20 mg of dried biomass was extracted from each sample in 3 mL of selenous H_2SO_4 at 420 °C and analyzed as previously described. Given the large volumes required, only one replica was performed for each test.

The removal efficiency in percentage (RE, %) was calculated according to the following equation:

$$RE\ (\%) = [(C_0 - C_1)/C_0] \times 100 \quad (1)$$

where C_0 and C_1 are the nutrient concentrations after the algal inoculum (0) and one day after the inoculum, respectively [67].

To estimate the quantity of nitrogen that was removed from the medium and accumulated inside the microalgae biomass, firstly, the nitrogen content in biomass per liter of culture (N_B, g_N L^{-1}) was calculated as follows:

$$N_B = \%_N \times DW \quad (2)$$

where $\%_N$ is the percentage of N inside the biomass (g_N in 100 g_{DW}); DW is the dry weight (g_{DW} L^{-1}). Then, total N concentration in the medium at the beginning of the experiments (N_{T0}) and the percentage of N removed from the medium and accumulated inside the biomass (N_A) were calculated as follows:

$$N_T = N\text{-}NH_4^+ + N\text{-}NO_3^- \quad (3)$$

$$N_A\ (\%) = [(N_{B20} - N_{B0})/(N_{T0})] \times 100 \quad (4)$$

where N_{B20} and N_{B0} represent the nitrogen content in biomass at the end of the experiment and on the day of inoculum, respectively.

Finally, the nitrogen that was removed from the medium and was not accumulated inside the biomass, called volatilized nitrogen (N_V), was calculated as follows:

$$N_V\ (\%) = [(N_{T0} - N_{T20})/(N_{T0})] \times 100 - N_A \quad (5)$$

where N_{T20} and N_{T0} are the sums of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations at end and at the beginning of the experiments, respectively.

For pH measurement, a Jenway mod. 3510 (Staorshire, Stone, UK) bench pH meter was employed.

After the inoculation, on the third and last days of the outdoor experiment and the last day of the indoor experiment, the culture media were analyzed to investigate the concentrations of *E. coli*, BOD_5, COD, and TSS. The high volumes required for these analyses allowed them to be carried out for indoor cultivation only at the end of the experiment. Since the algal cells naturally deposited, as described in paragraph 3.1, leaving a clear supernatant, culture medium was collected before manual resuspension. Samples for the outdoor culture were collected after the inoculation of algae, on the third day and at the end of cultivation. For analyses of the samples gathered after the inoculation and on the third day, culture media were collected through centrifugation (2500× *g*, 10 min) to separate algal biomass from the medium. On the last day of the experiment, as algal biomass naturally deposited leaving a clear supernatant as described in paragraph 3.2, samples of culture media were collected before manual resuspension. Analyses were conducted as reported previously.

2.8. Statistical Analyses

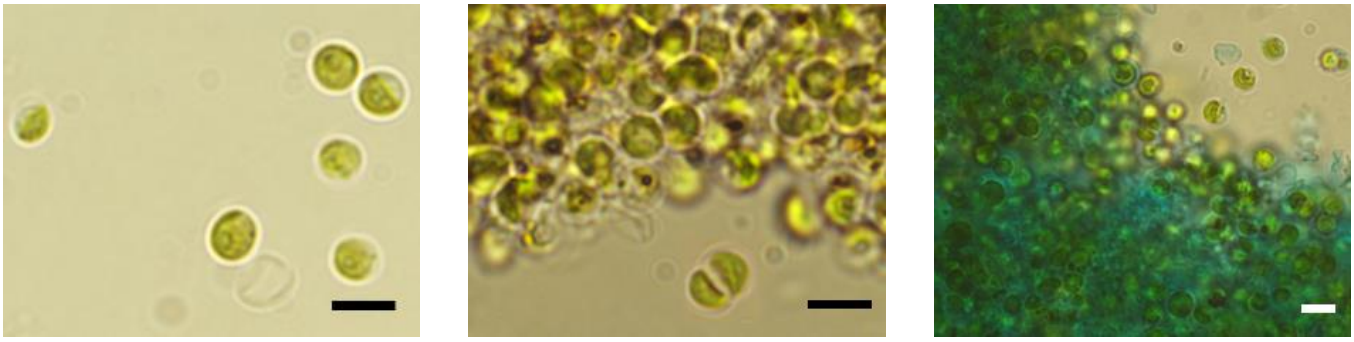
Data were processed with Origin 9.9 software (OriginLab, Northampton, MA, USA). A parametric one-way analysis of variance (ANOVA) was used to verify the presence of significant changes ($p \leq 0.05$) over time and compared to the initial values, followed by a Tukey test to find where the difference occurred. Data are expressed as means ± standard deviations.

3. Results

3.1. Indoor Setup

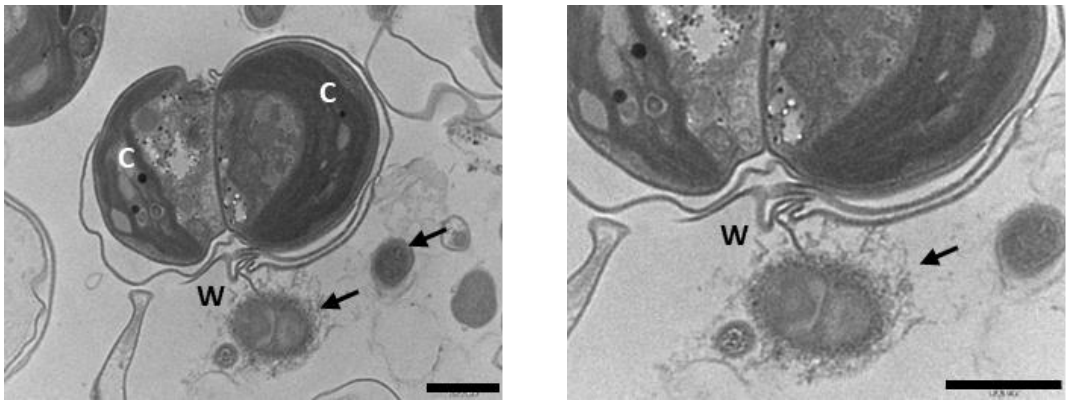
After the inoculation, the microalgae were suspended in the culture medium (Figure 2a). However, by day 14 the microalgae tended to form large aggregates (Figure 2b) that settle to the bottom of the pond, leading to the formation of a clear supernatant ($OD_{750} = 0.03$) within few minutes after manual mixing. High production of acidic mucopolysaccharides

was recorded in the aggregates, as shown in Figure 2c. As shown by TEM and SEM images, other microorganisms were also included in the aggregates (Figures 3 and 4).



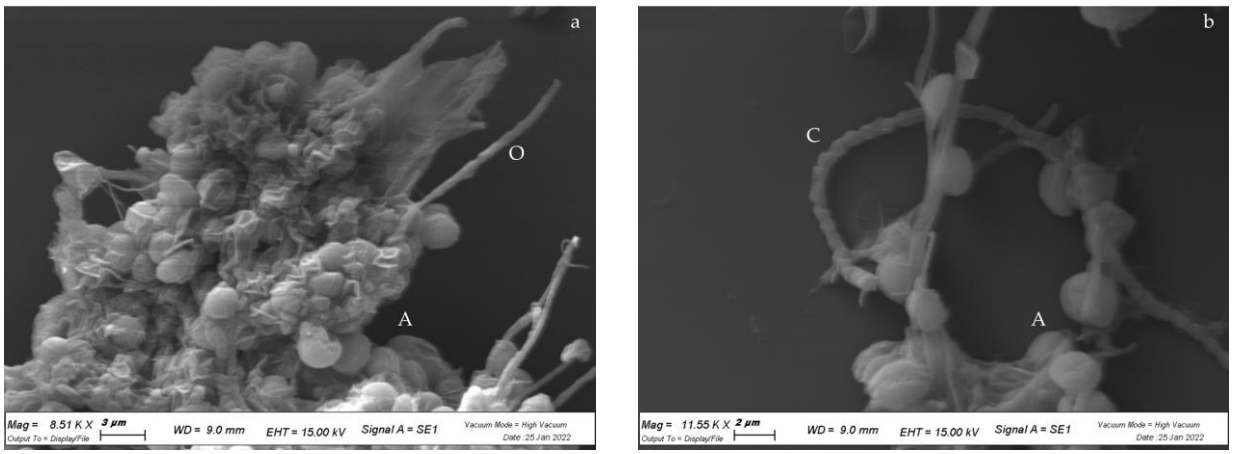
(**a**) (**b**) (**c**)

Figure 2. Light microscopy view of microalgae grown in the indoor setup at 0 (**a**) and 14 days (**b**) of cultivation. In (**c**), the presence of acidic mucopolysaccharides on the 14th day is highlighted thanks to Alcian Blue staining. Bars, 5 μm.



(**a**) (**b**)

Figure 3. Transmission electron images of the microalgae grown indoor on the 20th day. Other organisms (arrows) are visible in the medium. In (**a**) two cells surrounded by multiple layers of an electron-dense outer wall (W). (**b**) Detail of the microalgae outer wall and one of the microorganisms present in the medium. C, chloroplast. Bars 1 μm.



(**a**) (**b**)

Figure 4. SEM images of the microalgae grown indoors on the 20th day. In (**a**) a large microalgae aggregate (A) is visible, along with some filamentous-like organisms (O). In (**b**) other organisms (C) are visible near an aggregate (A).

Biomass concentration at the end of cultivation (0.129 g_{DW} L^{-1}) was significantly higher than the initial value (0.108 g_{DW} L^{-1}; Figure 5). Another significant variation registered over time was the decrease between days 10 and 14, with the dry biomass concentration recorded on day 14 being not significantly different from the initial one. Experimental error due to the elevated aggregation of the biomass recorded on day 14 can be taken into account for the low value recorded on that day. For a report of the ANOVA analysis see Table S1 in the Supplementary Materials.

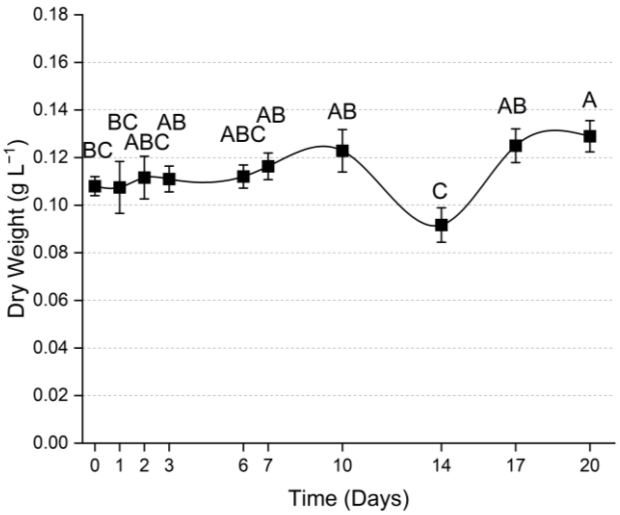
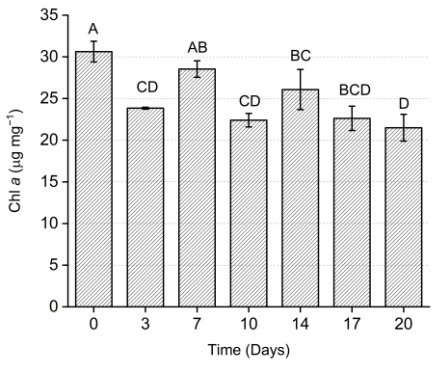
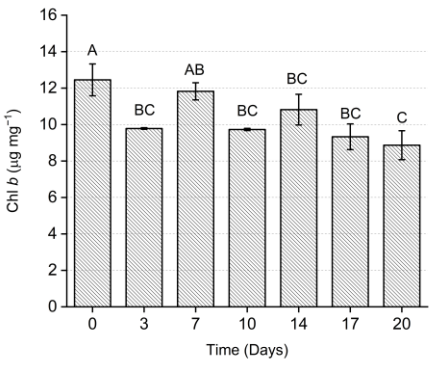


Figure 5. Biomass yield expressed as grams of algal dry weight per liter of the culture grown indoors. Data refer to means ± standard deviations (n = 3). Different letters highlight significant differences between groups ($p \leq 0.05$) found using a one-way ANOVA.

The concentration of all photosynthetic pigments decreased since the third day of the experiment, with no other significant variations over time, except for the increase recorded on day 7 for both Chl *a* and Chl *b*, with the concentration of both pigments not significantly different from that recorded at the beginning of the experiment (Figure 6a–c). The Chl/Car ratio fluctuated over time with values significantly higher than the initial one on days 7, 17, and 20. No significant changes were recorded in the Chl *a*/Chl *b* ratio (Figure 6d,e). Overall, the F_V/F_M ratio remained stable for the whole duration of the experiment, with a significant increase on the last day (Figure 6f). For a report of the ANOVA analyses, see Table S1 in the Supplementary Materials.



(**a**)



(**b**)

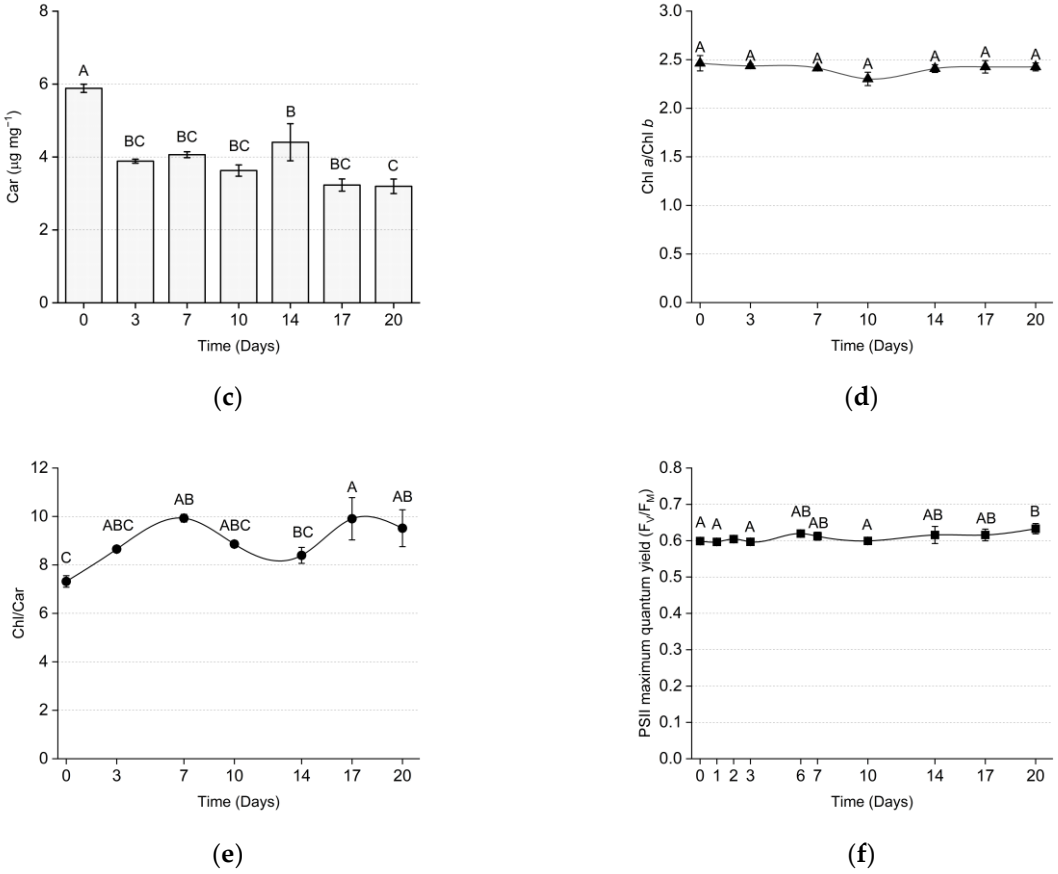


Figure 6. Photosynthetic pigment content (µg pigment mg^{-1} dry biomass) and PSII maximum quantum yield (Fv/Fm ratio) of microalgae cultivated indoors for 20 days. (**a**) Chlorophyll *a*, (**b**) chlorophyll *b*, (**c**) carotenoids, (**d**) chlorophyll *a* on chlorophyll *b* ratio, (**e**) total chlorophyll on carotenoid ratio, (**f**) PSII maximum quantum yield. Data refer to means ± standard deviations (n = 3). Different letters highlight significant differences between groups ($p \leq 0.05$) found using a one-way ANOVA.

Regarding the concentrations of N and P in the medium, different trends were recorded: nitrate concentration significantly decreased since day 3, while ammonium concentration significantly decreased only after day 14 (Figure 7a,b). In contrast, phosphate concentration fluctuated. In fact, a significant decrease was recorded only on day 3. Then, the P concentration increased, reaching significant peaks on days 10 and 14. The concentration recorded at the end of the experiment was higher than the initial one (Figure 7c). For a report of the ANOVA analyses, see Table S1 in the Supplementary Materials. P concentration in biomass also changed over time, reaching a value of around 2% on day 10 and decreasing after that day. The percentage of N in biomass also fluctuated around values of 7% (Figure 7d). Considering the differences between the initial and the final values of nitrogen in the medium and taking into account the amount absorbed by the algae (about 1.3% of the N initial content), approximately 50% of the nitrogen was removed from the medium by day 20 (Figure 7e). During the 20 days of experiments, the pH of the medium decreased gradually, as shown in Figure 7f.

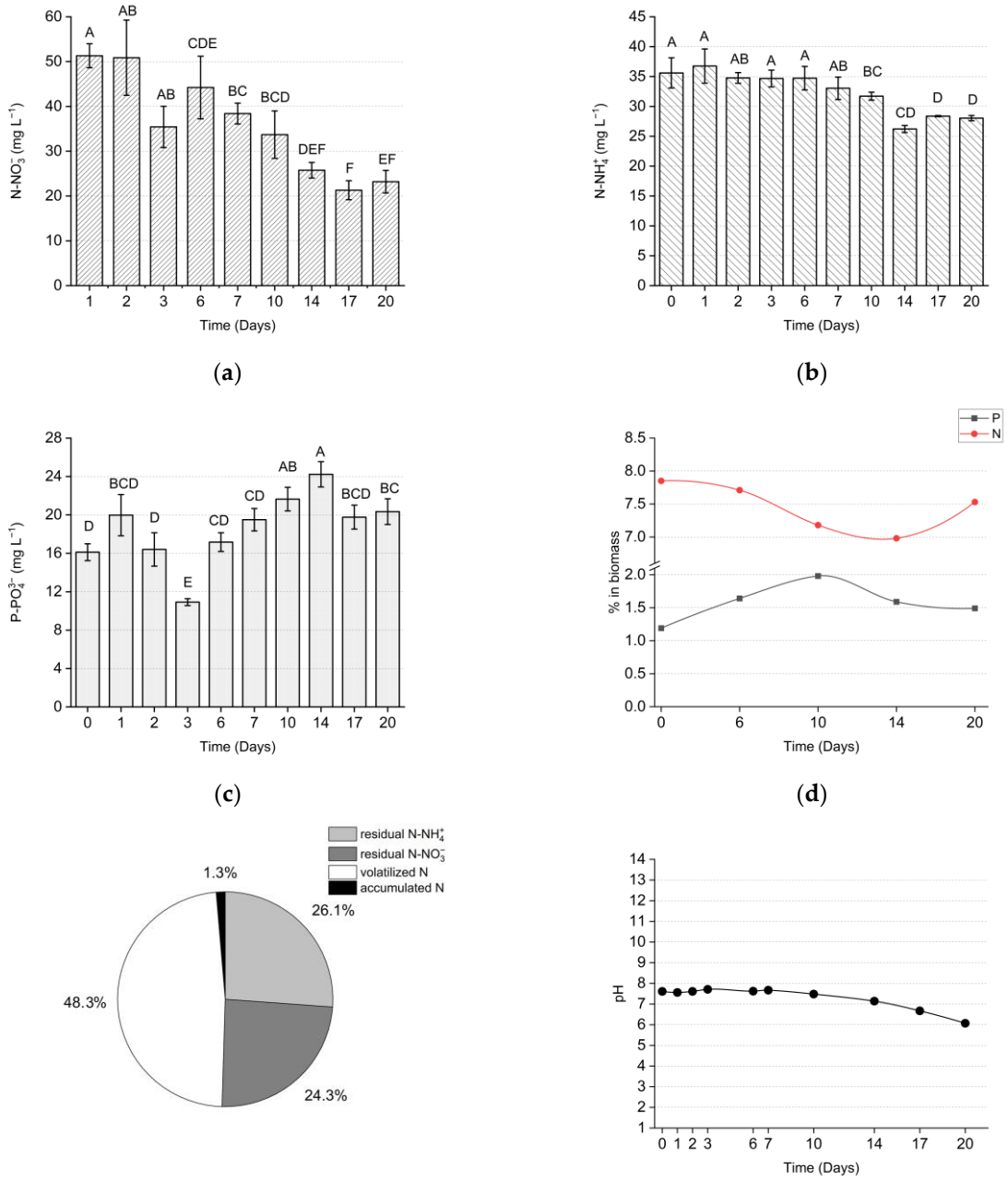


Figure 7. (**a**–**c**) Nutrient concentrations (N-NO_3^-, N-NH_4^+ and P-PO_4^{3-}; mg L^{-1}) evolution during the 20 days of the indoor experiment. Data refers to means ± standard deviations (n = 3) Different letters highlight significant differences between groups ($p \leq 0.05$) found using a one-way ANOVA. (**d**) Trends in N and P concentrations in biomass (n = 1). (**e**) Estimate in N mass balance at 20 days of experiment. (**f**) pH trends in culture media (n = 1).

As reported in Table 3, along with a reduction in COD, BOD_5, and total suspended solids, almost no residual *E. coli* was found at the end of the experiment.

Table 3. Estimated media composition after the inoculum in the indoor experiment, calculated considering dilution, and media composition at the end of the indoor experiment. At the end of cultivation, the media was collected before the manual resuspension, and it was not centrifuged.

Parameter	Unit	Day 0	Day 20
COD	O_2 mg L^{-1}	64	38

BOD_5	O_2 mg L^{-1}	15	<10
Total suspended solids	mg L^{-1}	38	8
Escherichia coli	CFU/100 mL	48,880	<2

3.2. Outdoor Setup

The microalgae in the outdoor setup did aggregate and settle like the indoor setup, but the culture stayed homogeneous for a few hours after manual mixing, with only a few larger aggregates that settled rapidly. Before manual resuspension, the supernatant appeared clear (OD_{750} = 0.03), while the culture remained opaque for a few hours after mixing ($OD_{750} \approx$ 0.30 every experimental day). As shown in Figure 8, chlorophyll autofluorescence changed, resulting in dimmer cells. Alcian Blue staining showed the presence of acidic mucopolysaccharide in the aggregates (Figure 8f). TEM observation showed the presence of bacteria and cell wall remnants in the culture medium. Both TEM observation (Figure 9b) and NR staining (Figure 8e) showed the presence of lipid droplets in some cells.

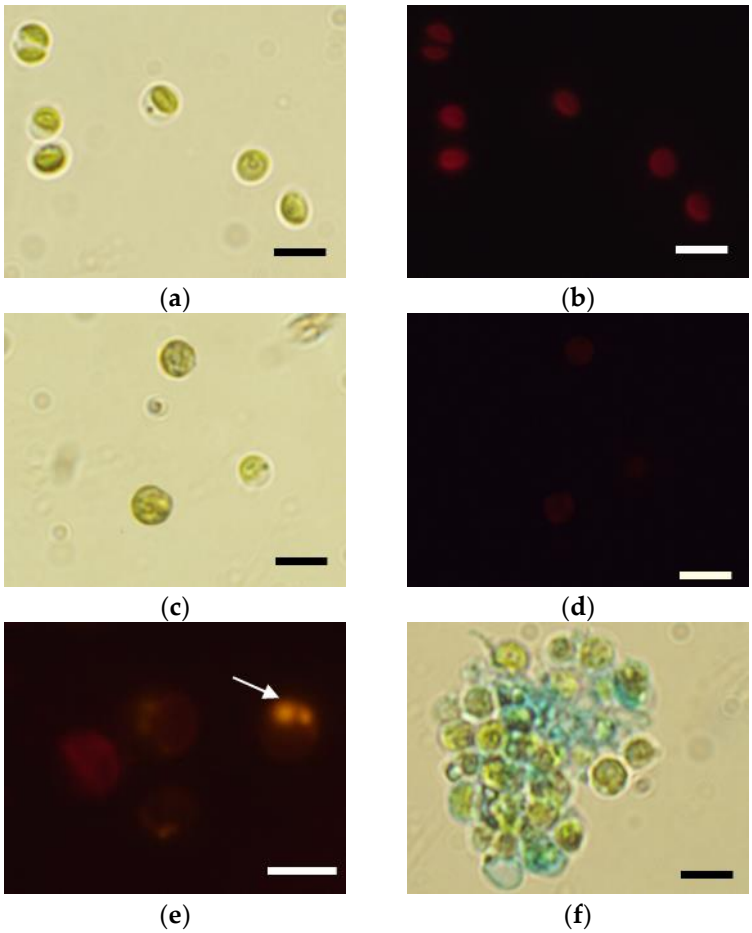
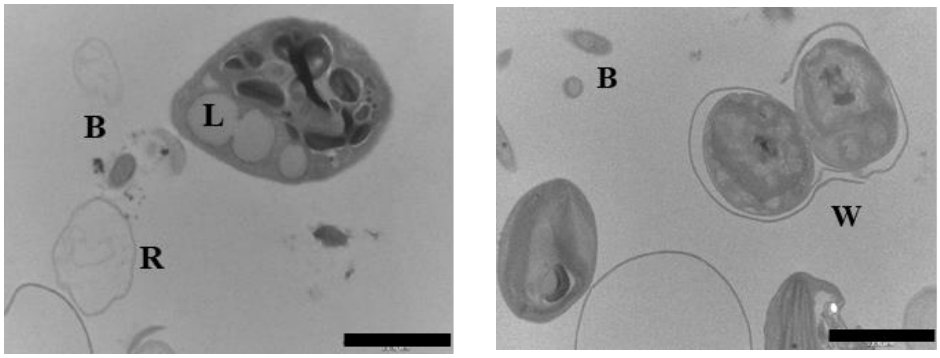


Figure 8. Light and fluorescence microscopy view of microalgae grown in the outdoor setup at 0 (**a**,**b**) and 14 (**c**,**d**) days of cultivation. Autofluorescence after 14 days decreased, resulting in dimmer cells. In (**e**) the presence of lipid droplets (arrow) inside the cells is highlighted thanks to Nile Red staining. In (**f**), the presence of acidic mucopolysaccharides on the 14th day is highlighted thanks to Alcian Blue staining. Bars, 5 μm.



(**a**) (**b**)

Figure 9. Transmission electron microscopy images of the microalgae grown outdoors on the 20th day. In (**a**) a cell with lipid droplets (L) and cell wall remnants (R) in the media are visible. In (**b**) two cells with a thick outer wall (W) are visible. In both images, other organisms (B) are visible in the medium. Bars, 2 μm.

The concentration of dry biomass at the end of the experiment (0.099 g_{DW} L^{-1}) was not significantly different from the initial one (0.090 g_{DW} L^{-1}), but some fluctuations were recorded. In particular, the concentrations recorded on day 3 (0.100 g_{DW} L^{-1}) and day 17 (0.102 g_{DW} L^{-1}) were significantly higher than the initial value (Figure 10). For a report of the ANOVA analysis, see Table S2 in the Supplementary Materials.

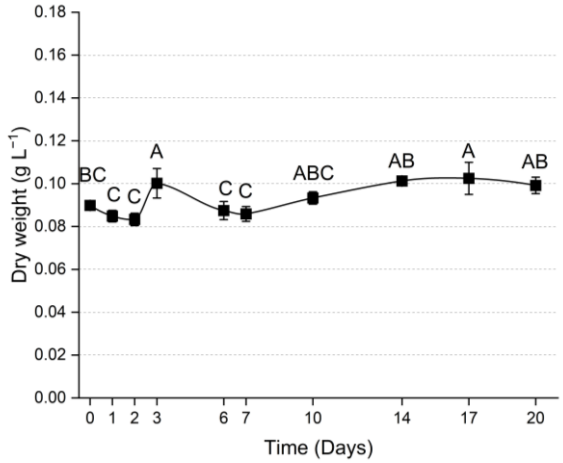


Figure 10. Microalgal biomass yield expressed as grams of algal dry weight per liter of the culture grown outdoors. Data refers to means ± standard deviations (n = 3). Different letters highlight significant differences between groups ($p \leq 0.05$) found using a one-way ANOVA.

The concentration of photosynthetic pigments dropped since day 3. Two other significant decreases were recorded in Chl *a* and Car concentrations on days 14 and 17 compared with the concentrations recorded on the previous days (Figure 11a,c). The variations in pigment concentrations affected both the Chl *a*/Chl *b* and Chl/Car ratio, with a significant increase in the Chl *a*/Chl *b* ratio since day 14 and a significant drop in the Chl/Car ratio since day 17 (Figure 11d,e). The PSII maximum quantum yield decreased since the first day, reaching its lowest value on days 10–14. Interestingly, after these days the F_V/F_M value increased with significant changes every day (Figure 11f). For a report of the ANOVA analyses, see Table S2 in the Supplementary Materials.

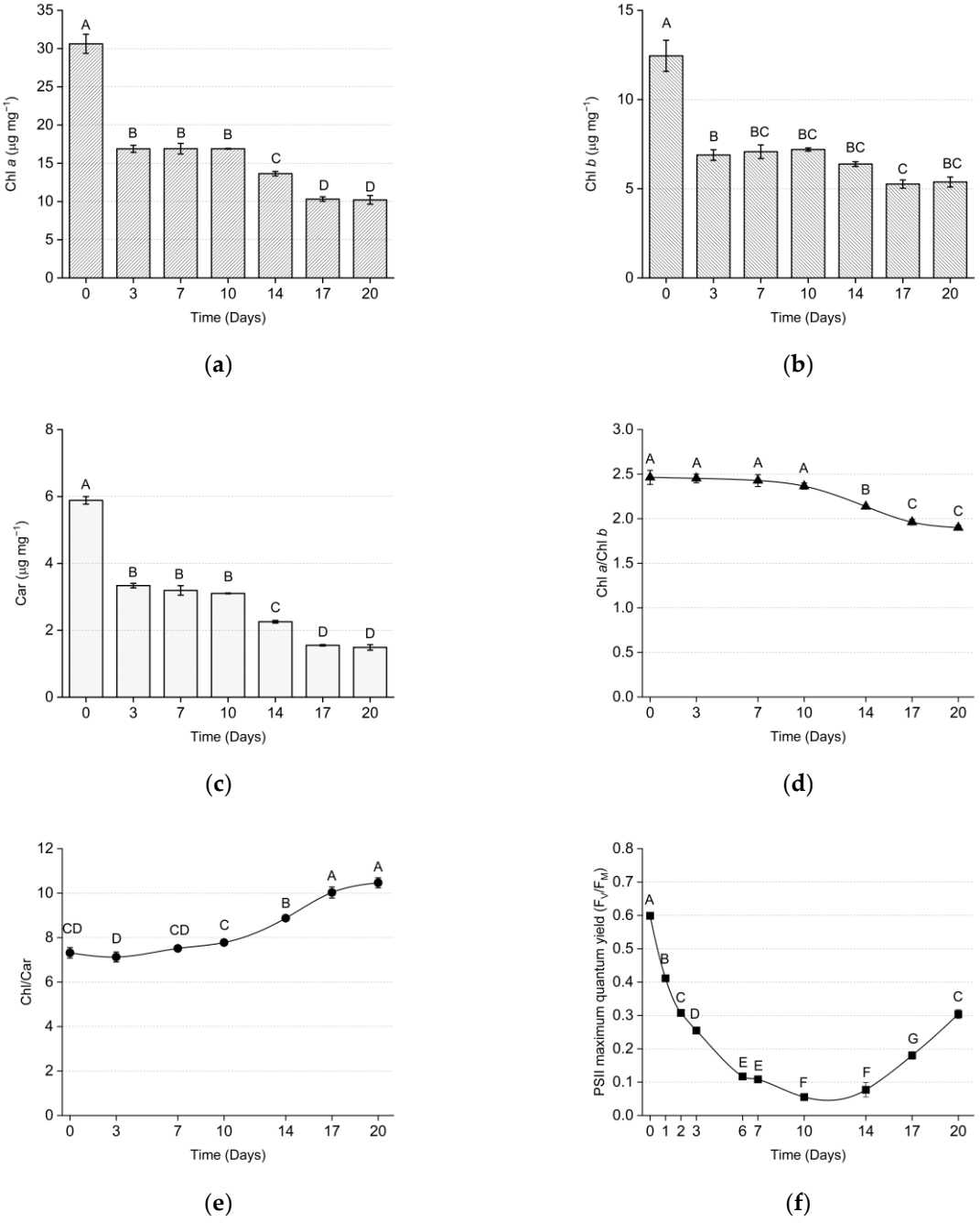


Figure 11. Photosynthetic pigment content (µg pigment mg^{-1} dry biomass) and PSII maximum quantum yield (F_V/F_M ratio) of microalgae cultivated outdoors for 20 days. (**a**) Chlorophyll *a*, (**b**) chlorophyll *b*, (**c**) carotenoids, (**d**) chlorophyll *a* on chlorophyll *b* ratio, (**e**) total chlorophyll on carotenoid ratio, (**f**) PSII maximum quantum yield. Data refers to means ± standard deviations (n = 3). Different letters highlight significant differences between groups ($p \leq 0.05$). Found using a one-way ANOVA.

The pH of the medium fluctuated over the 20 days, first with a decrease on the day after inoculation, a rapid increase on day 3, a second decrease between days 7 and 10, and a slight increase on the last days (Figure 12f). Ammonium concentration decreased significantly from day 1 and remained stable until the end of the experiment, apart from an increase registered on day 6, which was not significant compared to any other day (Figure 12b). Nitrate concentration decreased significantly since day 2, apart from a nonsignificant increase on day 3, and underwent three more significant drops on days 6, 10, and 14

(Figure 12a). For a report of the ANOVA analyses see Table S2 in the Supplementary Materials. P concentration in the medium fluctuated with the only significant difference recorded compared to the initial value being the increase registered on day 7 (Figure 12c). The percentage of P in the microalgal biomass increased over time, reaching the highest value (1.6%) at the end of the experiment. On the other hand, the concentration of N in the biomass dropped in the first days and then increased, reaching a value similar to the initial one at the end of the experiment (Figure 12d). Considering the differences between the initial and the final values of nitrogen in the medium and taking into account the amount absorbed by the algae (about 1.7% of the N initial content), approximately 68% of the nitrogen was removed from the medium by day 20 (Figure 12e).

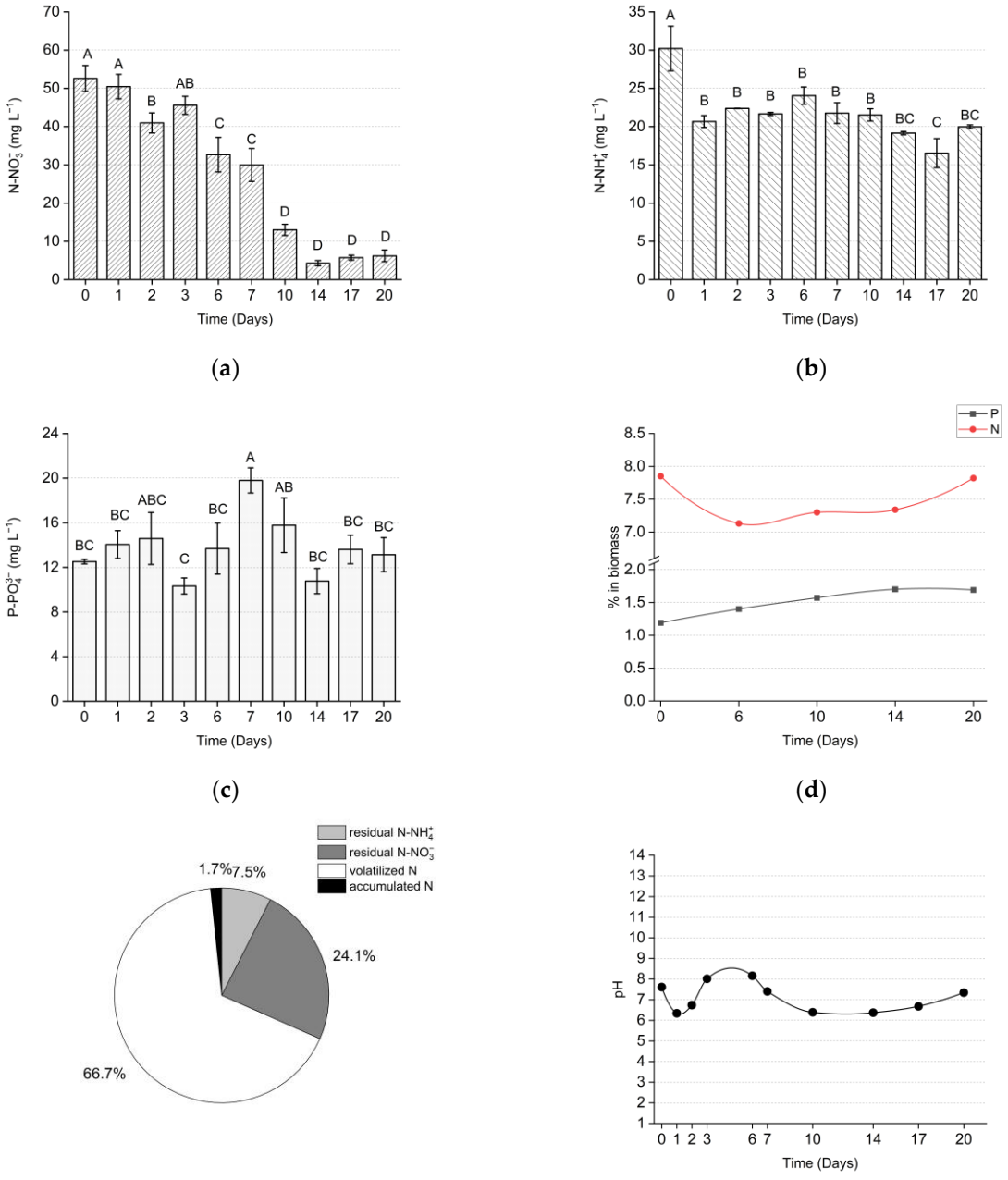


Figure 12. Variations in the concentrations (mg L^{-1}) of $N\text{-}NO_3^-$ (**a**), $N\text{-}NH_4^+$ (**b**), and $P\text{-}PO_4^{3-}$ (**c**) during the 20 days of the outdoor experiment. Data refers to means ± standard deviations (n = 3). Different letters highlight significant differences between groups ($p \leq 0.05$) found using a one-way ANOVA.

(**d**) Trends in N and P concentrations in biomass (n = 1). (**e**) Estimate in N mass balance at 20 days of experiment. (**f**) pH trends in culture media (n = 1).

With respect to the *E. coli* load in the medium, 3 days after the inoculum it dropped under the law's recommended limit of 5000 CFU/100 mL, with an even lower concentration recorded the last day (72 CFU/100 mL) (Table 4). COD values remained stable for the duration of the experiment, while BOD_5 values became lower than the detection limit by day 3 (Table 4). The total suspended solids value decreased after 3 days of cultivation and remained stable for the rest of the experiment (Table 4).

Table 4. Media composition at the end of the outdoor experiment. For analyses after the inoculum and on the 3rd day, the media was collected through centrifugation. On the last day, the media was collected before the manual resuspension, and it was not centrifuged.

Parameter	Unit	Day 0	Day 3	Day 20
COD	O_2 mg L^{-1}	61	62	59
BOD_5	O_2 mg L^{-1}	13	<10	<10
Total suspended solids	mg L^{-1}	32	26	27
Escherichia coli	CFU/100 mL	36,000	2600	72

4. Discussion

4.1. Effects of the Environmental Parameters on Microalgae Growth and Metabolism

Winter conditions can be a challenge for WW phytoremediation. Several studies report that both exposure to simulated winter conditions [37] and outdoor cultivation during the cold season [56,86,87] result in lower growth rates and limited nutrient removal in microalgae [50,51]. Interestingly, in the outdoor setup, no significant biomass production was recorded at the end of the experiments, while in the indoor setup, the concentration of dry biomass at the end of cultivation was slightly but significantly higher than the initial value. These results suggest that other factors, but not temperature, influence microalgae growth. Indeed, in addition to the difference in average temperature in indoor and outdoor conditions, the indoor temperature remains stable over time while it is externally variable in outdoor conditions. Probably the reduced photoperiod (and low light availability in general) could be the main cause of the negative effect on microalgal biomass production in both experimentations [40,55,88–90]. Similar results have been reported by other researchers, highlighting that photoperiod is a crucial parameter to consider for microalgae cultivation during cold seasons [37,40]. However, it cannot be excluded that some differences in the experimental responses are due to the different types of the two photobioreactors (e.g., the cultivation volumes) [46].

Cold stress can reduce photosynthetic activity either by reducing the irradiance threshold for photoinhibition or by slowing down the repair of the photosynthetic apparatus [52,55,91]. In the outdoor experiment, severe photoinhibition occurred, as indicated by the decrease in PSII maximum quantum yield [92]. Interestingly, after 14 days the F_V/F_M value increased, although it remained below the optimal value of 0.6 [84], suggesting acclimation to the new growth conditions. Adaptation to a low-light environment is also suggested by the decrease in the Chl *a*/*b* ratio and the increase in the Chl/Car ratio at the end of the outdoor experiment [93,94]. It is also important to note that in the indoor experiment, the F_V/F_M remained stable for the entire experiment, probably due to the absence of such a severe temperature as in outdoor cultivation [52,55,95]. Adaptation to the new light environment may also be suggested in the indoor experiment, considering the variations in the Chl/Car ratio [93,94,96]. Considering the trends of pigment concentrations in both cultures, it is possible to assume that the initial decrease was due to adaptation to

new culture conditions and reduced shading effect, while the concentrations at the end of the experiments were affected considerably by the photoperiod [97]. Different algal species accumulate more pigments with different combinations of irradiance and photoperiod, with some preferring high irradiance and reduced hours of light, while others prefer a lower irradiance and more hours of light [97–99]. However, it is also important to consider the negative effect that cold stress may have had on the pigment concentration of the microalgae cultivated outdoors [90]. Moreover, cold stress is also related to lipid accumulation in microalgae [100] and may explain the presence of lipid droplets in some cells cultivated outdoors, as shown by both NR staining and TEM images. Notably, a lipid-rich biomass can be used for other biotechnological applications, such as biofuel production [31,81,101].

It is important to note that the outdoor experiment was performed in a tank situated 1 m above ground level, whilst usually the ASP tanks are built into the ground. This construction difference can lead to significant temperature variations since soil temperature can be up to 10 °C away from air temperature [102]. For a real implementation in a WWTP, a ground-level tank can be constructed to reduce the impact of air temperature fluctuations [103,104].

4.2. Aggregate Production and Effluent Disinfection

The formation of large aggregates through the production of extracellular polymeric substances (EPS) may be another response to reduced photoperiod and low light [37,105,106]. Considering that unsterilized WW was used as a culture medium for both experiments, the role of bacteria in flocculation can be taken into account [107]. Vu and colleagues [108] demonstrated the formation of more compact aggregates when the self-flocculating microalga *Ettlia* sp. was grown in co-culture with bacteria thanks to the production of bacterial filamentous EPS. An even greater difference in flocculating activity has been reported by Lee and colleagues [109] when a strain of *Chlorella vulgaris* was grown in axenic rather than xenic culture, with a flocculation activity of 94% and 2%, respectively. The high flocculating activity and high sedimentation recorded in our experiment are worth noticing since they make it possible to separate and recover both algal biomass and treated wastewater easily and cost-effectively [110]. In large-scale applications, though, culture mixing cannot be performed manually so automated systems, e.g., aeration or paddle wheel, should be considered for culture mixing when biomass separation from the medium is not needed, increasing maintenance costs [45,46,111].

It is also been reported that pathogenic bacteria like *E. coli* can be trapped in the EPS matrix and thus removed from the treated WW, contributing to effluent disinfection [36]. This could explain the decrease in *E. coli* concentration reported in both indoor and outdoor setups, but other interactions, e.g., competition and metabolite production, cannot be excluded [36,112]. Moreover, cold stress can also affect bacteria growth and both EPS production and composition, explaining the smaller aggregates in the outdoor culture, compared to the indoor one [113,114]. The decrease in the *E. coli* concentration, along with the entrapment of other organisms in EPS, as evidenced by SEM images, are promising results that pave the way for the study of the disinfecting capabilities of this microalga.

4.3. Nutrient Removal

P concentrations in dry biomass suggest that this nutrient was retained or only partially accumulated by the microalgae in both experiments, but it does not explain the increase in P concentration in the medium recorded, in particular in the indoor trial, where the highest concentration on P in the biomass was registered the day with one of the highest P concentrations in the medium. Since P adsorption in the biomass is necessary for the removal of this nutrient, biomass production and P accumulation are needed, with studies

highlighting the importance of irradiance and photoperiod in the P uptake [29,115–117]. An increase in the P concentration of the medium during phytoremediation tests in winter time has already been reported by other studies such as [56]. In their experiments, those authors reported that, in the winter season, an increase in P concentration in deeper and unlit tanks resulted and that this trend was linked to low biomass production and net phosphorous release from decaying biomass. However, no significant decrease in biomass concentration was recorded, suggesting that multiple phenomena, e.g., P release by bacteria [14], could be happening at the same time. In fact, under anaerobic conditions, PAOs break down polyphosphate and release orthophosphate, increasing its concentration in the medium [14]. During microalgae cultivation, low dissolved oxygen conditions could happen, and this could help explain the increase in P concentration in the medium even if no net release from the microalgae was recorded, so the contribution of the native bacteria cannot be excluded [49,118].

In both experiments, N was largely removed from the medium, with better results achieved in the outdoor setup compared to the indoor setup, approximately 70% and 50%, respectively. This could be explained by the higher amount of light hours in the outdoor experiments (16 h) compared to the indoor experiment (9 h) [50,88]. As demonstrated by Ferro and colleagues [37], different microalgae strains are affected differently by temperature and photoperiod. In their study, the authors reported that the growth of the employed strain of *Scenedesmus obliquus* was more affected by the temperature than the photoperiod but still achieved 29% N removal even though no growth was recorded. On the contrary, the growth of the strain of *Chlorella vulgaris* was more affected by reduced photoperiod, resulting in the absence of biomass production and limited N removal when cultured at 25 °C and 3:21 h L:D photoperiod [37]. As a result of microalgae and bacteria metabolism, nitrate concentration may rise, as previously reported in a study that used the same microalgae [67] or by other researchers [49]. Since both ammonia and nitrate are subject to limits imposed by Italian law [119], it is quite noticeable that, in the conditions proposed in this research, nitrate was largely depleted.

Since the N percentage in biomass did not change in both indoor and outdoor experiments and limited growth was recorded only in the indoor experiment, only a fraction of N was assimilated by the microalgae, suggesting that most were removed from the medium through different mechanisms. Ammonia stripping is one of the phenomena usually implied in N reduction in phytoremediation. Some researchers report up to 80% of total $N\text{-}NH_4^+$ removal thanks to this phenomenon [47]. Unfortunately, the environmental conditions required for ammonia stripping, e.g., pH values higher than 10 and high temperature, have never been met in either outdoor or indoor setups [120–122]. Considering that unsterilized WW was employed, the effect of microorganisms naturally present in the medium can be considered. Indeed, microbial activities such as nitrification and denitrification could also be responsible for N depletion [12]. Oxygen produced through photosynthesis can be used by nitrifying bacteria to oxidize ammonia and produce nitrite and nitrate, and it has been reported that under low O_2 conditions, e.g., during the dark phase, denitrifying bacteria are not inhibited and can convert nitrate into molecular nitrogen [123]. Low dissolved oxygen is needed for denitrification to happen, but it has been reported that it could still occur at reduced rates even at medium dissolved oxygen concentrations [124]. As reported by Xu and colleagues [49], the oxygen produced by microalgae in an unaerated photobioreactor could be just enough to fulfill microalgal respiration and microbial nitrification, leading to an overall low dissolved oxygen concentration. Considering this, in these experiments, an alternative Anammox process, called algal anaerobic ammonium oxidation (Algammox), could be proposed. In this process, microalgae provide the oxygen for ammonia oxidation to nitrate, thus reducing aeration costs, and denitrification still happens [125]. At the end of both Anammox and Algammox reactions, N

leaves the medium in molecular form [9]. Similar reactions could have taken place in both indoor and outdoor setups, explaining the overall reduction in N concentration in the absence of the conditions usually required for ammonia stripping [120–122,125].

5. Conclusions

The results obtained in this preliminary research, in particular N removal (about 50% in the indoor setup and about 70% in the outdoor setup) and the decrease in *E. coli* content in the medium (>99% in both setups), suggest that this alga can efficiently depurate and disinfect WWs in the winter season when suboptimal environmental conditions happen. Even if the results regarding P removal were unsatisfying, it is important to note that the increase in P concentration in the medium of both experiments was not directly ascribable to microalgae metabolism. Therefore, more research is needed to understand the interactions between the microalgae and the native microbial consortium. Moreover, fine-tuning of environmental parameters is needed, with particular regard to photoperiod, which proved to be crucial for biomass production. Nonetheless, even if no biomass production was recorded in the outdoor setup, the presence of lipids inside the cells paves the way for biomass reuse after effluent depuration, such as biofuel production.

Considering the results obtained, in particular, in the outdoor culture, semi-continuous cultivation may be considered for future tests, with the discharge of effluent after proper depuration has happened and the addition of fresh WW. Furthermore, more tests are needed to evaluate the potential use of this microalga for WW remediation and disinfection throughout the year and for a further scale-up of the system.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/app15084235/s1, Table S1: Results of the parametric one-way analyses of variance (ANOVA) analyses for the indoor experiments.; Table S2: Results of the parametric one-way analyses of variance (ANOVA) analyses for the outdoor experiments.

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Abbreviations

The following abbreviations are used in this manuscript:

WW	Wastewater
N	Nitrogen
P	Phosphate
WWTP	Wastewater treatment plants
ASP	Active sludge process
PAOs	Polyphosphate accumulating organisms

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