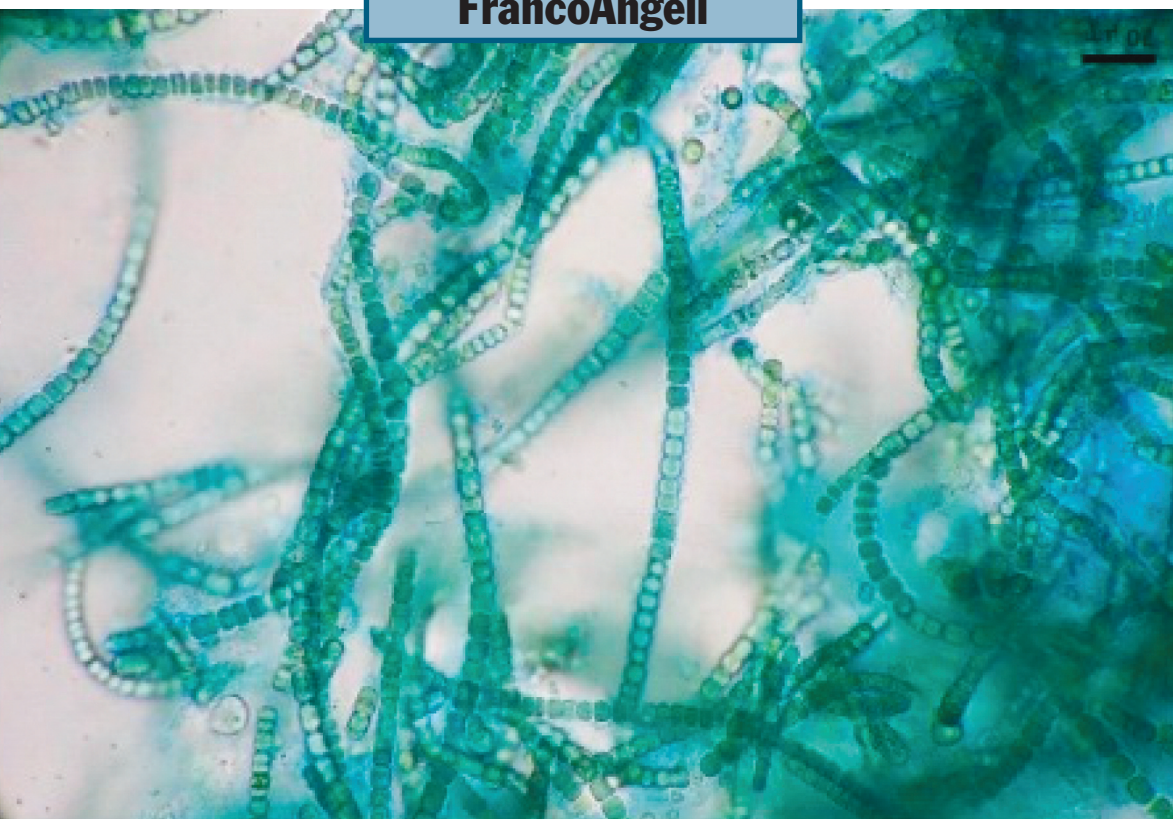
Fiammetta Costa, Attilio Nebuloni
(edited by)

THE JETSONS' KITCHEN

A ZERO-MILE SYSTEM FOR WASTE WATER RECYCLING AND CULTIVATION

FrancoAngeli



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The name Jetsons’ Kitchen comes from a quotation by Luciana Migliore.

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Appendix

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

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Abstract

Microbial consortia are effective biofilters to treat wastewaters, allowing for resource recovery and water remediation. To re-use and save water in the domestic cycle, we assembled a suspended biofilm, a 'biofilter' to treat dishwasher wastewater. Bacterial monocultures of both photo- and hetero-trophs were assembled in an increasingly complex fashion to test their nutrient stripping capacity. This 'biofilter' is the core of an integrated system devoted to re-using and upcycling of reconditioned wastewater, partly in subsequent dishwasher cycles and partly into a vertical garden for plant food cultivation.

The biofilter has been 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 has been 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 to the reuse and saving of water in household appliances.

Keywords: biofilter, cyanobacteria, dishwasher wastewater treatment, heterotrophic bacteria, microbial consortia, *Trichormus variabilis*

1. 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 (WW) treatment process. Conventional techniques rely on interconnected, bacteria-based complex and multistep 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 WW 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 partners, by means of organic matter degradation, release CO_2 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, pollutant and pathogen removal from WW.

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) are 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 practiced 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 lab-scale closed environment system. Consortium members were: (i) the filamentous cyanobacterium, *Trichormus variabilis*, and (ii) selected aerobic bacteria isolated from DWW. This approach allows to test a microbial association highly improbable in nature in a bioremediation challenge to ameliorate DWW.

2. Materials & Methods

2.1. 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, $BG11_0$) at 18 °C and 30 µmol photons m^{-2} s^{-1} irradiance, L:D cycle 12:12 (Di Pippo *et al.*, 2012; Bellini *et al.*, 2018).

Before the experiment, a sample of the stock culture has been acclimated for two weeks at 80 µmol photons m^{-2} s^{-1} 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 hrs.

2.2. Trichormus variabilis growth experiments

Aliquots of the exponentially growing culture used as inoculum were prepared for *T. variabilis* growth experiments by centrifuging 50 ml (2200 *g*, 10 min) and resuspending the pellet in $BG11_0$ to an optical density of 0.5 at 665 nm. Culture growth was tested in DWW as it 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. 3 replicates for each experiment were set and culture growth was measured as *in vivo* chlorophyll *a* absorbance and culture turbidity (OD, Optical Density, at 665 nm 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.

2.3. Identification of the dishwasher wastewater microbial community

The dishwasher wastewater has been collected 16 times from November 2017 to February 2018. From each dishwasher wastewater sample, 10 µl were plated on three solid media: TSA (Tryptic Soy Agar); PSA (Pseudomonas Agar Base); MCA (Mac Conkey 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 dH_2O, 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 PCR, using COM1 (forward 5'-CAGCAGCCGCGGTAATAC-3'; position 519-536) and COM2 (reverse 5'-CCGTCAATTCCTTTGAGTTT-3'; position 907-926), selective primers for the 16S rRNA gene, identifying the variable region V4 and V5 of ribosomal RNA. 10 µl of the PCR solution contain: 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.

2.4. 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 tablets 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 waste are also reported (see Table S2, Supplementary material).

2.5. 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 guaranteeing the best performance.

Each bacterial isolate from the dishwasher wastewater was seeded on a TSA (Tryptic Soy Agar) 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 CFU. The bacterial suspensions were gently vortexed and then centrifuged (6804 *g*, 10 min), the supernatant discarded, and the pellet resuspended in the same amount of $BG11_0$.

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 hrs, over 12 days (time to reach stationary phase) by recording the absorbance values at the wavelengths of 665 and 730 nm.

2.5.1. Co-culture one-to-one, one-to-two and one-to-three

The development of the engineered consortium, planned to be composed by *T. variabilis* and three bacterial isolates from the dishwasher wastewater, has been built in a step by step process of association. The co-cultivation of the cyanobacterium with each bacterial strain has been the first step of the challenge of *T. variabilis* with 16 bacterial isolates (*one-to-one* consortia). 5 ml bacterial

suspension was mixed with 5 ml of *T. variabilis* in $BG11_0$ medium up to a final volume of 30 ml. The growth performance of each *one-to-one* consortium was evaluated by measuring every 48 hrs the absorbance at 665 nm and 730 nm. The second step has been the challenge of *T. variabilis* with two of the selected isolates in *one-to-two* consortia, and the third step has been 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 were mixed with 4.17 ml of each bacterial suspension; while in *one-to-three* tests 8.33 ml of *T. variabilis* suspensions were 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 hrs the absorbance at 665 nm and 730 nm.

2.6. 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 % waste, diluted in $BG11_0$ medium), in order to evaluate viability and growth, both by spectrophotometric measurements, in triplicate, at the wavelengths of 665 nm and 730 nm, every 48 hrs and microscopy observation, using a ZEISS Axioskop light microscope at 400 and 1000x magnification.

2.6.1. 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 sample of dishwasher wastewater as it is (100%) or 75% diluted in $BG11_0$ after 24 - 48 hrs. The analyses were performed according to the Italian official protocol (APAT IRSA-CNR, 2003). 10 ml samples of 100 or 75 % wastewater were collected immediately before the start of the co-culture experiments and after 24 and 48 hrs treatment, in triplicate. The samples were centrifuged (3400 *g*, 10 min) and the supernatant transferred into new tubes. 2.8 ml of oxidizing solution (50 g $K_2S_2O_8$ - Merck n. 5092, 30 g H_3BO_3, 14 g NaOH in 1 l of deionized water) were 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 $NaNO_3$ in distilled water at 0-1-5-10 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_2, 3 ml formic acid in a final volume of 500 ml dH_2O) and 0.6 ml of reagent mixture (0.34 g $KOOC(CHOH)_2COOSb$ ½ H_2O, 8.1 g $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 100 ml H_2SO_4 concentrated, density 1.84, in a final volume of 500 ml dH_2O) were added to each sample, which were 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_2PO_4 in distilled water at levels of 0-0.25-0.50-1 µg/l P, subjected to oxidation as described.

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

3. Result and Discussion

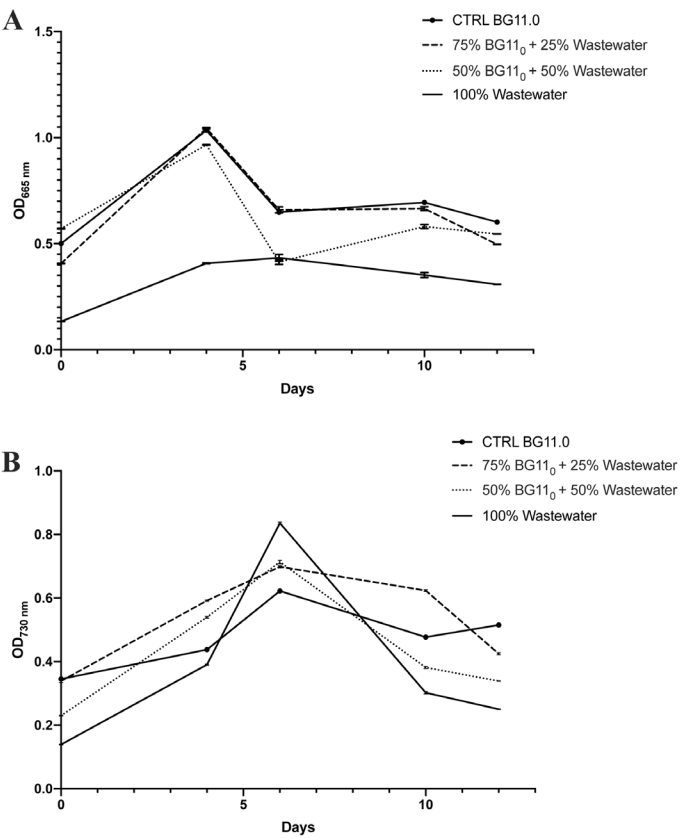
The synergistic relationship between photosynthetic and heterotrophic microorganisms is a key issue for remediation of wastewaters. In this study *Trichormus variabilis*, a promising oxygen evolving candidate for dishwasher wastewater (DWW) remediation did not survive in the DWW. Thus, we elaborated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

3.1. T. variabilis in DWW

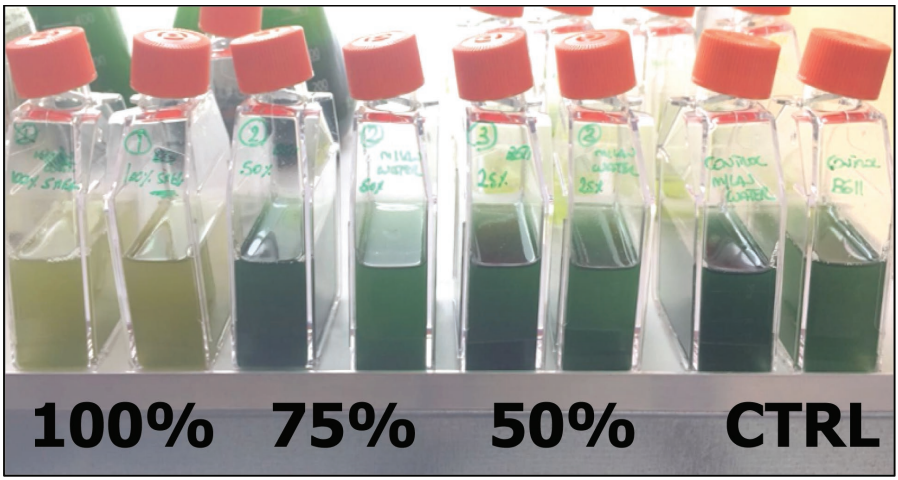
T. variabilis ability to survive and grow in dishwasher wastewater was evaluated recording the absorbance of *in vivo* chlorophyll *a* (OD at 665 nm) and culture turbidity (OD at 730 nm), over 12 days in 100, 75 and 50 % wastewater dilutions.

Chlorophyll *a* (Figure 1A) shows that 100% wastewater significantly reduces cyanobacterium growth (ANOVA, $p<0.05$), as confirmed by the loss of pigmentation of the culture (Figure 2). Conversely, *T. variabilis* is able to thrive in 50 and 75 % (ANOVA, $p>0.05$; Figure 1A), indicating its capability of growing under these DWW concentrations. On the other hand, culture turbidity (Figure 1B)

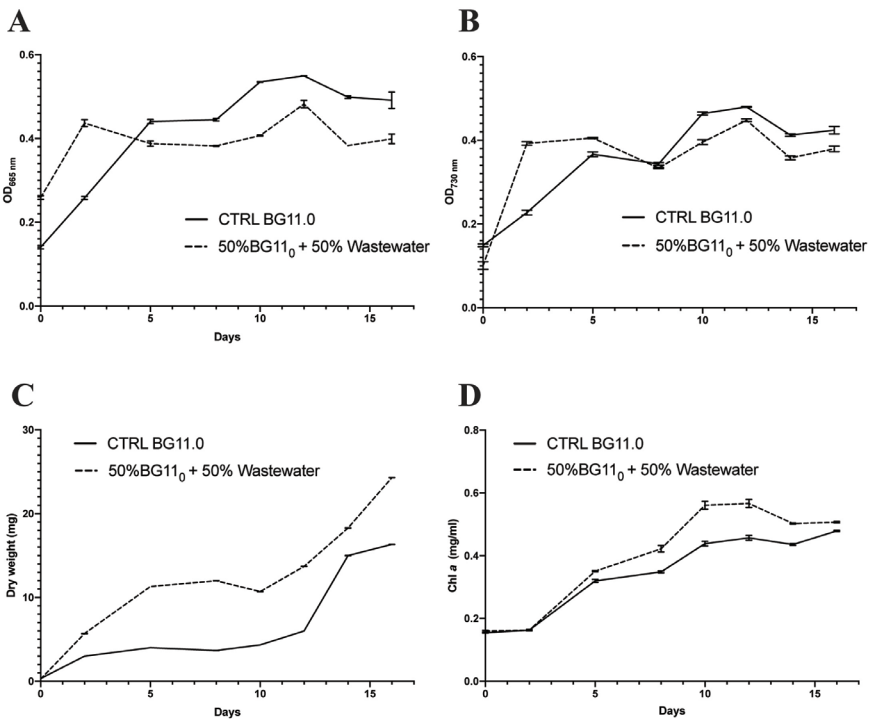
increases in all the cultures especially at 100% DWW, with a peak at day 6, probably due to heterotrophic bacteria. No lag phases occurred at all dilutions. Overall, these results suggest that DWW may contain growth-inhibiting compounds/conditions or may lack some essential components which affected *T. variabilis* growth. As a further test, 50% DWW was used for a growth experiment with air insufflation 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*-Sudent test, $p<0.05$). Nevertheless, dry weight, chlorophyll *a* and turbidity values are similar to the control ones (*t*-Student test, $p>0.05$) (Figure 3). This indicates that even a slight modification of DWW composition may allow *T. variabilis* growth.



[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.



[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.

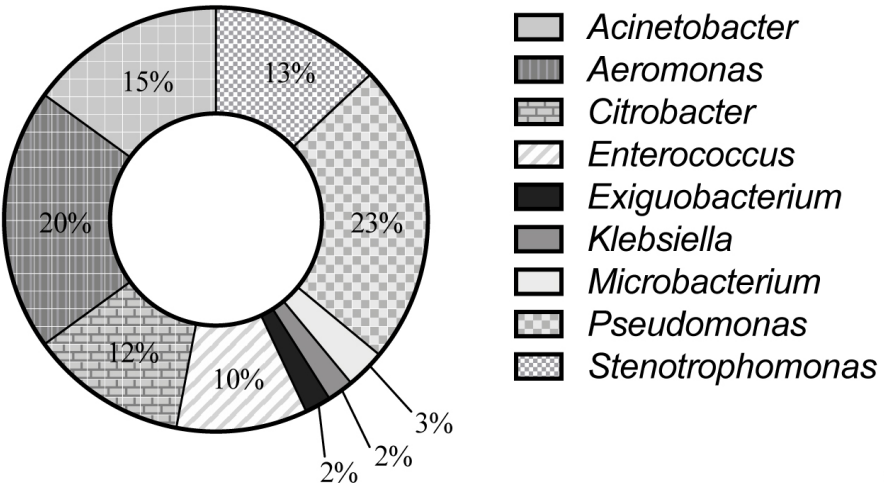
3.2. Isolation of the DWW microbial colonizers

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 10^3 cells/ml on MCA. From these microbial cultures 41 bacterial strains were isolated the basis of their different morphology and taxonomically identified by Sanger sequencing. The main colonizers were Proteobacteria of to the Gamma-Proteobacteria class (34 isolates), followed by Firmicutes of the class Bacilli (6) and Actinobacteria (1). The Proteobacteria are: Aeromonadales (8, all *Aereomonas* genus), Enterobacteriales (6, 5 *Citrobacter* and 1 *Klebsiella*), Pseudomonadales (15, 6 *Acinetobacter* and 9 *Pseudomonas*) and Xantomonadales (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 extracellular polymeric substances (EPS) conferring tolerance properties (Limoli *et al.*, 2015).

16 isolates were selected to be challenged in co-culture with *T. variabilis*, they are listed in bold in Table 1.

[Table 1] Taxonomical identification of the bacterial isolates. In bold those chosen for the co-culture experiments with T. variabilis

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



[Figure 4] Taxonomy and frequency of the DWW isolates.

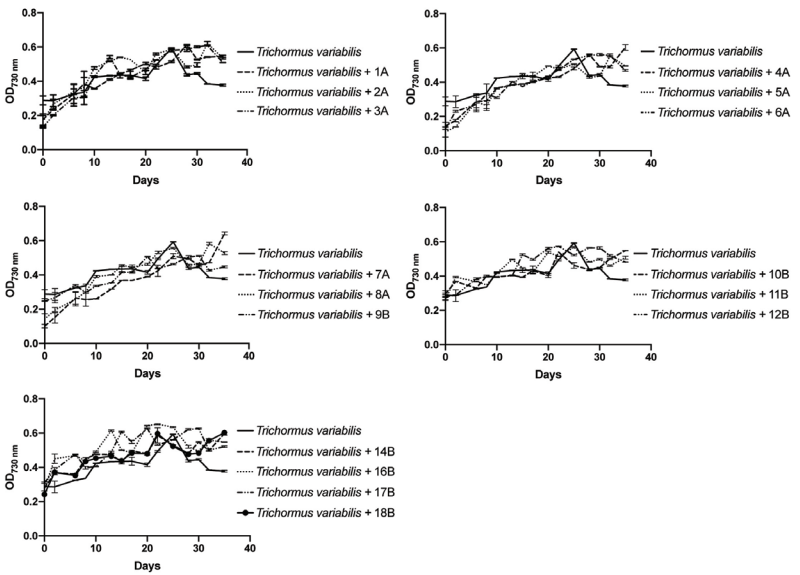
3.3. Co-culture experiments for the engineering of the microbial consortium

The development of the microbial consortium bases on the possibility to produce a functional consortium, including *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 has been applied, the first step is 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 $BG11_0$.

3.3.1. 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, 2 *Acinetobacter*, 5 *Aeromonas*, 2 *Citrobacter*, 2 *Enterococcus*, 1 *Exiguobacterium*, 1 *Klebsiella* and 3 *Pseudomonas* are used (Table 2, bold). Strains of these genera are often used for activated sludge-based WW treatment: *Pseudomonas* degrades carbon by oxidation, *Citrobacter* contributes to floc formation, *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 to establish pathogeny or risks for human health, never recorded for home appliances. The co-culture allowed to evaluate the best growth of *T. variabilis* 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, Supplemental 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.

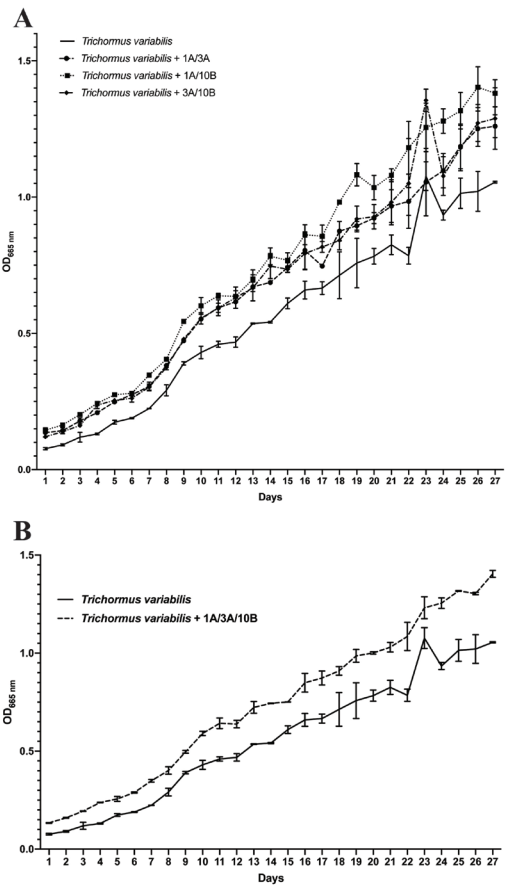


[Figure 5] One-to-one co-culture (n=3) of T. variabilis and one of the heterotrophic isolates in $BG11_0$. The absorbance values at 665 nm, corresponding to in vivo chlorophyll a signal, of each consortium are reported.

3.3.2. 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, *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.



[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* $BG11_0$*. A: Absorbance values of each one-to-two consortium. B: Absorbance values of the one-to-thee consortium (both at 665 nm, indicating in vivo chlorophyll a).*

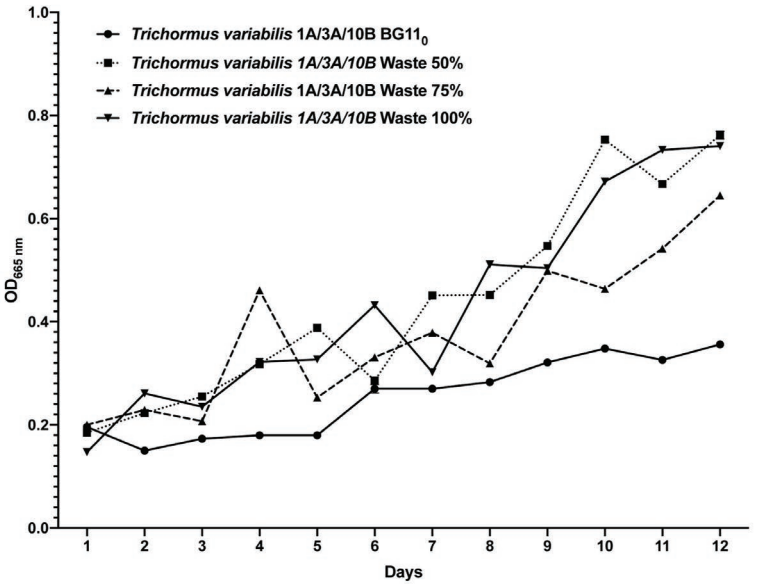
The *one-to-two* consortia grew better than *T. variabilis* alone (control; Figure 6), particularly the *T. variabilis* +1A/10B consortium.

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

3.4. One-to-three consortium in different DWW concentration

To demonstrate the *one-to-three* consortium efficacy it has been grown in DWW as it is (100%) or diluted (75 and 50%) in $BG11_0$ 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 7A). The result demonstrates the cooperative 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 7B shows a further important emergent property of the consortium, its three-dimensional organization as floating microbial aggregates (a sort of 'green sausages'), not adhering to flask surfaces. These 3D structures are reversible associations that upon strong flask manual shaking disassociate in a homogeneous green suspension, they quickly reconstitute (a couple of hours) when the flask is left to rest. Hence, the 3D structure this microbial consortium must be an 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 WW treatment plants is well known (Congestri *et al.*, 2006; Roeselers *et al.*, 2007; Congestri, 2008; Di Pippo *et al.*, 2014).

A



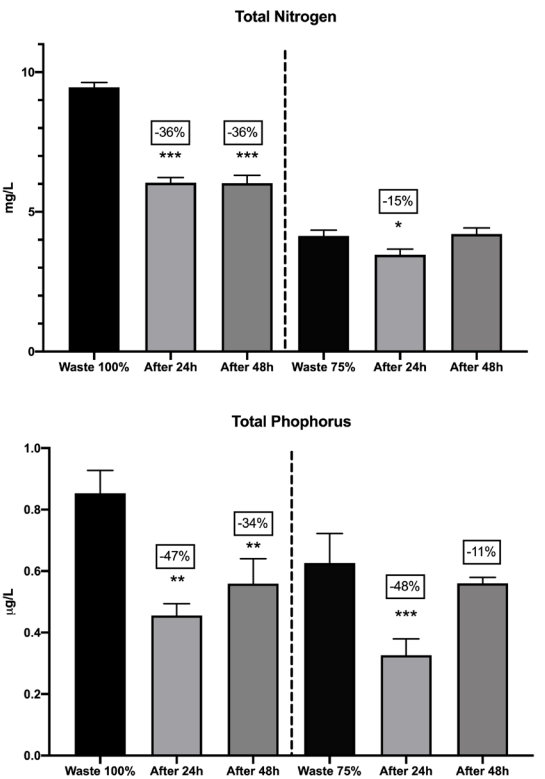
B

[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, 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).*

3.4.1. DWW nutrient removal by the one-to-three consortium

The efficiency of the microbial consortium in ameliorating DWW as it is, 100% or diluted at 75% in $BG11_0$, has been evaluated as removal of total nitrogen and phosphorus after 24 or 48 hrs (Figure 8). In DWW as it is nitrogen is reduced of 36 % after 24 and 48 hrs, this reduction is significant (ANOVA, $p<0.001$). Conversely, in 75% DWW the reduction was of 15 % after 24 hrs (ANOVA $p<0.05$), no nitrogen variation was found after 48 hrs.

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



[Figure 8] Nitrogen and phosphorus removal by the one-to-three microbial consortium in DWW (as it is, 100%, or diluted at 75% in $BG11_0$) after 24 and 48 hrs. Asterisks indicate significance levels (= p<0.05; **= p<0.01; ***= p<0.001). In brackets the percent removal.*

4. Conclusions

The engineered microbial consortium made by the filamentous cyanobacterium *Trichormus 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 has been planned to be the functional core of a prototype, called Zero Mile System® (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 valorisation 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 ratio behind both applications is that DWW reuse becomes more economically feasible if the point of reuse is close to the point of production.

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Supplementary materials

[Table S1] Safety Data Sheet of "Vivi Verde - Coop" dishwasher tablets: ingredient composition. The dishwasher detergent includes a proteolytic enzyme of bacterial origin. Subtilisin is a biodegradable protein that shows good solubility and poor stability in water. This enzyme reaches the maximum activity in alkaline conditions (optimal pH range: 9-11) and is inactivated during the wash cycle (in the range 55-65 °C; HERA, 2007, Human & Environmental Risk Assessment on ingredients of household cleaning products. Subtilisins (Protease). Available from: https://www.heraproject.com/files/22-F-07_PROTEASE_HERA_Final%20Edition%20(unsecured%20-%20PDFA-1b).pdf.).

SCHEDA DATA DI SICUREZZA (REGOLAMENTO (CE) N° 1907/2006 - REACH) **Data: 13/07/2016 Page 2/7**
Versione: N° 1 (18/05/2015) **Revisione: n° 6 (18/05/2015)**
McBride S.p.A. (Bagnatica)
Coop Pastiglie lavastoviglie ecolabel - 16129731 - 3001883

P305 + P351 + P338	IN CASO DI CONTATTO CON GLI OCCHI: sciacquare accuratamente per parecchi minuti. Togliere le eventuali lenti a contatto se è agevole farlo. Continuare a sciacquare.
P332 + P313	In caso di irritazione della pelle: consultare un medico.
P337 + P313	Se l'irritazione degli occhi persiste, concultare un medico.

2.3. Altri pericoli

La miscela non contiene alcune delle "Sostanze estremamente preoccupanti" (SVHC) >= 0,1% pubblicate dall'Agenzia Europea per le Sostanze Chimiche (ECHA) ai sensi dell'articolo 57 del REACH: http://echa.europa.eu/fr/candidate-list-table
La miscela non risponde ai criteri applicabili alle miscele PBT e vPvB, ai sensi dell'allegato XIII del regolamento REACH (CE) n. 1907/2006.

SEZIONE 3: COMPOSIZIONE /INFORMAZIONI SUGLI INGREDIENTI

3.2. Miscele

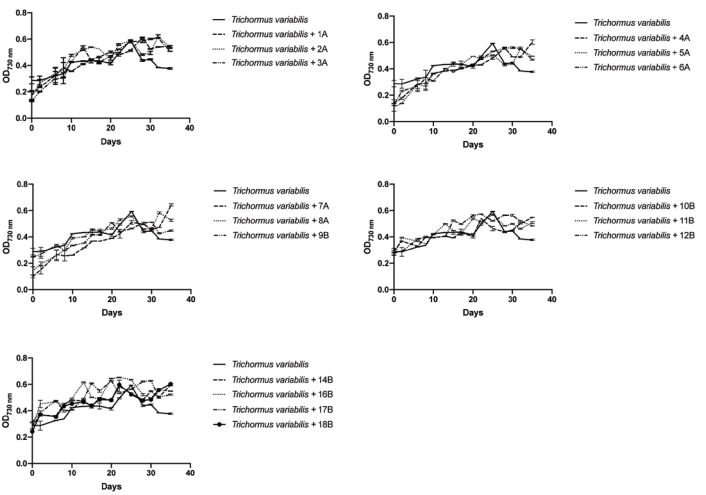
Composizione:

Identificazione	(CE) 1272/2008	67/548/CEE	Nota	%
INDEX: 011-005-00-2 CAS: 497-19-8 EC: 207-838-8 REACH: 01-2119485498-19- SODIO CARBONATO	GHS07 Wng Eye Irrit. 2, H319	Xi Xi;R36		25<=x%<50
INDEX: 1001124 CAS: 15630-89-4 EC: 239-707-6 REACH: 01-2119457268-30 DISODIUM CARBONATE, COMPOUND WITH HYDROGEN PEROXIDE (2:3) (SODIUM CARBONATE PEROXIDE)	GHS07, GHS05, GHS03 Dgr Ox. Sol. 3, H272 Acute Tox. 4, H302 Eye Dam. 1, H318	Xn,O Xn;R22 Xi;R41 O;R8		10<=x%<25
INDEX: 1002122 CAS: 1344-09-8 EC: 215-687-4 REACH: 01-2119448725-31 SILICIC ACID, SODIUM SALT (2.6 MR 3.2)	GHS07 Wng Skin Irrit. 2, H315 Eye Irrit. 2, H319 STOT SE 3, H335	Xi Xi;R36/37/38		10<=x%<25
INDEX: I77_92_9 CAS: 77-92-9 EC: 201-069-1 REACH: 01-2119457026-42- CITRIC ACID	GHS07 Wng Eye Irrit. 2, H319	Xi Xi;R36		2.5<=x%<10
INDEX: 647-012-00-8 CAS: 9014-01-1 EC: 232-752-2 REACH: 01-2119480434-38- SUBTILISINA	GHS08, GHS05, GHS07 Dgr STOT SE 3, H335 Skin Irrit. 2, H315 Eye Dam. 1, H318 Resp. Sens. 1, H334	Xn Xn;R42 Xi;R37/38-R41		0<=x%<2.5

*[Table S2] Main physico-chemical parameters of wastewater (WW) and control water, CW, samples (analyses on 3 l of WW and CW, the tap water used for the washing cycles). Standard Italian and European methods were used. Legend: WW1/WW2 = wastewater samples; CW = control (drinking) water sample. *Data from: https://www.milanoblu.com/la-tua-acqua/lacqua-di-milano/ access date: September 2019*

Parameter	Unit	CW*	WW1	WW2	Analytical method
pH	-	6,9	9.3	9.6	APAT-IRSA 2060, 2003
Conductivity	µS/cm 20 °C	585	6600	3300	APAT-IRSA 2030, 2003
Potassium	mg/l	2	4.3	7.9	UNI EN ISO 17294-1 2007 e 17294-2 2005
Sodium	mg/l	34	1608	901	UNI EN ISO 17294-1 2007 e 17294-2 2005
Calcium	mg/l	109	140	59	UNI EN ISO 17294-1 2007 e 17294-2 2005
Magnesium	mg/l	18	33	13	UNI EN ISO 17294-1 2007 e 17294-2 2005
Iron	mg/l	0.012	0.054	0.094	UNI EN ISO 17294-1 2007 e 17294-2 2005
Manganese	µg/l	2,1	6,1	14	UNI EN ISO 17294-1 2007 e 17294-2 2005
Alkalinity	mg $CaCO_3$/l	235	613	941	SM 2320B, 2012
Nitrate	mg/l	31	29	9,8	APAT IRSA CNR 4020 Man 29/2003
Sulphate	mg/l	61	72	79	APAT IRSA CNR 4020 Man 29/2003
Phosphate	mg P/l		< 0.05	< 0.05	MU 201, 2006
Total phosphorus	mg P/l		0.255	1.6	MU 201, 2006
Total COD	mg O_2/l		730	2600	MU 201, 2006
Soluble COD	mg O_2/l		665	1550	MU 201, 2006
Total Nitrogen	mg N/l		5.8	17	MU 201, 2006
Anionic Surfactants	mg MBAS/l		0.99	5.4	MU 201, 2006
Non-Anionic Surfactants	mg TAS/l		98	173	MU 201, 2006
BOD_5	mg O_2/l		210	1500	DIIAR/POP. 99. 421 Agg.1
Total Suspended Solids (TSS)	mg/l		72	700	APAT IRSA 2090B, 2003

[Figure S1] One-to-one co-culture in $BG11_0$ of T. variabilis *and one of the heterotrophic bacteria isolated by dishwasher wastewater. The absorbance values of each consortium are reported (in spectrophotometry at 730 nm, corresponding to the turbidity of each culture)*



The book aims to investigate the up-cycling of domestic effluents for plant production, bringing together a series of considerations by an interdisciplinary group of researchers from the Politecnico di Milano, Università Statale di Milano and Università di Roma Tor Vergata, ranging from biology to design through sociology and architectural composition.

Integrating vegetable cultivation in the domestic environment with reusing kitchen wastewater for irrigation is a promising strategy for reducing freshwater consumption, limiting the amount of wastewater to be treated producing healthy plant food and, ultimately, raising environmental awareness among citizens. A first step in this direction is the experimental project to reuse dishwasher effluents in living spaces (kitchen, household, and community level), as described in the book. Dishwasher effluents were chosen as an initial bench test because of their high nutrient content, low harmful elements and constant wastewater quantity and quality, where treatment may consist of a combination of several chemical, physical and biological processes. Studies for the development of a domestic biofilter containing a consortium of microalgae and heterotrophic bacteria are also presented.

Fiammetta Costa, PhD in Industrial Design and researcher at the Design Department of Politecnico di Milano. Principal areas of her research interests are user research methods and environmental design. She has been teaching Ergonomics and Industrial design since 2000 at the School of Design of Politecnico di Milano.

Attilio Nebuloni, PhD, is assistant professor in Architectural and Urban Composition at the Design Department of Politecnico di Milano. His research interests are sustainability and computational design. He teaches architectural composition at the School of Architecture, Urban Planning and Construction Engineering.

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