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# Developing a microbial consortium for removing nutrients in dishwasher wastewater: towards a biofilter for its up-cycling

R. Congestri , S. Savio, S. Farrotti, A. Amati, K. Krasojevic, N. Perini, F. Costa and L. Migliore

## ABSTRACT

Microbial consortia are effective biofilters to treat wastewaters, allowing for resource recovery and water remediation. To reuse and save water in the domestic cycle, we assembled a suspended biofilm, a 'biofilter' to treat dishwasher wastewater. Bacterial monocultures of both photo- and heterotrophs were assembled in an increasingly complex fashion to test their nutrient stripping capacity. This 'biofilter' is the core of an integrated system (Zero Mile System) devoted to reusing and upcycling of reconditioned wastewater, partly in subsequent dishwasher cycles and partly into a vertical garden for plant food cultivation. The biofilter was assembled based on a strain of the photosynthetic, filamentous cyanobacterium *Trichormus variabilis*, selected to produce an oxygen evolving scaffold, and three heterotrophic aerobic bacterial isolates coming from the dishwasher wastewater itself: *Acinetobacter*, *Exiguobacterium* and *Pseudomonas* spp. The consortium was constructed starting with 16 isolates tested *one-to-one* with *T. variabilis* and then selecting the heterotrophic microbes up to a final *one-to-three* consortium, which included two dominant and a rare component of the wastewater community. This consortium thrives in the wastewater much better than *T. variabilis* alone, efficiently stripping N and P in short time, a pivotal step for the reuse and saving of water in household appliances.


**Key words** | biofilter, cyanobacteria, dishwasher wastewater treatment, heterotrophic bacteria, microbial consortia, *Trichormus variabilis*

## HIGHLIGHTS

- Microbial consortia can be efficiently used as biofilter for wastewater remediation
- Integration of photo- and heterotrophic bacteria allows for nutrient stripping in wastewater treatment.
- An ad hoc engineered consortium was built using *Trichormus variabilis* (*Cyanobacteria*) and three dishwasher wastewater isolates.
- The development of the consortium was built in a step-by-step process of association in laboratory closed conditions.
- The consortium proved to be able to thrive in raw dishwasher wastewater and reduce its nutrient load, self-assembling in suspended aggregates.

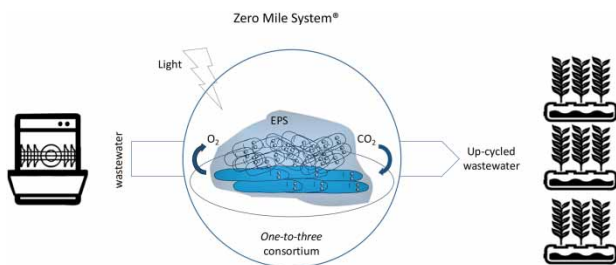
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## GRAPHICAL ABSTRACT



## INTRODUCTION

Water demand and amount of wastewater produced are continuously increasing worldwide. Hence, wastewater management towards reuse, recycle and resource recovery is a stringent need (WWAP 2017).

In this context, biological treatment is a key step of wastewater treatment processes. Conventional techniques rely on interconnected, bacteria-based complex and multi-step operations (e.g. activated sludge systems) with high costs and energy input. More recently, biological filtering and bioremediation strategies based on the synergistic relationship between photosynthetic and heterotrophic microorganisms, ‘microbial consortia’, proved to be a more sustainable wastewater treatment approach both in terms of treatment and cost efficiencies (Posadas *et al.* 2017). The consortium partner microalgae/cyanobacteria provide oxygen, through their photosynthetic activity, to the heterotrophic bacteria for chemical oxygen demand reduction, while the bacterial partner, by means of organic matter degradation, releases CO<sub>2</sub> and mineral nutrients used by microalgae when exposed to light, resulting in increased pollutant removal efficiency and biofiltration ability (Gonçalves *et al.* 2017).

Biofilters based on microbial consortia can form biofilms, complex heterogeneous communities occurring either suspended or attached, that proved promising in advanced remediation of municipal wastewater (Posadas *et al.* 2017). Indeed, cooperative interactions in the biofilms between bacteria and microalgae/cyanobacteria promote the establishment of stable communities in which simultaneous autotrophic and heterotrophic metabolism support nutrient excess, and pollutant and pathogen removal from wastewater.

Microbial interactions in the biofilms, both spatial and functional, are possible thanks to presence of the extracellular polymeric (extracellular polymeric substances, EPS) matrix that embeds biofilm cells, mediating their cohesion

and exchanges. The matrix gel-like network has high retentive properties, serves in the immobilization and accumulation of particulate and noxious compounds – acting as a natural molecular sieve or an ion exchanger of xenobiotics, entraps particulate matter and exposes exoenzymes for organic matter degradation (Di Pippo *et al.* 2009; Guzzon *et al.* 2019).

Dishwasher WW (DWW) is often nutrient-rich; urban agriculture could absorb these nutrients and has historically done so. Despite the high nutrient content and the very low presence of pathogens, heavy metals and pharmaceuticals, the reuse of this wastewater is not practised in modern society, because it is produced by point sources in small amounts.

The goal of this work is to build up, in a gradually increasing complexity mode, a microbial consortium based on autochthonous heterotrophic dishwasher bacteria and a photosynthetic EPS network builder. The feasibility of this engineered consortium was checked by studying its structure and function in a laboratory-scale closed environment system. Consortium members were (i) the filamentous cyanobacterium *Trichormus variabilis* and (ii) selected aerobic bacteria isolated from DWW. This approach allows testing of a microbial association highly improbable in nature in a bioremediation challenge to ameliorate DWW.

## MATERIALS AND METHODS

### *Trichormus variabilis* culture

The strain of the heterocytic cyanobacterium *Trichormus variabilis* (Kützing ex Bornet & Flahault) Komárek & Anagnostidis (VRUC168) was isolated from sediment biofilms of a Mediterranean coastal lagoon (Cabras lagoon, Sardinia, Italy). It is maintained as monoalgal culture in

the Tor Vergata Rome University Collection (VRUC) in liquid culture medium (Blue Green Medium – Nitrogen, BG11<sub>0</sub>) at 18 °C and 30 μmol photons m<sup>-2</sup> s<sup>-1</sup> irradiance, light:dark cycle 12:12 (Di Pippo *et al.* 2012; Bellini *et al.* 2018).

Before the experiment, a sample of the stock culture was acclimated for 2 weeks at 80 μmol photons/(m·s) irradiance and 25 °C temperature conditions, and then used for the production of the experimental inoculum. *T. variabilis* inoculum was maintained in exponential growth phase (log phase) by adding fresh (semi-continuous) culture medium every 48 h.

### **Trichormus variabilis growth experiments**

Aliquots of the exponentially growing culture used as inoculum were prepared for *T. variabilis* growth experiments by centrifuging 50 ml (2,200 g, 10 min) and resuspending the pellet in BG11<sub>0</sub> to an optical density of 0.5 at 665 nm. Culture growth was tested in DWW as is (100%) and diluted at 75 and 50% in culture medium. Experiments were performed in two settings: (i) ventilated flasks – static cultures, and (ii) aerated (air bubbling) flasks, to facilitate culture mixing and gas exchanges. Three replicates for each experiment were set and culture growth was measured as *in vivo* chlorophyll *a* absorbance and culture turbidity (optical density (OD)) at 665 and 730 nm, respectively; spectrophotometer ONDA UV-20). Culture chlorophyll *a* concentration was quantified, after extraction in 90% methanol (Wellburn 1994), along with dry weight, to evaluate cyanobacterium viability and growth at the experimental conditions.

### **Identification of the dishwasher wastewater microbial community**

The dishwasher wastewater was collected 16 times from November 2017 to February 2018. From each dishwasher wastewater sample, 10 μl was plated on three solid media: TSA (tryptic soy agar); PSA (Pseudomonas agar base); MCA (MacConkey agar). Based on the morphological characteristics of the colonies grown on the media, each different strain was isolated on TSA (24 h at 30 °C) and a sample of each pure culture suspended in 200 μl of sterile distilled water (dH<sub>2</sub>O), gently vortexed and heated at 95 °C for 5 min. Each sample was then centrifuged (10,000 g, 5 min) and the supernatant, containing the bacterial DNA, recovered to be identified by Sanger sequencing. To this end, bacterial DNA was amplified by polymerase chain reaction (PCR), using COM1 (forward 5'-CAG-CAGCCGCGTAATAC-3'; position 519–536) and COM2

(reverse 5'-CCGTC AATTCCTTTGAGTTT-3'; position 907–926) selective primers for the 16S rRNA gene, identifying the variable region V4 and V5 of ribosomal RNA. Ten micro-litres of the PCR solution contained: 5 μl of EmeraldAmp GT PCR Master Mix 2X, 2 μl of bidistilled water, 1 μl of forward primer COM1 (20 mmol), 1 μl of reverse primer COM2 (20 mmol) and 1 μl of above prepared bacterial DNA (~2 ng/μl). Amplified DNA samples were sent to BioFab Research (Rome, Italy) to be sequenced by Sanger method; results were analysed using RDP Classifier.

### **Dishwasher wastewater**

A household dishwasher (Energy Class A) was used, setting the 'eco' program as washing cycle; as cleaning product, an EU Ecolabel certified dishwasher tablet detergent containing only non-toxic mineral substances and subtilisin was chosen (CAS No.: 9014-01-1; for the composition see Table S1, Supplementary material). The physico-chemical characteristics of the wastewater are also reported (see Table S2, Supplementary material).

### **Co-culture experiments to assemble the final consortium**

To produce the engineered microbial consortium, as a first step the bacterial strains isolated from the dishwasher wastewater were challenged with *T. variabilis* in co-culture experiments. In these co-cultures, both the growth of *T. variabilis*, as chlorophyll *a* concentration and *in vivo* absorbance, and co-culture development, as turbidity, were estimated to identify those strains guaranteeing the best performance.

Each bacterial isolate from the dishwasher wastewater was seeded on a TSA plate; from each plate a colony was transferred into a 50 ml sterile tube containing 15 ml of TSB (tryptic soy broth). The isolates were incubated overnight under stirring, at 30 °C. Then, the OD was measured at 600 nm and TSB added to reach the OD value of 0.5 in a final volume of 20 ml. The number of bacteria in each suspension was further quantified by plating 10 μl of each bacterial suspension and counting the resulting colony-forming units. The bacterial suspensions were gently vortexed and then centrifuged (6,804 g, 10 min), the supernatant discarded, and the pellet resuspended in the same amount of BG11<sub>0</sub>.

The *T. variabilis* suspensions were also prepared, OD 665 nm of 0.5, and used in the co-culture experiments.

The growth of each co-culture was evaluated every 24 h, over 12 days (time to reach stationary phase) by recording the absorbance values at the wavelengths of 665 and 730 nm.

### Co-culture one-to-one, one-to-two and one-to-three

The development of the engineered consortium, planned to be composed of *T. variabilis* and three bacterial isolates from the dishwasher wastewater, was built in a step-by-step process of association. The co-cultivation of the cyanobacterium with each bacterial strain was the first step of the challenge of *T. variabilis* with 16 bacterial isolates (*one-to-one* consortia). Five millilitres of bacterial suspension was mixed with 5 ml of *T. variabilis* in BG11<sub>0</sub> medium up to a final volume of 30 ml. The growth performance of each one-to-one consortium was evaluated by measuring every 48 h the absorbance at 665 and 730 nm. The second step was the challenge of *T. variabilis* with two of the selected isolates in *one-to-two* consortia, and the third step was the challenge of the final *one-to-three* consortium. To maintain the same density ratio among the microbes, in one-to-two tests 8.33 ml of *T. variabilis* suspensions was mixed with 4.17 ml of each bacterial suspension, while in one-to-three tests 8.33 ml of *T. variabilis* suspensions was mixed with 2.78 ml of each bacterial suspension. The growth performance of each consortium was then evaluated over 35 days, by measuring every 48 h the absorbance at 665 and 730 nm.

### Growth test of the one-to-three consortium in the dishwasher wastewater

The co-culture of the engineered consortium, consisting of *T. variabilis* and the selected three bacterial strains, was tested at different concentrations of wastewater (100, 75 and 50% wastewater, diluted in BG11<sub>0</sub> medium), in order to evaluate viability and growth, both by spectrophotometric measurements, in triplicate, at the wavelengths of 665 and 730 nm, every 48 h and microscopy observation, using a Zeiss Axioskop light microscope at 400 and 1,000× magnification.

### Nitrogen and phosphorus removal by the one-to-three consortium

The efficiency of the one-to-three consortium to modify the concentration of total nitrogen and total phosphorus was assessed in samples of dishwasher wastewater as is (100%) or 75% diluted in BG11<sub>0</sub> after 24–48 h. The analyses were performed according to the Italian official protocol (APAT IRSA-CNR 2000). Ten-millilitre samples of 100 or 75% wastewater were collected immediately before the start of the co-culture experiments and after 24 and 48 h treatment, in triplicate. The samples were centrifuged

(3,400 g, 10 min) and the supernatant transferred into new tubes. An aliquot of 2.8 ml of oxidizing solution (50 g K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, Merck n. 5092, 30 g H<sub>3</sub>BO<sub>3</sub>, 14 g NaOH in 1 l of deionized water) was added to the samples and autoclaved (120 °C, 30 min) and then left at room temperature.

After oxidation, total nitrogen was quantified spectrophotometrically at 220 nm in 2 ml of each sample. The data were calculated against a calibration curve built with a standard solution of NaNO<sub>3</sub> in distilled water at 0, 1, 5 and 10 mg/l N, subjected to oxidation as previously described.

After oxidation, to quantify total phosphorus, 0.6 ml of reducing solution (35 g L-ascorbic acid, 0.150 g EDTA-Na<sub>2</sub>, 3 ml formic acid in a final volume of 500 ml dH<sub>2</sub>O) and 0.6 ml of reagent mixture (0.34 g KOOC(CHOH)<sub>2</sub>COOSb ½H<sub>2</sub>O, 8.1 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>4H<sub>2</sub>O, 100 ml H<sub>2</sub>SO<sub>4</sub> concentrated, density 1.84, in a final volume of 500 ml dH<sub>2</sub>O) were added to each sample, which was then incubated for 15 min. Total phosphorus content was spectrophotometrically measured at 882 nm. The data were calculated against a calibration curve built with a standard solution of KH<sub>2</sub>PO<sub>4</sub> in distilled water at levels of 0, 0.25, 0.50 and 1 µg/l P, subjected to oxidation as described.

All the instruments were cleaned for 24 h in a specific phosphorus-free detergent, and then rinsed with distilled water.

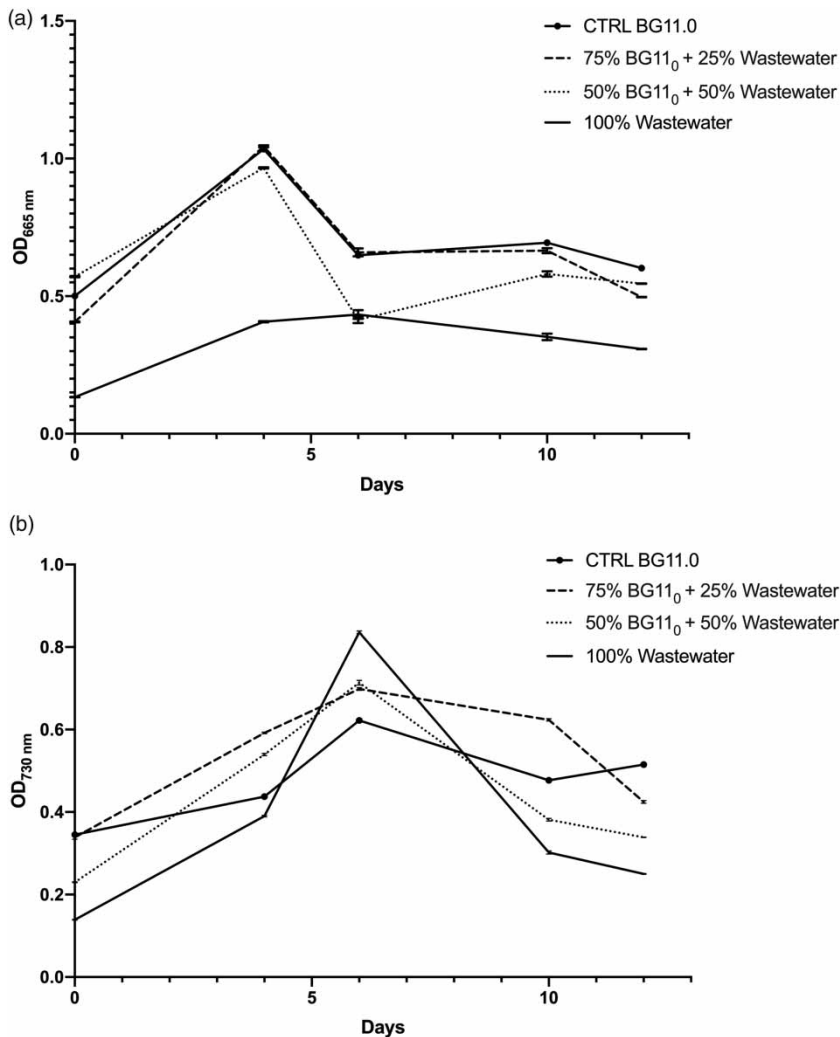
## RESULTS AND DISCUSSION

The synergistic relationship between photosynthetic and heterotrophic microorganisms is a key issue for remediation of wastewaters. In this study *T. variabilis*, a promising oxygen evolving candidate for dishwasher wastewater remediation did not survive in the DWW. Thus, we elaborated a process to develop a microbial engineered consortium able to thrive in this DWW and reduce the concentration of nutrients.

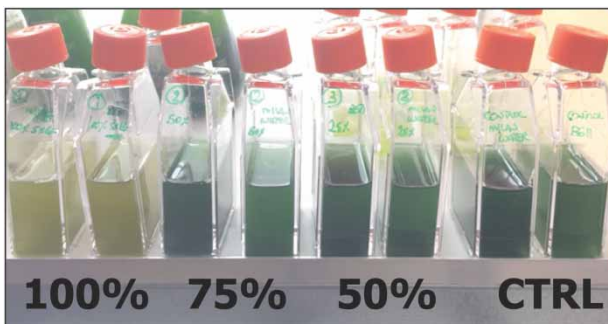
### *T. variabilis* in DWW

*T. variabilis* ability to survive and grow in dishwasher wastewater was evaluated recording the *in vivo* chlorophyll *a* absorbance (OD at 665 nm) and culture turbidity (OD at 730 nm), over 12 days in 100, 75 and 50% wastewater dilutions.

Chlorophyll *a* (Figure 1(a)) shows that 100% wastewater significantly reduces cyanobacterium growth (analysis of variance (ANOVA),  $p < 0.05$ ), as confirmed by the loss of pigmentation of the culture (Figure 2). Conversely,



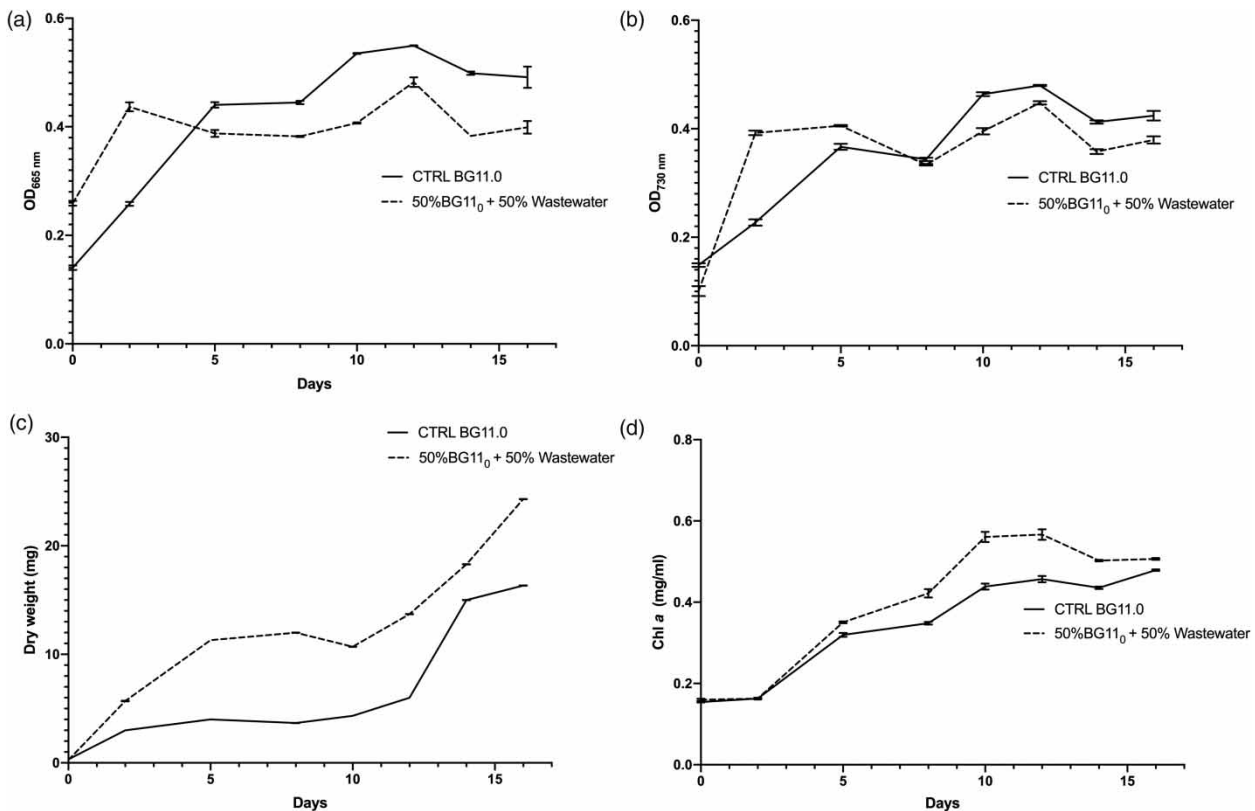
**Figure 1** | *T. variabilis* culture growth ( $n = 3$ ), static conditions, in DWW at three dilutions: 100, 75 and 50%. (a) Absorbance values at 665 nm, indicating *in vivo* chlorophyll *a*; (b) absorbance values at 730 nm, indicating culture turbidity.



**Figure 2** | Image of *T. variabilis* cultures after 7 days. The loss of pigmentation of the cultures is evident in 100% DWW.

*T. variabilis* is able to thrive in 50 and 75% (ANOVA,  $p > 0.05$ ; Figure 1(a)), indicating its capability of growing under these DWW concentrations. On the other hand,

culture turbidity (Figure 1(b)) increases in all the cultures especially at 100% DWW, with a peak at day 6, probably due to heterotrophic bacteria. No lag phases occurred at all dilutions. Overall, these results suggest that DWW may contain growth-inhibiting compounds/conditions or may lack some essential components which affected *T. variabilis* growth. As a further test, 50% DWW was used for a growth experiment with air bubbling to enable culture mixing and improve abiotic conditions (i.e. illumination and gas exchanges); growth curves clearly evidenced that *T. variabilis* is able to thrive in these conditions although at significantly lower rate than the control ( $t$ -test,  $p < 0.05$ ). Nevertheless, dry weight, chlorophyll *a* and turbidity values are similar to the control ones ( $t$ -test,  $p > 0.05$ ) (Figure 3). This indicates that even a slight modification of DWW composition may allow *T. variabilis* growth.



**Figure 3** | *T. variabilis* culture growth, mixing conditions ( $n = 3$ ), evaluated by four descriptors. (a) Absorbance values at 665 nm, indicating *in vivo* chlorophyll *a*; (b) absorbance values at 730 nm, indicating culture turbidity; (c) dry weight values; (d) chlorophyll *a* concentration in the extracts.

### Isolation of the DWW microbial colonizers

The cultivable aerobic heterotrophic microbial community from 16 wastewater samples grew on three solid media (TSA, PSA and MCA; Table 1); the microbial load ranged from  $10^7$  cells/ml on PSA and TSA to  $10^5$  cells/ml on MCA. From these microbial cultures 41 bacterial strains were isolated on the basis of their different morphology and taxonomically identified by Sanger sequencing. The main colonizers were *Proteobacteria* of the *Gammaproteobacteria* class (34 isolates), followed by *Firmicutes* of the class *Bacilli* (6) and *Actinobacteria* (1). The *Proteobacteria* are: *Aeromonadales* (8: all *Aeromonas* genus), *Enterobacteriales* (6: 5 *Citrobacter* and 1 *Klebsiella*), *Pseudomonadales* (15: 6 *Acinetobacter* and 9 *Pseudomonas*) and *Xanthomonadales* (5: all *Stenotrophomonas*). The *Firmicutes* are *Bacillales* (1: *Exiguobacterium* genus) and *Lactobacillales* (5: all *Enterococcus*). The only *Actinobacteria* belongs to *Microbacterium* genus (Figure 4). As expected, the microbial colonizers of DWW are heterotrophic aerobic generalists, which tolerate the limiting

environmental conditions of the DWW. They have been already found in dishwasher biofilms: the *Exiguobacterium* strains, tolerant to wide temperature ( $-12$  to  $+55$  °C), salinity (up to 13%), and pH (5–11) ranges; the *Acinetobacter* strains, able to thrive in a wide range of temperatures and pH (Vishnivetskaya et al. 2009; White et al. 2013; Raghupathi et al. 2018); *Enterococcus*, common human colonizers, have also been found in the home microbiome (Dannemiller et al. 2016), although their presence in extreme conditions is not reported. *Enterococcus* presence in dishwasher biofilms is possible because of the protection provided by EPS conferring tolerance properties (Limoli et al. 2015). Sixteen isolates were selected to be challenged in co-culture with *T. variabilis*; they are listed in bold in Table 1.

### Co-culture experiments for the engineering of the microbial consortium

The development of the microbial consortium was based on the possibility to produce a functional consortium, including

**Table 1** | Taxonomical identification of the bacterial isolates

Isolate ID	Taxonomical identification	Isolate ID	Taxonomical identification
3A	<i>Acinetobacter</i> [100%]	17B	<i>Enterococcus</i> [90%]
4A	<i>Acinetobacter</i> [100%]	21B	<i>Enterococcus</i> [85%]
15A	<i>Acinetobacter</i> [100%]	22A	<i>Enterococcus</i> [90%]
16A	<i>Acinetobacter</i> [100%]	1A	<i>Exiguobacterium</i> [100%]
20B	<i>Acinetobacter</i> [100%]	11B	<i>Klebsiella</i> [56%]
21A	<i>Acinetobacter</i> [100%]	15B	<i>Microbacterium</i> [85%]
3B	<i>Aeromonas</i> [100%]	7A	<i>Pseudomonas</i> [89%]
6A	<i>Aeromonas</i> [100%]	10A	<i>Pseudomonas</i> [86%]
8A	<i>Aeromonas</i> [100%]	12A	<i>Pseudomonas</i> [83%]
9B	<i>Aeromonas</i> [100%]	13B	<i>Pseudomonas</i> [93%]
10B	<i>Aeromonas</i> [100%]	14B	<i>Pseudomonas</i> [91%]
11A	<i>Aeromonas</i> [100%]	16B	<i>Pseudomonas</i> [90%]
14A	<i>Aeromonas</i> [100%]	17A	<i>Pseudomonas</i> [93%]
18B	<i>Aeromonas</i> [100%]	18A	<i>Pseudomonas</i> [81%]
5A	<i>Citrobacter</i> [45%]	20A	<i>Pseudomonas</i> [80%]
9A	<i>Citrobacter</i> [45%]	1B	<i>Stenotrophomonas</i> [93%]
12B	<i>Citrobacter</i> [63%]	5B	<i>Stenotrophomonas</i> [100%]
13A	<i>Citrobacter</i> [60%]	6B	<i>Stenotrophomonas</i> [100%]
19A	<i>Citrobacter</i> [58%]	7B	<i>Stenotrophomonas</i> [100%]
1A	<i>Enterococcus</i> [96%]	8B	<i>Stenotrophomonas</i> [100%]
2B	<i>Enterococcus</i> [87%]		

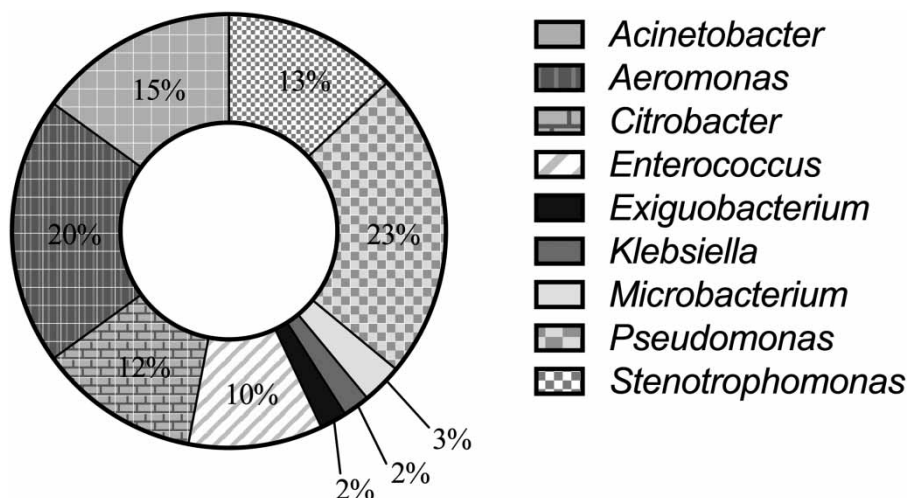
In bold, those chosen for the co-culture experiments with *T. variabilis*.

*T. variabilis* and three bacterial isolates from DWW, able to thrive in and clean up the wastewater. A step-by-step procedure in co-cultivation was applied, the first step was

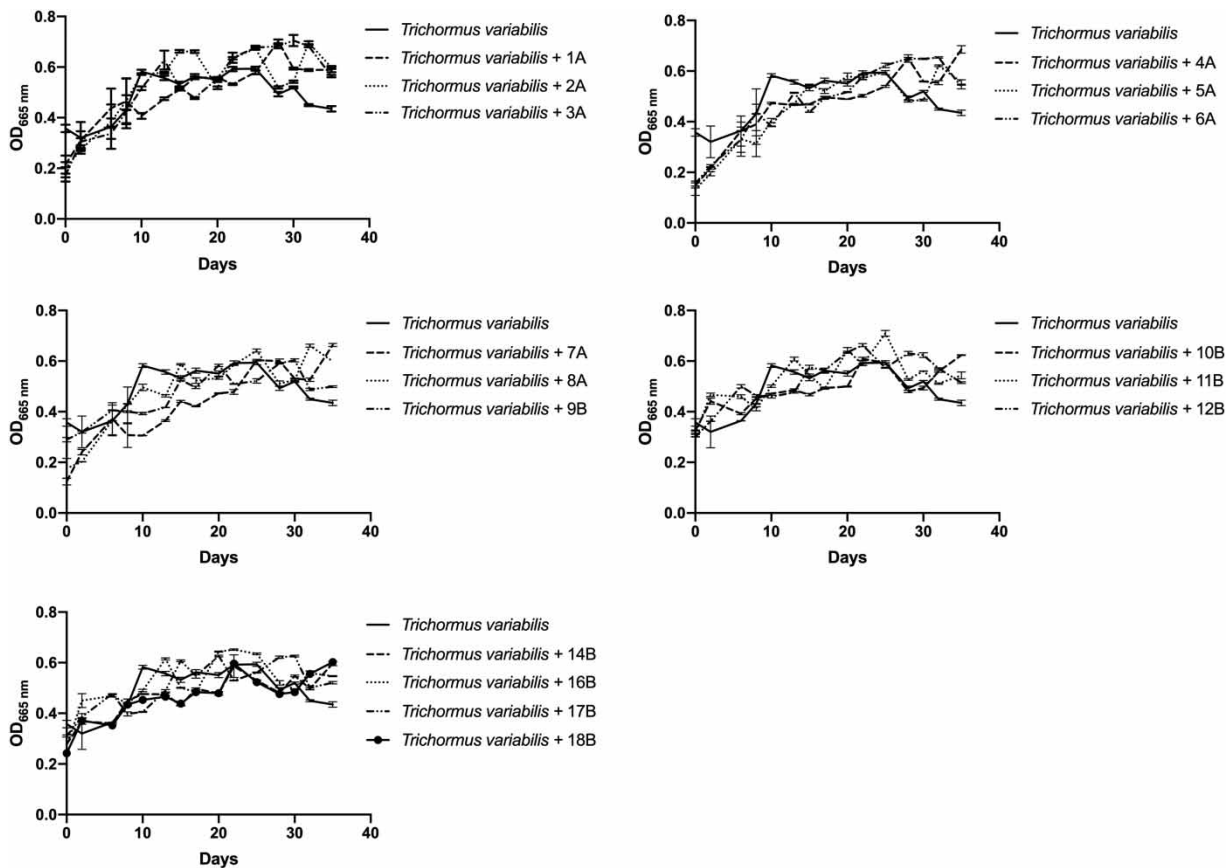
to challenge *T. variabilis* with one bacterial isolate in one-to-one consortia, followed by progressive integration in one-to-two consortia, to end up with a one-to-three consortium. These co-cultures were all grown in BG11<sub>0</sub>.

### One-to-one consortia

The first step of co-cultivation involves *T. variabilis* and the 16 bacterial isolates from DWW selected according to their best taxonomic identification by Sanger sequencing. In one-to-one challenges, two *Acinetobacter*, five *Aeromonas*, two *Citrobacter*, two *Enterococcus*, one *Exiguobacterium*, one *Klebsiella* and three *Pseudomonas* are used (Table 1, bold). Strains of these genera are often used for activated sludge-based wastewater treatment: *Pseudomonas* degrades carbon by oxidation, *Citrobacter* contributes to floc formation, and *Acinetobacter* and *Klebsiella* accumulate phosphorus, removing it from the medium. Conversely, *Microbacterium* and *Stenotrophomonas* were excluded because of their potential harm to human health. The taxonomic identification at the genus level does not allow establishment of pathogeny or risks for human health, never recorded for home appliances. The co-culture allowed the best growth of *T. variabilis* to be evaluated with the aim to select the bacterial isolates to be included in the next steps of the consortium building. One-to-one consortium performances are evaluated as *in vivo* chlorophyll *a* (Figure 5) or turbidity (Figure S1, Supplementary material). *T. variabilis* grows well in all the challenges (no significant difference with controls, ANOVA,  $p > 0.05$ ), showing the potential application of all the bacterial isolates.

**Figure 4** | Taxonomy and frequency of the DWW isolates.





**Figure 5** | One-to-one co-culture ( $n = 3$ ) of *T. variabilis* and one of the heterotrophic isolates in BG11<sub>0</sub>. The absorbance values at 665 nm, corresponding to *in vivo* chlorophyll *a* signal, of each consortium are reported.

### One-to-two and one-to-three consortia

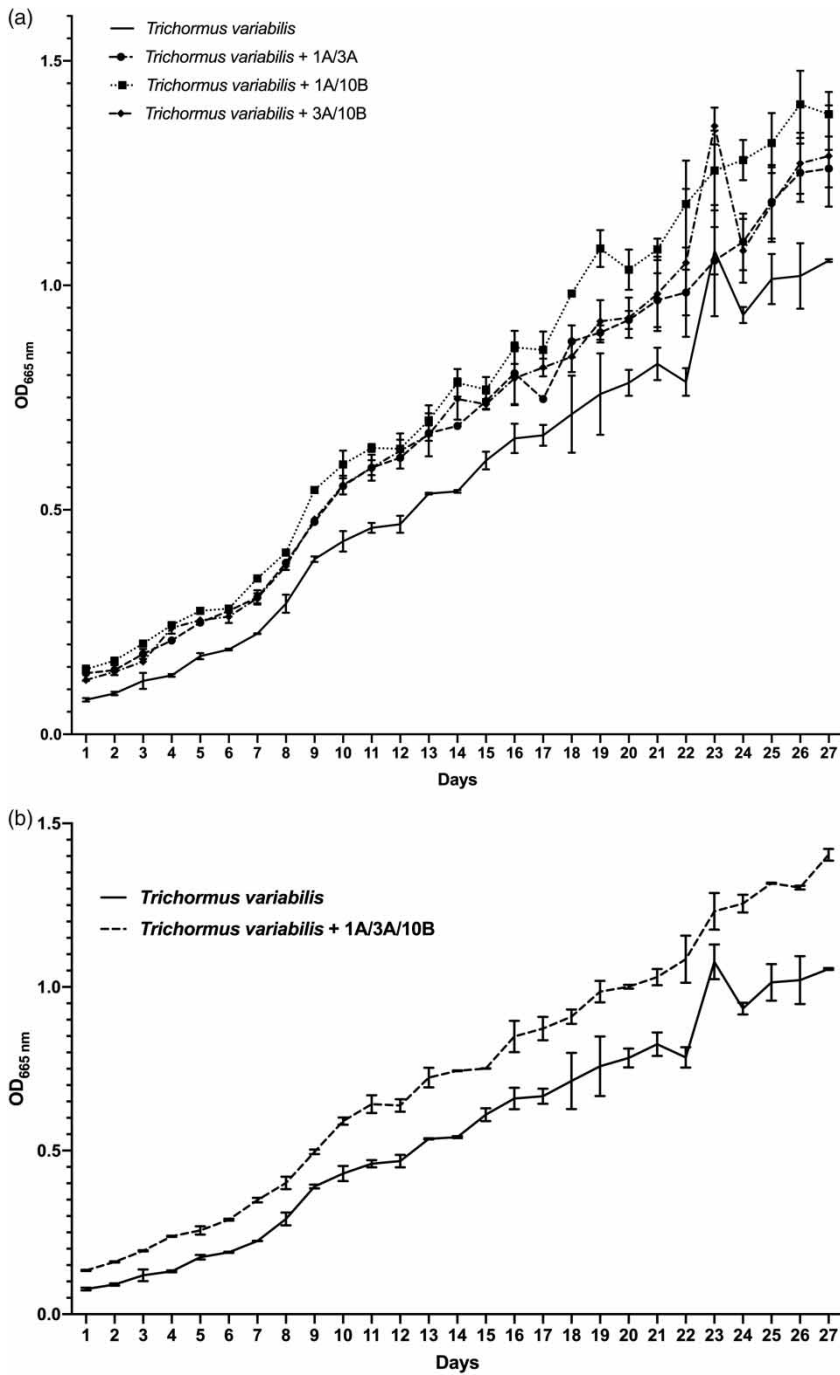
The one-to-two co-cultures of *T. variabilis* with couples of heterotrophic isolates from DWW were carried out by selecting the microbes on the basis of their frequency in the DWW and literature data. Since no isolate significantly favoured the growth of *T. variabilis*, the three strains necessary for the construction of the consortium were chosen according to a dominance/rarity criterion: among the isolates from wastewater, two were chosen as the most frequent isolates (probably dominant species among the DWW colonizers) and one because of its rarity, as it was found only in one sample. These three bacterial isolates are respectively: *Acinetobacter* and *Aeromonas* for the dominant component and *Exiguobacterium* for the rare one; according to the isolate IDs in Table 1, the three strains are 3A, 10B and 1A. In biotechnological application, *Acinetobacter* (Liu et al. 2017) and *Exiguobacterium* (Kasana & Pandey 2018) were used in co-cultivation with cyanobacteria.

The one-to-two consortia grew better than *T. variabilis* alone (control; Figure 6), particularly the *T. variabilis* +1A/10B consortium.

As a final step, the three isolates (1A, 3A and 10B) were challenged in co-culture with *T. variabilis* in the one-to-three consortium, which showed an enhanced growth of the cyanobacterium, suggesting an effective application of this consortium in a DWW biofilter (Figure 6).

### One-to-three consortium in different DWW concentration

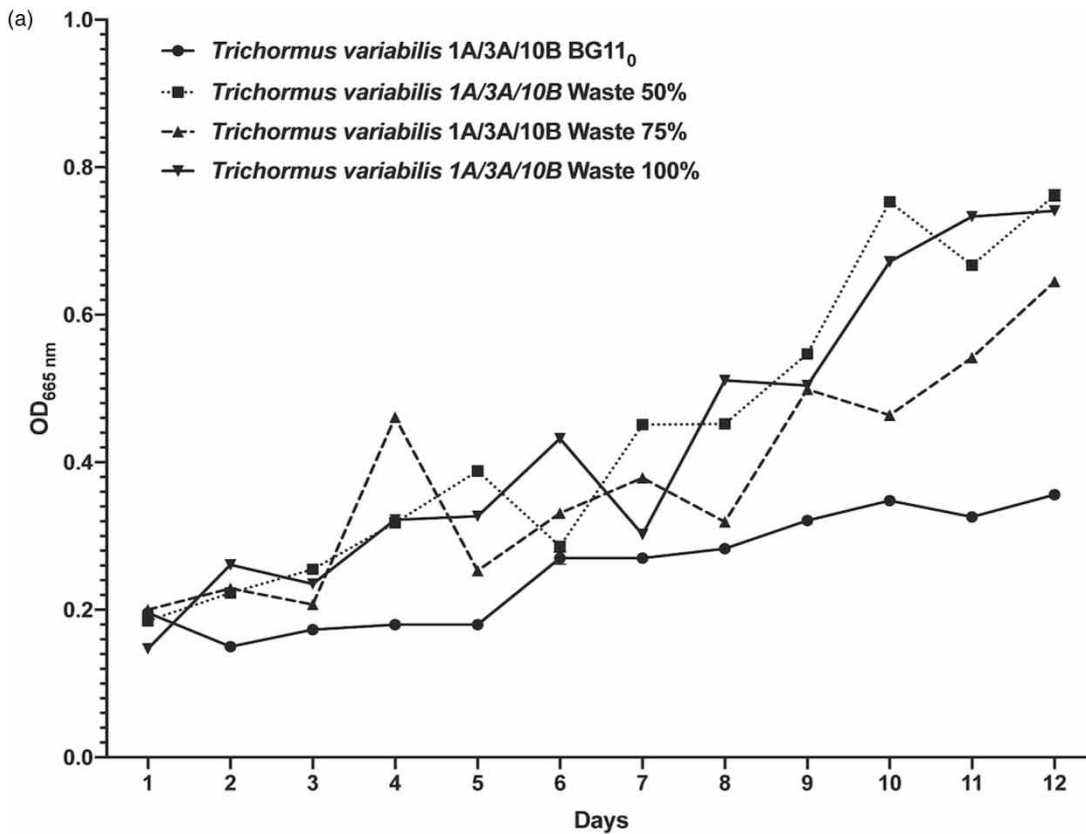
To demonstrate the one-to-three consortium efficacy it was grown in DWW as is (100%) or diluted (75 and 50%) in BG11<sub>0</sub> culture medium. The growth curves of the consortium, measured as *in vivo* chlorophyll *a* absorbances, show that DWW promotes *T. variabilis* photosynthetic activity and biomass accumulation at any dilution (Figure 7(a)). The result demonstrates the cooperative



**Figure 6** | One-to-two ( $n = 3$ ) and one-to-three ( $n = 3$ ) co-cultures of *T. variabilis* and the selected isolates (1A: *Exiguobacterium* sp., 3A: *Acinetobacter* sp., 10B: *Aeromonas* sp.) in BG11<sub>0</sub>. (a) Absorbance values of each one-to-two consortium. (b) Absorbance values of the one-to-three consortium (both at 665 nm, indicating *in vivo* chlorophyll *a*).

interaction between cyanobacteria and heterotrophic bacteria in the consortium, leading to a stable community where coordinated autotrophic and heterotrophic metabolism supports nutrient removal from DWW. Figure 7(b) shows a further important emergent property of the consortium, its three-dimensional organization as floating microbial aggregates (a

sort of 'green sausage'), not adhering to flask surfaces. These three-dimensional (3D) structures are reversible associations that upon strong manual shaking of the flask disassociate in a homogeneous green suspension: they quickly reconstitute (a couple of hours) when the flask is left to rest. Hence, the 3D structure of this microbial consortium must be an



**Figure 7** | One-to-three co-culture growth of *T. variabilis* with isolates in DWW (1A: *Exiguobacterium* sp., 3A: *Acinetobacter* sp., 10B: *Aeromonas* sp.). (a) Absorbance values at 665 nm, indicating *in vivo* chlorophyll *a* ( $n = 3$ ); (b) microbial consortia in DWW (0, 100, 75, 50%), at 100% the 3D structures resemble 'green sausages' ( $n = 2$ ). Please refer to the online version of this paper to see this figure in colour: <http://dx.doi.org/10.2166/wst.2020.325>.

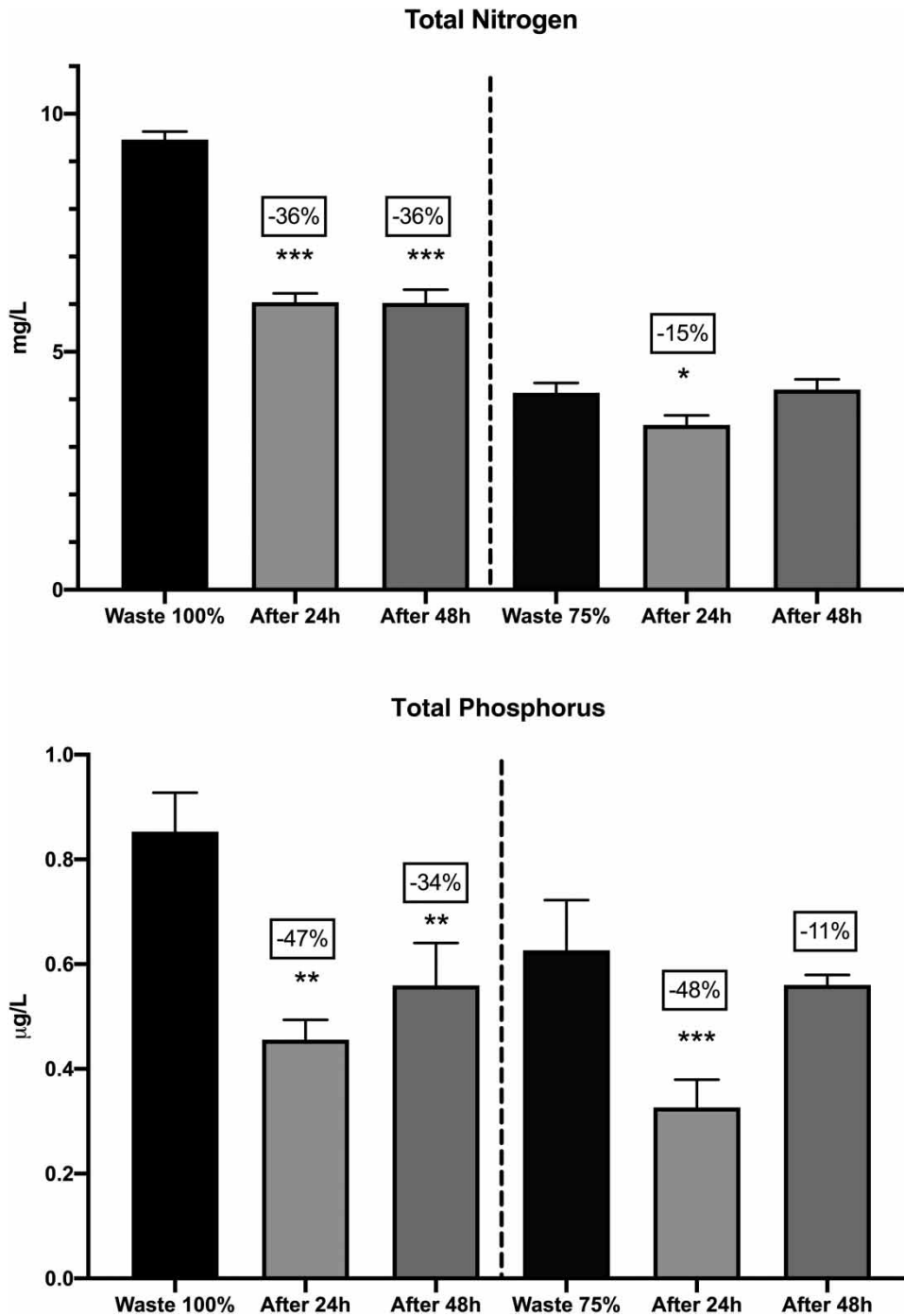
advantageous, stable type of association, although *T. variabilis* is known to form compact aggregates attached as biofilms to exposed surfaces (Di Pippo *et al.* 2012). The development of

cyanobacterial-bacterial consortia in wastewater treatment plants is well known (Congestri *et al.* 2006; Roeselers *et al.* 2007; Congestri 2008; Di Pippo *et al.* 2014).

### DWW nutrient removal by the one-to-three consortium

The efficiency of the microbial consortium in ameliorating DWW as is, 100% or diluted at 75% in BG11<sub>0</sub>, was evaluated as removal of total nitrogen and phosphorus after 24

or 48 h (Figure 8). In DWW as is, nitrogen is reduced by 36% after 24 and 48 h; this reduction is significant (ANOVA,  $p < 0.001$ ). Conversely, in 75% DWW the reduction was 15% after 24 h (ANOVA  $p < 0.05$ ); no nitrogen variation was found after 48 h.



**Figure 8** | Nitrogen and phosphorus removal by the one-to-three microbial consortium in DWW (as is, 100%, or diluted at 75% in BG11<sub>0</sub>) after 24 and 48 h. Asterisks indicate significance levels (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). In brackets, the percent removal.

In DWW as is, phosphorus is reduced by 47 and 34% after 24 and 48 h, respectively, both values are significant (ANOVA,  $p < 0.001$  and  $p < 0.01$ ). In 75% DWW the reduction was 48 and 11% after 24 and 48 h respectively; differences are significant only at 24 h (ANOVA,  $p < 0.01$ ).

## CONCLUSIONS

The engineered microbial consortium made by the filamentous cyanobacterium *T. variabilis*, selected to produce an oxygen evolving scaffold, and the three heterotrophic aerobic isolates from DWW, *Acinetobacter*, *Exiguobacterium* and *Pseudomonas* spp., proved to be able to thrive in raw DWW and reduce its nutrient load. In addition, the engineered microbial consortium self assembles in suspended aggregates and this is particularly promising for its application in DWW processing. This consortium was planned to be the functional core of a prototype, called Zero Mile System<sup>®</sup> (Costa et al. 2018), which integrates a dishwasher, a microbial biofilter (the one-to-three consortium) and a distribution system for the treated DWW that can be both reused in subsequent dishwashing cycles and upcycled in a vertical garden to produce vegetal food. Zero Mile is conceived to allow the simultaneous reduction of water use and DWW production coupled with the conversion of the organic load into food. It will support the DWW resource recovery and valorization as envisaged by the circular economy paradigm, intended as a restorative or regenerative system by intention and design (Ellen MacArthur Foundation 2013).

Zero Mile is dimensioned at individual household size for own plant irrigation, but the further aim is to scale it up to the dimension of a restaurant or a set of households sharing services, such as in a co-housing environment. The rationale behind both applications is that DWW reuse becomes more economically feasible if the point of reuse is close to the point of production.

## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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