

# Exploiting symbiotic interactions between *Chlorella protothecoides* and *Brevundimonas diminuta* for an efficient single-step urban wastewater treatment

Martina Pastore and Eleonora Sforza

## ABSTRACT

The application of microalgal bacteria consortia to the treatment of wastewater is receiving increasing attention, meeting the demand for new green and efficient technologies for water remediation. The specificity of the consortium, however, may strongly affect the performance of the treatment. In fact, even though a general exploitation of the O<sub>2</sub>/CO<sub>2</sub> exchange between microalgae and bacteria is effective, some specific interactions may increase the pollutant removal. With this aim, the co-cultivation of *Chlorella protothecoides* and *Brevundimonas diminuta* was tested, with particular attention to the removal capability of nitrogen, phosphorus and chemical oxygen demand (COD) from wastewater. Batch experiments were carried out both for the consortium and, separately, for the bacteria and microalgae alone, in order to compare their performances. *B. diminuta* showed a remarkable capability for removing organic substances and transforming organic nitrogen to ammonium. *C. protothecoides* efficiently removed nitrogen and phosphorus. As the specific growth rates of the two organisms are different, the co-cultivation was also carried out also in a continuous system, and the effect of hydraulic retention time (HRT) on the steady-state biomass concentration and nutrient removal efficiency was verified. Residence time was found as the main operating variable for obtaining a significant reduction of pollutants from wastewater.

**Key words** | consortium, continuous reactor, microalgae, mixotrophy, steady state

### Martina Pastore

**Eleonora Sforza** (corresponding author)  
Interdepartmental Centre Giorgio Levi Cases,  
Via Marzolo 9, 35131 Padova,  
Italy  
E-mail: [eleonora.sforza@unipd.it](mailto:eleonora.sforza@unipd.it)

### Eleonora Sforza

Department of Industrial Engineering DII,  
University of Padova,  
Via Marzolo 9, 35131 Padova,  
Italy

## INTRODUCTION

In the last few years it has become imperative to study more efficient and environmental friendly methods to recycle fresh water and recover nutrients from wastewaters. Even though conventional biological wastewater treatment plants (WWTPs), based on activated sludge technology, have been set up and constantly improved, some issues still remain unresolved. In this type of plant, the nitrogen and phosphorous recovery efficiency is usually low and requires additional steps that often involve the addition of chemicals, with a consequent increase of costs and environmental impact. The most energy-consuming step is the use of blowers for the oxygenation of the aerobic tank, which constitutes almost 50% of the total operative cost of conventional WWTPs. In addition, from a circular economy perspective, more efficient and green technologies for resource recovery are being investigated. Many researchers have recently been focusing on the use of algal–bacteria consortia: it is known that gas exchange between

microalgae and bacteria may act as a growth-promoting agent for both cultures. Microalgae are also able to reduce nitrogen and phosphorous concentrations in wastewaters, thus avoiding any other chemical steps (Beltràn-Rocha *et al.* 2017). In the natural environment, most microalgae are associated with either aerobic or anaerobic microorganisms and, in an industrial setting, using a system that is self-sustaining in terms of oxygen has potential not only for wastewater bioremediation but also for the production of commercially valuable by-products (Subash-chandrabose *et al.* 2011; Beltràn-Rocha *et al.* 2017).

Interactions between microorganisms may be either positive or negative (Fukami *et al.* 1997) and this may strongly affect treatment performance: for example, *Azospirillum brasilense* produces indole-3-acetic acid (IAA), among other compounds, which is a plant hormone able to act as a growth-enhancing compound for *Chlorella vulgaris* (Gonzalez-Bashan *et al.* 2000). On the other hand, other bacterial

strains, such as *Pseudomonas putida*, are used as biological control agents against algal blooms (Doucette *et al.* 1999; Kang *et al.* 2005). Bacteria can also affect microalgal growth by limiting the access to light of these photosynthetic microorganisms (Tricolici *et al.* 2014). Even microalgae may have an influence on bacterial growth, in both negative and positive ways. As an example, several microalgal species secrete compounds with specific antibacterial activity (Natrah *et al.* 2014), or that are able to interfere with bacterial quorum sensing (Natrah *et al.* 2011). In summary, the specificity of the consortium should be carefully considered in biological processes and, possibly, controlled.

Some examples of natural and synthetic consortia with plant-growth-promoting bacteria have been already reported in the literature, as in the case of *A. brasilense* (de-Bashan *et al.* 2002, 2004; Palacios *et al.* 2016b) and *Brevundimonas diminuta* (Park *et al.* 2008). Biochemical mechanisms exploited by these bacteria to promote microalgal growth are still unknown, despite many authors reporting that *B. diminuta* may protect plants from the detrimental effects of heavy metal pollution (Ji *et al.* 2016), arsenic stress (Singh *et al.* 2016) and the ability to degrade pesticides based on organophosphates (Parthasarathy *et al.* 2017). *B. diminuta* is also able to solubilize phosphates, produce siderophore and plant growth hormones such as IAA (Singh *et al.* 2016). In particular, it is known that the *Chlorella* genus and *B. diminuta* may have symbiotic interactions in the aquatic environment (Park *et al.* 2008) and that *Chlorella* sp. is able to secrete thiamine, which is an important cofactor of the enzyme responsible for IAA biosynthesis and in many other metabolic ways (Palacios *et al.* 2016b). *Chlorella* sp. is also shown to secrete tryptophan, an amino acid used as a precursor of IAA by bacteria (Palacios *et al.* 2016a, 2016b).

Aside from the mechanisms of interaction between the two species, which are still under investigation, the application of this consortium for wastewater treatment has not yet been tested. Accordingly, in this work the co-cultivation of *C. protothecoides* and *B. diminuta* was assessed, with particular attention to the simultaneous removal capability of nitrogen, phosphorus and chemical oxygen demand (COD) from wastewater. Both batch and continuous experiments were carried out: batch experiments were performed separately for bacteria and microalgae, in order to compare the performance of the consortium. As the specific growth rates of the two organisms are different, the co-cultivation was also carried out in a continuous system, and the effect of residence time (hydraulic retention time; HRT) on the steady-state biomass concentration and nutrient removal efficiency was investigated.

## METHODS

### Microalgal and bacterial species, growth medium and wastewater

*Chlorella protothecoides* 33.80 (obtained from SAG Goettingen, Germany) was maintained in liquid BG11 medium for the inoculum (Na<sub>2</sub>MgEDTA 1 mg L<sup>-1</sup>, ferric ammonium citrate 6 mg L<sup>-1</sup>, citric acid-H<sub>2</sub>O 6 mg L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 36 mg L<sup>-1</sup>, MgSO<sub>4</sub> 75 mg L<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 30.5 mg L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 2.86 mg L<sup>-1</sup>, MnCl<sub>2</sub>·4H<sub>2</sub>O 1.81 mg L<sup>-1</sup>, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.222 mg L<sup>-1</sup>, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.70 mg L<sup>-1</sup>, COCl<sub>2</sub>·6H<sub>2</sub>O 0.050 mg L<sup>-1</sup>, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.391 mg L<sup>-1</sup>, Na<sub>2</sub>CO<sub>3</sub> 20 mg L<sup>-1</sup>, NaNO<sub>3</sub> 0.943 mg L<sup>-1</sup>, HEPES 1M pH = 8).

*Brevundimonas diminuta* LMG 02089 (obtained from Belgian Coordinated Collections of Microorganisms, BCCM) was maintained in liquid flasks with Luria broth, and inoculated in fresh medium at 30 °C one day before each experiment, as a preinoculum.

To avoid the influence of the environmental changes of real wastewater on the performances of the consortium, a synthetic formula was used to obtain a medium with constant quality. A standard synthetic wastewater (<http://www.oecd.org/chemicalsafety/testing/>) was slightly modified in order to have an average characterization similar to local urban wastewater (peptone 80 mg L<sup>-1</sup>, meat extract 110 mg L<sup>-1</sup>, NH<sub>4</sub>Cl 40 mg L<sup>-1</sup>, CH<sub>3</sub>COONa 159 mg L<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 23 mg L<sup>-1</sup>, NaCl 7 mg L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 4 mg L<sup>-1</sup> and Mg<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O 2 mg L<sup>-1</sup>). The final characterization of the synthetic sewage was the following: ammonium (NH<sub>4</sub><sup>+</sup>-N) 13 mg L<sup>-1</sup>, total nitrogen (N<sub>tot</sub>) 29.5 mg L<sup>-1</sup>, orthophosphate (PO<sub>4</sub><sup>3-</sup>-P) 3.75 mg L<sup>-1</sup>, COD 310 mg L<sup>-1</sup>.

### Experimental set-up and procedures

Both batch and continuous experiments with *C. protothecoides* and *B. diminuta* grown in synthetic wastewater were carried out in order to investigate their growth and their nutrient removal capacity under continuous light.

Batch growth curves were carried out in Drechsel bottles of 250 mL, with 5 cm diameter, continuously mixed by a magnetic stirrer. Each run started with an initial biomass inoculation of OD<sub>750</sub> = 0.6. Before starting each experiment, the volume of pre-inoculum was centrifuged for 5 min at 1,500 rpm in order to remove the preinoculum medium.

Four conditions were studied in order to better understand the effect of non-limiting gas supply and the possible

exploitation of gas produced by the two populations in the case of consortium:

- C. protothecoides* alone with air–CO<sub>2</sub> bubbling;
- C. protothecoides* alone without bubbling;
- C. protothecoides* and *B. diminuta* cultivated together without bubbling;
- B. diminuta* alone without bubbling.

Aeration (air enriched with 5% v/v of CO<sub>2</sub>) was provided to the microalgae as a control, at a total flow rate of 1 L h<sup>-1</sup>. The temperature was controlled at 24 °C in an incubator (Frigomeccanica Andreaus, Padova, Italy) and artificial light (white neon lamps OSRAM) was supplied continuously at an intensity of 30 μmol photons m<sup>-2</sup> s<sup>-1</sup> of photosynthetic active radiation (PAR), measured by a photoradiometer (Model LI-189, LI-COR, USA).

Continuous experiments were carried out in a 300 mL flattened glass tank, with 6.5 cm thickness, under 150 μmol m<sup>-2</sup> s<sup>-1</sup> of irradiation. The reactor was initially inoculated with the consortium and it was first run in batch mode, allowing cells to enter an exponential phase of growth, so as to reach a sufficient biomass concentration and avoid the occurrence of washout when the operation mode was shifted to continuous mode. After activating the peristaltic pump (Sci-Q 400, Watson Marlow, USA), continuous operating conditions were achieved. Fresh medium was continuously supplied from an external sterilized stirred bottle by means of the peristaltic pump, at a constant volumetric flow-rate  $Q$  (mL d<sup>-1</sup>). The culture volume was kept constant by the presence of an overflow tube, placed at the opposite side with respect to the fresh medium inlet, from which biomass was continuously collected at the same volumetric flow-rate. The HRT of the reactor is calculated as:

$$\tau = \frac{V_R}{Q} \quad (1)$$

where  $V_R$  is the reactor volume (mL). The flow-rate was regulated in order to obtain the desired residence time. As no biomass separation and recirculation was done, the solid retention time (SRT) was equal to the HRT.

After a transition period of acclimatization, steady state was reached, with a constant biomass concentration, which depends on residence time and nutrients in the inlet (de Farias Silva *et al.* 2017). Subsequently, flow-rate was changed, resulting in a different residence time. This resulted in a new transition period followed by a further steady state.

## Analytical methods

The microalgal and bacterial concentration was indirectly measured as absorbance, by spectrophotometric analysis of the optical density (OD; measured at 750 nm, by double beam spectrophotometer UV-Visible UV 500 from Spectronic Unicam, UK), which however did not allow discrimination between bacteria and microalgae, as it is just a measure of light scattering. Accordingly, microalgal cell concentration was also counted with a Bürker Chamber (HBG, Germany). Microalgal growth rates in the batch experiments were measured by linear regression of 6–8 experimental points of cell concentration during the logarithmic phase of growth, from two or three independent biological replicates. The specific growth rate was calculated as the slope on graph of logarithm of cells as a function of time.

At the beginning and the end of the growth curve the final biomass concentration of each experiment was also measured as dry weight (DW) in terms of g L<sup>-1</sup>. This was done gravimetrically on biomass filtered (0.22 μm) and dried at 100 °C in a laboratory oven for 2 hours. Nitrate (NO<sub>3</sub><sup>-</sup>-N), nitrites (NO<sub>2</sub><sup>-</sup>-N), NH<sub>4</sub><sup>+</sup>-N, N<sub>tot</sub>, PO<sub>4</sub><sup>3-</sup>-P and COD were determined at time 0 and at the end of each growth curve, in order to verify the consumption by microalgae, bacteria or consortium, after biomass removal by filtration, to measure dissolved compounds only. Nitrate, nitrites, ammonium and orthophosphate were analysed using Hydrocheck Spectratest kits by Reasol<sup>®</sup>, while an Aquanal<sup>®</sup> kit by Sigma-Aldrich was used for COD. N<sub>tot</sub> was measured as nitrate, after persulfate digestion in an autoclave for 1 hour.

Dissolved oxygen (DO) concentration was measured by means of a Handylab Ox 12 SCHOTT<sup>®</sup> oximeter connected to a PC by using Multi/ACHAT II software provided by WTW.

In continuous experiments, biomass was sampled at least five times at steady state, and all analytical measures reported before were carried out, including N, P and COD in the outlet stream.

## Statistical analysis

Student's *t*-tests were applied to ascertain meaningful differences in nutrient consumption and growth curves (both OD values and cell numbers) in the continuous system at three different HRT values. The level of statistical significance was assumed for  $p < 0.05$ , and significantly different results are highlighted with letters in the figures. Biomass samples

were collected at least five times for each residence time and were found to be normally distributed.

## RESULTS AND DISCUSSION

### Batch experiments of co-cultivation of the consortium

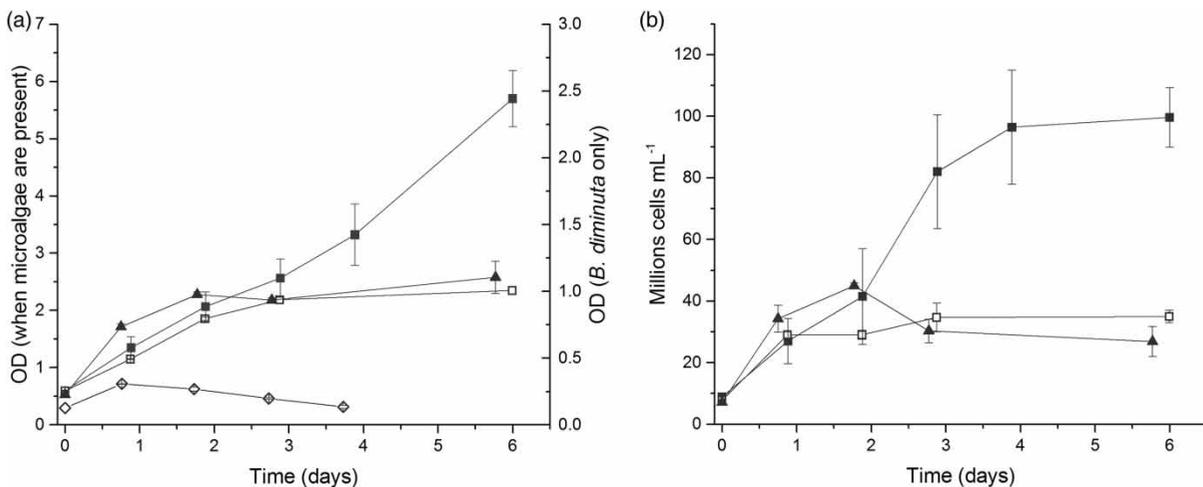
To ascertain the possible mutual interactions between *C. protothecoides* and *B. diminuta* the two species were cultivated in batches, both individually and in consortium, and the nutrient removal was measured and compared. As shown in Figure 1, the presence of bacteria increased the performance of microalgal growth. In fact (Figure 1(a)) a higher specific growth rate of microalgae can be calculated by the trend of cell concentration, when bacteria are present: the specific growth rate of *C. protothecoides* increased from  $0.689\text{ d}^{-1}$  to  $0.995\text{ d}^{-1}$  in the presence of *B. diminuta*.

A number of authors have suggested that one of the mechanisms behind the higher performance of the bacterial–microalgal consortium is related to gas exchange (Subashchandrabose *et al.* 2011; Boelee *et al.* 2014), where oxygen produced by photosynthesis is supplied to the bacteria, while  $\text{CO}_2$  can be exploited as carbon source by the microalgae. To find out if gas exchange is the main reason of the increased performances of the consortium, some other experiments were carried out by bubbling air– $\text{CO}_2$  (5% v/v) to the axenic culture of *C. protothecoides* (Figure 1), in order to compare non-limiting gas conditions with the performance of the mixed culture. Even though the algal

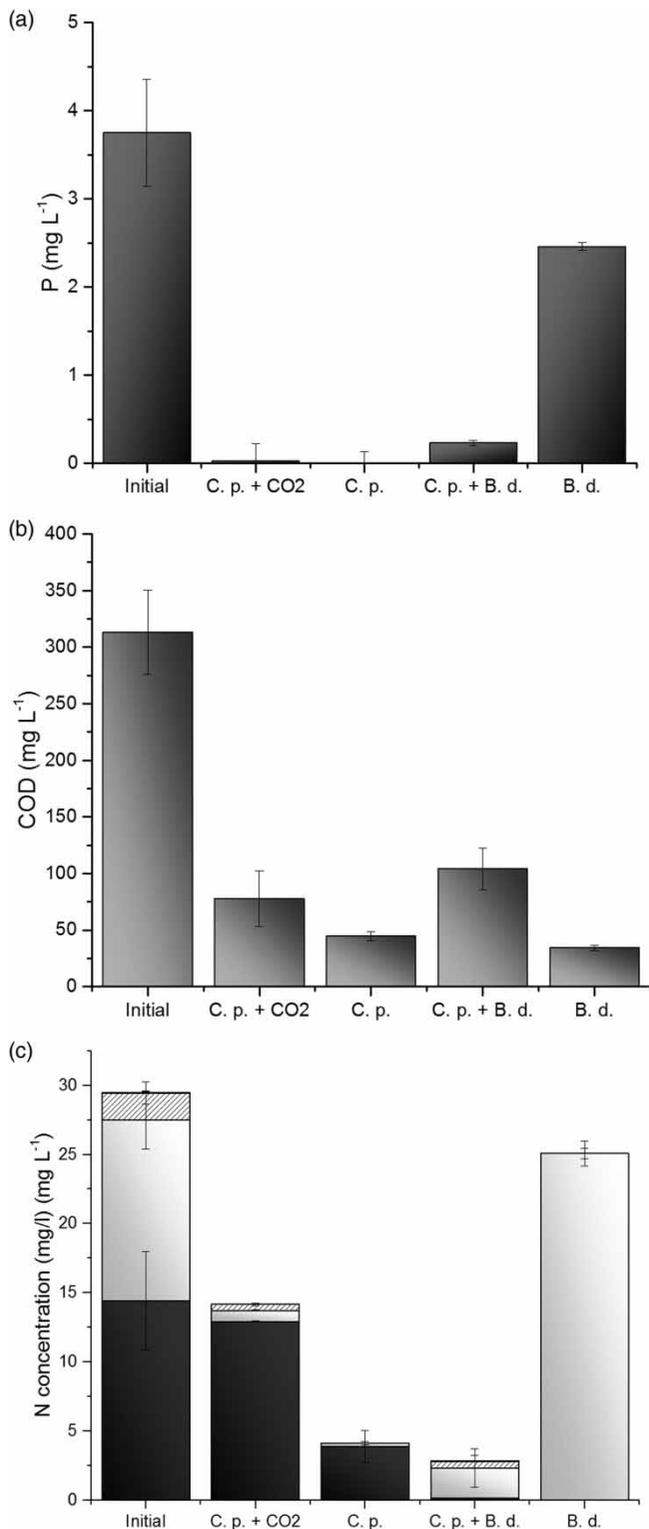
biomass concentration reached higher values when external  $\text{CO}_2$  was provided, the specific growth rate was lower than in the co-culturing condition ( $0.808$  and  $0.995\text{ d}^{-1}$ , respectively). This supports the conclusion that the mutual positive interaction between the two populations is not limited to a simple gas exchange, but is the result of an actual symbiotic interaction. The positive interactions between microalgae and bacteria may occur with a wide range of mechanisms (Fukami *et al.* 1997; Subashchandrabose *et al.* 2011; Unnithan *et al.* 2014), and they may involve nutrient exchange as well (Park *et al.* 2008; Kouzuma & Watanabe 2015; Palacios *et al.* 2016b), facilitating, for instance, nitrogen assimilation by microalgae (Le Chevanton *et al.* 2013).

Nutrient consumption data are shown in Figure 2, for all the conditions tested. One interesting result relates to phosphorus reduction, which reached 100% when microalgae were present. In the case of co-cultivation, about 94% of the P was removed, which is remarkable considering that, in current wastewater treatment, the conventional method to reduce this pollutant requires a chemical precipitation step. This P uptake capability is well known and described for many algal species, including luxury uptake (Solovchenko *et al.* 2016). *B. diminuta*, along with other bacterial species, is not able itself to consume all the phosphorus present in wastewaters: the reduction was only about 34% in the axenic culture of this species.

On the other hand, the bacterial species showed a remarkable capability for organic carbon removal, higher than the mixotrophic capability of microalgae. From Figure 2(b) it is clear that microalgae can consume organic carbon by mixotrophy, as already demonstrated for this species in previous



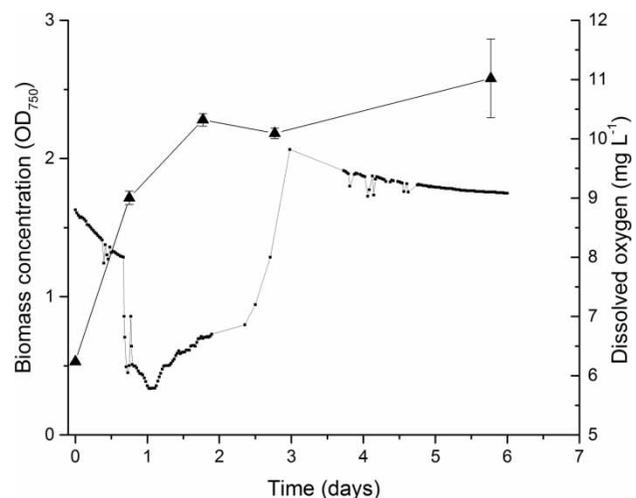
**Figure 1** | Growth curve (OD data in (a), cell counts in (b)) of *C. protothecoides*–*B. diminuta* consortium (dark triangles), microalgae alone without bubbling (open squares) and with non-limiting  $\text{CO}_2$  supply (dark squares) and *B. diminuta* alone (open diamonds) in synthetic wastewater. In (a), data of cultures with bacteria only (open diamonds) relate to the right Y axis, for graphical reasons only.



**Figure 2** | Initial (first column) and final concentration of phosphorus (a), COD (b) and nitrogen (c) of growth curve in synthetic wastewater of *C. protothecoides* alone without bubbling (C. p.) and with non limiting CO<sub>2</sub> supply (C. p. + CO<sub>2</sub>), microalgal–bacterial consortium (C. p. + B. d.) and bacteria alone without bubbling (B. d.). In (c), grey portions represent N-NH<sub>3</sub>, dark N<sub>org</sub> and striped N-NO<sub>3</sub>.

work, to an extent that depends on CO<sub>2</sub> concentration and light availability (Sforza *et al.* 2012). COD removal in the case of co-cultivation was slightly lower than for *B. diminuta* alone, suggesting that some possible competition for nutrients (including micronutrients), or other kinds of interactions may occur. For instance, as the specific growth rate of the two organisms were quite different, so that it affects the consumption of nutrients in a batch system, in particular if only the final concentration is measured. A decoupled growth of bacteria and microalgae is indeed the possible explanation of this, as confirmed also by the trend of oxygen availability in the mixed growth curve (where the two organisms are cultivated together, see Figure 3 for details). In fact, the dissolved oxygen concentration in the culture strongly decreased in the first day of growth, which corresponds to the exponential phase of bacteria alone (Figure 1). Accordingly, during this phase, bacteria are actively growing, thus consuming all the oxygen available. After that, bacteria reach a stationary phase, but microalgae are still active, as confirmed by the trend of oxygen, with increasing concentration up to 9 mg L<sup>-1</sup> in the following days. Thus, a sequential growth pattern occurs, with a first phase of bacterial predominance, followed by microalgal growth. Therefore, the kinetics of the two organisms is a key aspect that may influence co-cultivation and nutrient removal, in particular when moving to a real continuous system.

A remarkable result relates to the total nitrogen consumption: as shown in Figure 2(c), it is clear that *B. diminuta*, cultivated axenically, was able to remove all the organic nitrogen and to convert it to ammonium. No nitrification or denitrification were observed, and this species was



**Figure 3** | Dissolved oxygen (dots) in culture medium during the batch experiment of co-cultivation (triangles). Biomass concentration is reported as optical density.

also not able to consume all the ammonia produced. On the other hand, even though *C. protothecoides* was able to mixotrophically consume a fraction of the organic nitrogen, when cultivated alone, its removal capability was not as efficient as the removal of ammonium in the case of co-cultivation. It appeared that one advantage of co-culturing these two species lies in the transformation of nitrogen by *B. diminuta* to a chemical form which is more easily exploited by the microalgal species (Markou *et al.* 2014). As already shown in the literature, the growth of *Chlorella* sp. is promoted by co-cultivation with *B. diminuta* (Park *et al.* 2008), but the interaction is not fully understood. Our results suggest that nutrient exchange may play a role in this mutual interaction, which agrees with current literature (Park *et al.* 2008; Kouzuma & Watanabe 2015; Palacios *et al.* 2016b).

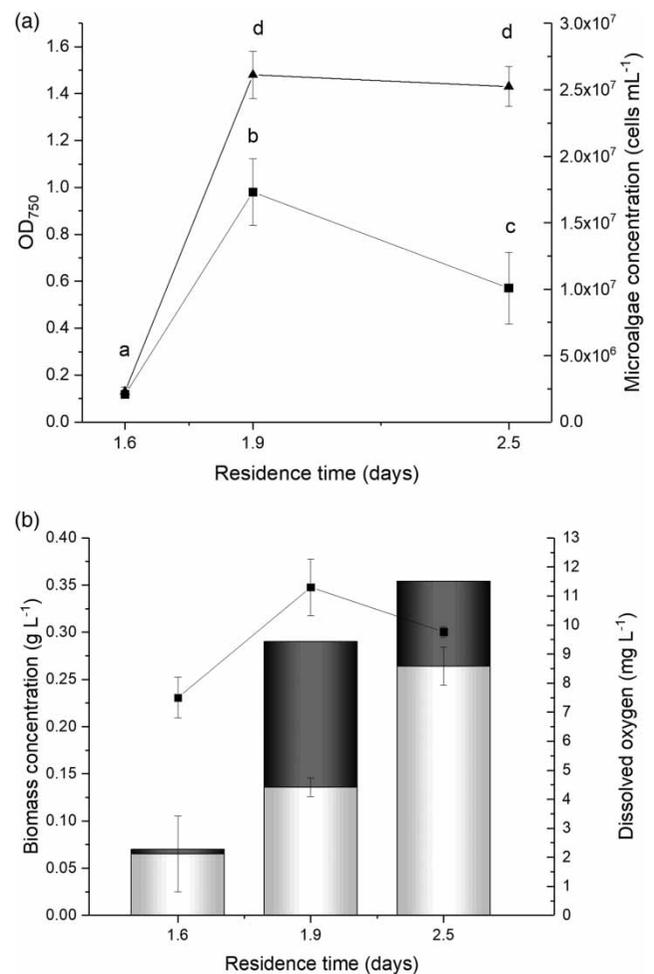
In summary, the co-cultivation of this consortium is very promising for bioremediation purposes, as it resulted in a reduction of 66.7, 69.4, 34.4% of C, N and P respectively, with final concentrations far below the limit for water discharge into surface water bodies (Council of the European Communities (1998) EUR-Lex - 31998L0015).

### Continuous experiments: effect of residence time on nutrient removal

As reported in the previous section, *B. diminuta* and *C. protothecoides* are characterized by different specific growth rates. This may actually influence the effectiveness of wastewater treatment in continuous reactors, where biomass is continuously removed from the system, depending on the flow-rate applied. In addition, if the residence time is too low, it may result in the washout of the biomass from the reactor, or an unbalanced microalgae:bacteria ratio. Thus, the growth and nutrient removal performances of the consortium were investigated in a continuous reactor running at steady state, with particular attention to the influence of the residence time.

Results of biomass concentration, in terms of optical density, microalgal cell concentration and dry weight are shown in Figure 4(a) and 4(b).

The overall biomass concentration (Figure 4(b)) increased with the residence time, which is a common result in biological continuous reactors (Sforza *et al.* 2015). On the other hand, as shown in Figure 4(a), at the lower residence time an almost complete removal of microalgal biomass occurred, as the microalgal cell number decreased and the colour of the culture turned from green to white (due to the turbidity of bacteria only). Continuous experiments with axenic culture of *C. protothecoides* were also



**Figure 4** | Biomass concentration data (OD data dark triangles, cell numbers dark squares) at different HRTs tested (a). Biomass concentration expressed as dry weight (bars) and dissolved oxygen concentration (dark squares) (b) at different HRT. In (b), grey portions refer to bacteria concentration, dark to microalgae. Different letters correspond to statistically significant results.

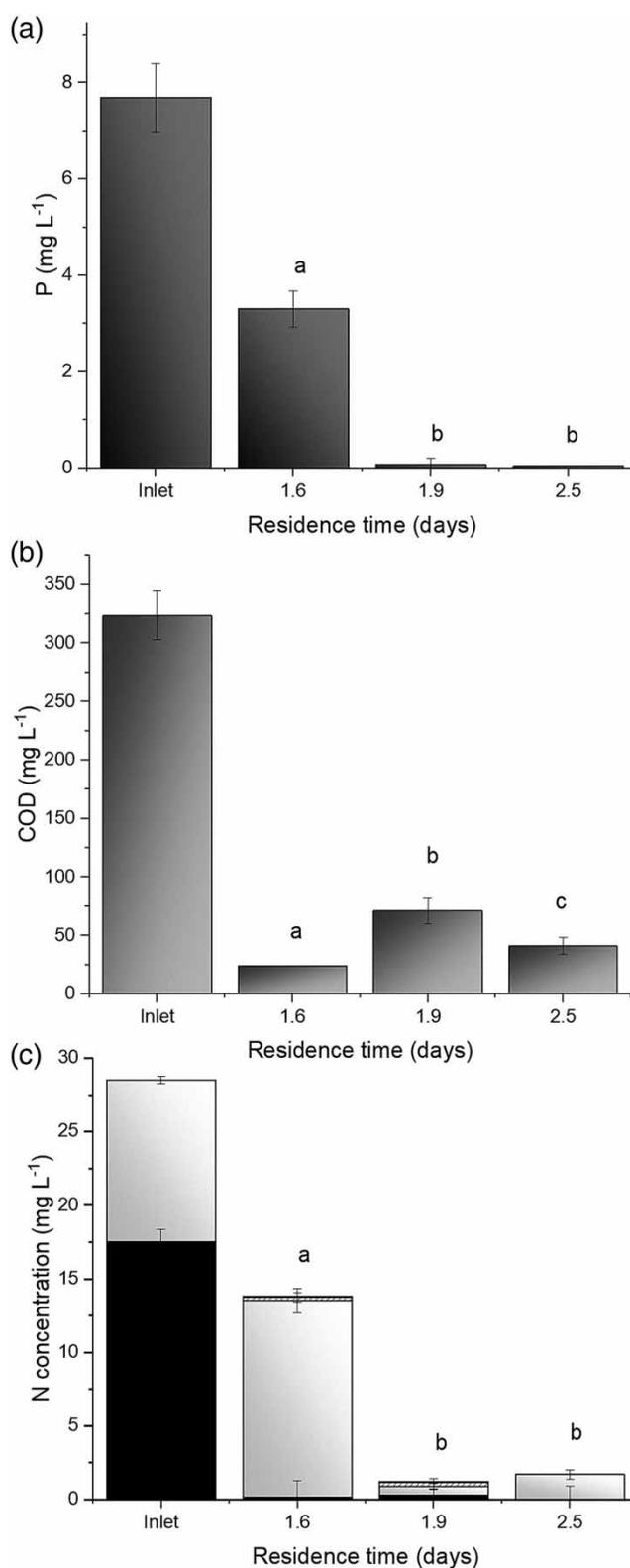
carried out as a control, at the same residence times tested with the consortium: an optical density of  $1.13 \pm 0.02$  and a biomass concentration of  $0.18 \pm 0.02 \text{ g L}^{-1}$  were obtained at 2.5 days, which are lower than for the consortium, while at lower residence times microalgal washout occurred (data not shown). Accordingly, this confirmed that, when *B. diminuta* is present, the growth performance of *C. protothecoides* is enhanced, and the washout occurs at a lower residence time, as the microalgal specific growth rate is enhanced. In the case of the consortium, however, the optical density remained comparable at both 1.9 and 2.5 days, even though the dry weight increased, as a result of the different extinction coefficients of the two organisms, as already clear from Figure 1(a). To better understand how the residence time influenced the population in the reactor, a correlation between dry weight and microalgal cell number

was applied (see supplementary material, Figure S1, available with the online version of this paper). As a rough indication, by applying the specific cellular weight to the cell concentration actually counted, it was possible to observe that the residence time also influenced the ratio of the two populations. Thus, if the bacterial concentration increased with residence time, microalgal biomass appeared to reach a maximum at 1.9 days. Of course an effective measure of the biomass concentration of the two distinct populations is challenging, but the correlation of microalgal dry weight may be applied as a rough indication. This trend of microalgal biomass concentration as a function of residence time is confirmed by the trend of the dissolved oxygen concentration measured in the reactor (Figure 4(b)), with a maximum at 1.9 days, where the microalgal:bacteria ratio is higher. This also affected the nutrient removal, as shown in Figure 5.

Remarkable removals were obtained, in particular related to P consumption, which was almost total at higher residence times (about 99%). At 1.6 days, on the other hand, the washout of microalgae caused an inefficient reduction of this nutrient, with a final concentration of about  $3.3 \text{ mg L}^{-1}$  (removal efficiency of 56%), since the removal of phosphorus is mainly performed by microalgae, as already observed in the case of batch experiments.

COD removal (Figure 5(b)) was satisfactory for all the residence times, with concentrations measured at the outlet being lower than the legal limit value. Removal efficiencies of about 93, 78 and 87% were obtained for 1.6, 1.9 and 2.5 days, respectively. As shown in Figure 5(b), differences in COD outlet concentrations, among the tested HRTs, are statistically significant. This trend, apparently unexplained, may be due to the ratio of the two populations at different residence times (see Figure 4(b)). In fact, it was reported in previous literature that microalgae may also release organic carbon compounds/matter during growth (Watanabe *et al.* 2005; Subashchandrabose *et al.* 2011; Natrah *et al.* 2014), including the molecules produced to sustain the specific interaction with *B. diminuta* (Palacios *et al.* 2016a, 2016b). Thus, *B. diminuta* itself was able to almost completely remove organic carbon alone, at  $\tau = 1.6$  days, when microalgae are washed out, while at 1.9 days microalgal concentration prevails, with a possible excretion of organic compounds. At 2.5 days, the ratio of bacteria:microalgae increased, and COD removal was enhanced accordingly. On the other hand, it is noteworthy that, in spite of the differences, the removal of organic carbon was very efficient in all cases.

Finally, it must be pointed out that the presence of microalgae is crucial for nitrogen removal. In fact, as



**Figure 5** | Inlet (first column) and outlet concentration of phosphorus (a), COD (b) and nitrogen (c) of continuous systems at three retention times. In (c), grey portions represent  $\text{N-NH}_3$ , dark  $\text{N}_{\text{org}}$  and striped  $\text{N-NO}_3$ . Different letters correspond to statistically significant results.

shown in Figure 5(c), the washout of microalgae at 1.6 days strongly affected the decrease of nitrogen concentration, which was actually converted from organic to ammonia form, but was not efficiently removed, similar to what was observed in batch experiments with *B. diminuta* cultivated alone. By contrast, at higher residence times, an almost complete removal of nitrogen occurred, suggesting that residence time is the main operating variable affecting the process.

In conclusion, the continuous cultivation of these two species is feasible and efficient in removing pollutants from wastewater, given the proper operating conditions. Clearly, a deeper understanding of the influence of light on microalgal growth, and the subsequent interactions with bacteria, should be pursued in future. In particular, the effect of day–night light cycles on the process should be assessed, with particular attention to the mixotrophic capabilities of microalgae. The capability of the consortium to maintain their interactions in a complex system such as a real WWTP should certainly be investigated, but the preliminary results here reported are promising in terms of an efficient, single-step wastewater treatment. In fact, as COD, nitrogen and phosphorus were simultaneously removed from the wastewater, a possible WWTP consisting of one single biological step may be proposed in the future, without the need for an external oxygen or CO<sub>2</sub> supply, with the consequent abatement of operational costs. A first scaling up should be carried out to validate it in a real system.

## CONCLUSIONS

The symbiotic interaction between *B. diminuta* and *C. protothecoides* was assessed with a view to enhancing nutrient removal efficiency from wastewater.

The presence of *B. diminuta* stimulated the growth of the microalgal species, and such an effect is not only due to the gas exchange between the two populations, as *C. protothecoides* in the presence of CO<sub>2</sub> bubbling did not show such a high nutrient removal efficiency. The positive interaction between *C. protothecoides* and *B. diminuta* was probably due to nutrient exchange between the two populations of microorganisms, in particular related to the capability of *B. diminuta* to convert organic nitrogen into ammonium species, which is more easily consumed by microalgae. Even though the two organisms have different specific growth rates, continuous co-cultivation is possible, as the mutual interaction allows an equilibrium to be achieved in the bacteria:microalgae ratio, which was found

to be strongly dependent on the residence time applied. Residence times that were too low caused a washout of the microalgae, with a consequent inefficient removal of nitrogen and phosphorus.

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