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Home Sweet Home: Setting the Best Thriving Conditions for the Ad Hoc Engineered Microbial Consortium in the Zero Mile System

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Abstract: Wastewaters from household appliances, such as dishwashers and washing machines, are an untapped resource of recoverable water and/or nutrients. The Zero Mile system has been developed to reuse/upcycle dishwasher wastewaters through bioremediation activity carried out by an ad hoc engineered phototrophic/heterotrophic microbial consortium. The choice of both suitable microorganisms for engineering consortia and detailed knowledge on their structure, behaviour and interaction are essential to optimising consortium culture conditions and drive the biofilter container design (structure and topology). To these aims, the effect of abiotic conditions (i.e., irradiance, pH and organic load) on the microbial consortium growth and its capability to survive and thrive in different dishwasher wastewater dilutions have been evaluated. At the same time, the crucial interplay between biological and design research has allowed us to define the characteristics of the biofilter container and plan its development for the industrial application of the Zero Mile system, bringing sustainability benefits as it moves household wastewater from a traditional linear model to a more sustainable, circular approach.

Keywords: autotrophic and heterotrophic biofilter; consortium taxonomic composition; consortium culture condition; wastewater upcycling; research through design

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Citation: Alabiso, A.; Frasca, S.; Bartolini, M.; Congestri, R.; D'Andrea, M.M.; Buratti, G.; Costa, F.; Meraviglia, M.; Nebuloni, A.; Migliore, L. Home Sweet Home: Setting the Best Thriving Conditions for the Ad Hoc Engineered Microbial Consortium in the Zero Mile System. *Sustainability* **2024**, *16*, 2227. https://doi.org/10.3390/su16062227

Academic Editors: Marc A. Rosen, Eun-Sung Chung and Charles Chang-Yu Hong

Received: 2 December 2023
Revised: 29 February 2024
Accepted: 5 March 2024
Published: 7 March 2024

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1. Introduction

The water demand problem and the consumption of water resources are crucial environmental issues. According to the 2030 Agenda targets of the Sustainable Development Goals #6 (Clean water and Sanitation [1]), wastewater management aimed at reuse, recycling and resource recovery is a critical requirement [2,3].

The consumption of water resources in household appliances is an increasing environmental problem, and, at the same time, the wastewaters from household appliances, such as dishwashers and washing machines, are rarely taken into account as a recoverable resource of water and/or nutrients. Although dishwasher wastewater (DWW), according to the 2030 Agenda definition [4], is classified as greywater, it represents an underestimated resource, generally flowing into blackwater. Conversely, kitchen wastewater reuse has been historically practised for irrigating vegetables [5], as it is enriched with nutrients from food leftovers. Furthermore, the advantage of DWW reuse is bolstered by its minimal levels of pathogens and contaminant content, including chemicals. Hence, a proper treatment can turn DWW into water of quite good quality [6–8].

From an environmentally friendly perspective, in recent decades, the biological approach has been extensively investigated and widely applied to wastewater treatments, such as those coming from domestic, industrial, agricultural and zootechnical sites [9–11].

The biological filtration and bioremediation strategies for water reclamation are increasingly based on microbial consortia, i.e., microbial communities formed by photosynthetic (cyanobacteria or microalgae) and heterotrophic (bacteria) microorganisms, living in a synergistic relationship. The combination of microorganisms with different metabolic activities, adapted to different environmental conditions, allows the formation of a resilient biological complex capable of functioning under a range of conditions with diverse pollutants and nutritional loads. Furthermore, the use of microbial consortia reduces oxygenation demand and costs along with carbon dioxide emissions [12,13].

The Jetsons' Kitchen project aims to reuse and upcycle household greywater, starting with DWW. To this end, the Zero Mile system, integrating dishwasher wastewater treatment and plant growing, has been conceived to reuse DWWs both in the following dishwasher cycles and by cultivating edible and ornamental plants ([7,14], Figure 1). The foundation of the Zero Mile system is a biofilter based on a microbial consortium expediently engineered, consisting of selected, different microorganisms able to process and mineralise the food leftovers. Specifically, this consortium is made up of different microbial partners: a photosynthetic, filamentous and nitrogen-fixing cyanobacterium and three heterotrophic aerobic bacterial strains, isolated from the DWW [8]. This consortium, challenged with different DWWs obtained utilising bio-detergents, demonstrated its bioremediation capability by significantly reducing N and P concentrations [8,14].

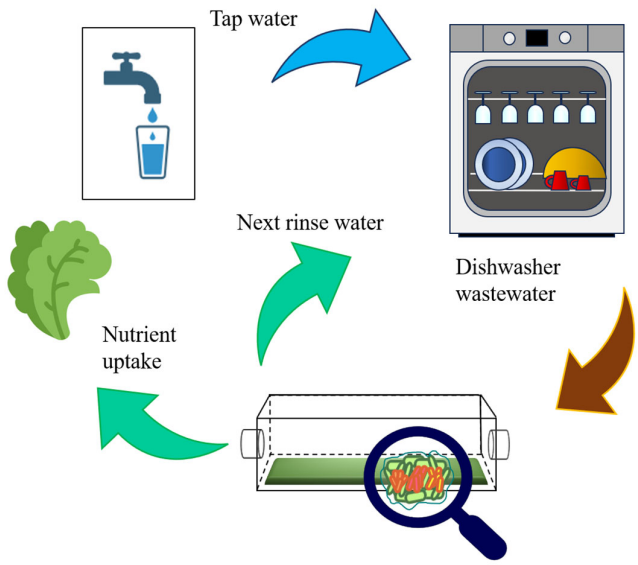


Figure 1. The main phases of water recovery in the Zero Mile system. Arrow in blue means the input of tap water, arrow in brown indicates the output of untreated dishwasher wastewater and the arrows in green indicate the reuse of bioremediated wastewater.

To gain an efficient wastewater treatment, both the selection of suitable candidates for engineering consortia and detailed knowledge on their structure, behaviour and interaction are essential [15]. At the same time, some parameters, such as light, temperature, pH, nutrient supply and mixing, need to be carefully monitored to guarantee successful microbial growth and remediation activity. It is well known that microbial growth can be influenced by several factors, both abiotic (light, temperature, pH, salinity, nutrient availability, dissolved-oxygen concentration and toxic compounds) and biotic (presence of pathogens and competition with other microorganisms) [9]; nowadays, the study and evaluation of their effects on the survival and efficiency of the consortia is a mandatory issue [16].

It is also important to parallel the study on the microbial consortium structure and function in view of its bioremediation potential with design research that involves demonstrators and prototypes, to support the experiments throughout the whole project. Specifically, we firstly used a demonstrator to identify technical and spatial relationships between the system's components and between the components and the context. This demonstrator, substantially characterised by the assembly of parts unrelated to each other, was aimed at evaluating the system's capability of reusing 3.6 L of treated DWW to irrigate 80 lettuce plants on a 3 sqm vertical surface, mimicking the average operative condition in the kitchen of an Italian family [7]. This first demonstrator was followed by the development of a more integrated prototype specifically designed for laboratory experiments with the possibility of being moved in different contexts [14]. Among the possible integrations of the different components of the system, that between the dishwasher and the biofilter was initially set as a priority in the design framework, significantly constraining and addressing Zero Mile as an integrated system. In contrast, a greater flexibility was allowed to the vertical garden consisting of hanging pots.

The interplay between biological and design research is of paramount importance for the industrial application of the Zero Mile system. On one hand, achieving a better understanding of the consortium physiological characteristics and optimising its culture conditions is needed, since the design of the biofilter container (structure and topology) is strictly linked to the microbial consortium characteristics and its growth demands. On the other hand, the research through a design approach involving the development of demonstrators and prototypes [17] is useful to support the microbial consortium experiments and to prefigurate application scenarios, ranging from kitchen furniture to collective wastewater upcycling and gardening solutions.

Thus, the present study aims to (i) assess the effect of abiotic conditions (i.e., irradiance, pH and organic load) on the microbial consortium growth, (ii) evaluate its capability to survive and thrive in different DWW dilutions in batch operational conditions, (iii) define the characteristics of the biofilter container and plan its development and (iv) update the implementation requirements previously defined [7] to drive the further development of the Jetsons' Kitchen project.

2. Materials and Methods

2.1. Trichormus variabilis

Since a photosynthetic partner is lacking in DWW, the strain of the heterocytous cyanobacterium *Trichormus variabilis* (Kützing ex Bornet and Flahault) Komárek and Anagnostidis, labelled as strain VRUC 168 in the Tor Vergata Culture Collection, isolated from sediment biofilms of a Mediterranean coastal lagoon (Cabras lagoon, Sardinia, Italy), was used to integrate the microbial consortium. The experimental inoculum was produced according to Congestri et al. [8]. A sample of the stock monoalgal non-axenic culture in $BG11_0$ (Blue-Green Medium—nitrogen-depleted) was acclimated for 2 weeks at 80 µmol photons m^{-1} s^{-1} irradiance (artificial light, OSRAM L30W/965 BIOLUX white lamp, OSRAM Licht AG, Munich, Germany) and 25 °C, and the exponential growth phase (log phase) was maintained by adding fresh culture medium every 48 h.

2.2. Assembling the Microbial Consortium

To test the assembling of the microbial consortium ad hoc engineered by Congestri et al. [8], *T. variabilis* and the same three heterotrophic isolates (*Aeromonas* sp., *Acinetobacter* sp. and *Exiguobacterium* sp.) were assembled in $BG11_0$ or in DWW, according to the authors' methodology.

The three bacterial strains were seeded separately on different TSA plates; from each plate, a single bacterial colony was transferred into a 1 L sterile conical flask containing 500 mL of TSB (Tryptic Soy Broth) to obtain three separated pure cultures. The flasks were incubated under magnetic stirring for 24 h at room temperature. Then, the optical density (OD) was measured at λ = 600 nm until the value of 0.15 was reached. Each bacte-

rial suspension was gently vortexed and centrifuged (6804× *g*; 10 min), the supernatant discarded and the pellet pooled and resuspended in $BG11_0$ or DWW, depending on the experimental set up. Also, the *T. variabilis* suspension was prepared and maintained until the OD at λ = 665 nm reached the value of 0.15. The inoculum was prepared in the same way as the bacterial one and then used in the co-culture experiments. The consortium was assembled by combining in a one-to-one ratio *T. variabilis* and the three heterotrophic bacteria, in $BG11_0$ or DWW. In vivo chlorophyll *a* absorbance and culture turbidity (OD at 665 and 730 nm) were used to measure the consortium growth with an ONDA UV-20 spectrophotometer.

2.3. Operational Conditions of the Consortium: Effect of Light, pH and Organic Load

The effects of light, pH and organic load on the consortium growth and viability have been analysed in small-scale experiments (50–100 mL). The tests were conducted in three replicates. In all the experiments, the consortium was grown at 25 °C under a light–dark (L:D) cycle of 12:12 h.

To evaluate the effect of light, the consortium was exposed to two light intensities: 60 and 130 μmol photons m^{-2} s^{-1} (artificial light, OSRAM L30W/965 BIOLUX white lamp). These irradiances are considered standard operating conditions for the growth of the selected cyanobacterium. The microbial consortium was assembled in $BG11_0$ (50 mL), and the test was conducted over a period of 21 days.

To evaluate the effect of pH on newly assembled or mature (30-day-old) consortia, two sets of tests were performed at three pH values, 4, 7.5 and 10: (i) consortium assembled just at the start of the experiment in $BG11_0$ (50 mL) and (ii) consortium assembled in $BG11_0$ (neutral pH) 30 days before the start of the experiment and then resuspended for the test at different pHs. Both tests lasted 21 days.

In both light irradiance and pH tests, the consortium growth was evaluated by measuring (i) the surface area of the consortium 3D structure by the ImageJ version 1.52a software (as pixel density) weekly, from the beginning of the experiment (after 6 h from the microbial assemblage), and (ii) the biomass of the consortium at the end of the experiment, as dry weight (obtained by maintaining filtered samples for 48 h at 37 °C).

To evaluate the capability to face organic load, the consortium was exposed to an easily available source of nutrients for heterotrophic bacteria, the TSB powder (TSB, a medium of known composition), at three concentrations (0.09, 0.27 and 0.54 g/L) in $BG11_0$ (100 mL). The tests lasted 30 days. In vivo chlorophyll *a* absorbance and culture turbidity were utilised as a proxy for the consortium growth.

2.4. Dishwasher Wastewater

For these experiments, a household Electrolux dishwasher (Energy Class A+++, Electrolux 859 EES69300L, Stockholm, Sweden) was used, selecting the 'eco' programme as the washing cycle, with an EU Ecolabel-certified dishwasher detergent (Everdrop tablets, Munich, Germany). The physical–chemical profiling of the input water (Rome tap water) as well as the wastewater has been previously analysed and the different experimental settings reported by Alabiso et al. [14].

2.5. Cultivation-Based Approach: Isolation of Dishwasher Wastewater Bacteria on Solid Media and Identification by Sanger Sequencing

The DWW was collected in September 2021. Soon after collection for each wastewater sample, (i) the microbial load was quantified by plating 10 μL of wastewater on two solid media, TSA (Tryptic Soy Agar) and PSA (Pseudomonas Agar Base), and enumerating the resulting colony-forming units (CFUs) after 24 h of incubation at 37 °C; (ii) the taxonomic composition of the cultivable bacteria was obtained by plating 10 μL of wastewater on the previously mentioned TSA and PSA. One colony for each observed morphology was picked up, isolated on TSA (24 h at 30 °C), checked for purity and stored at −70 °C in glycerol 20%. A loopful of each pure culture was suspended in 500 μL of sterile distilled water,

gently vortexed and heated at 100 °C for 5 min, then centrifuged (10,000× *g*; 5 min), and the supernatant containing the bacterial DNA was recovered for Sanger sequencing. Bacterial DNA was amplified by PCR, using EubB2 = 27F (f5′-GAGAGTTTGATYMTGGCTCAG-3′, position 2811–2832) and EubA2 = 1541R (5′-GAAGGAGGTGWTCCARCCGCA-3′, position 1287–1307) universal primers for the 16S rRNA gene. For the PCR procedure, 50 µL of the PCR solution was used (25 µL of Emerald Amp GT PCR Master Mix 2X, 16 µL of nuclease-free water, 3.5 µL of forward primer EubB2 = 27F, 3.5 µL (10 pmol/µL) of reverse primer EubA2 = 1541R (10 pmol/µL) and 2 µL of the above-prepared bacterial DNA, ~2 ng/µL). Five microlitres of amplified DNA were detected on 0.8% agarose gel in TAE 1X buffer stained with ethidium bromide. Amplified DNA samples were purified by the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), following the manufacturer's instructions. DNA samples were then sequenced at an external facility (Eurofins Genomics, Rome, Italy) by using amplification primers; the results were analysed using RDP Classifier and BLAST. After the microbial analyses, wastewater samples were stored at −20 °C in the dark.

Furthermore, swab samples from dirty and washed vessels were obtained by rubbing a cotton swab over the same tableware surface before and after the washing cycle in the dishwasher. The swabs were immediately sealed in sterile tubes, stored at 4 °C and seeded within 8 h on TSA Petri dishes. Colony-forming units were finally enumerated after 24 h of incubation at 30 °C.

2.6. DNA Metabarcoding Approach: Taxonomic Composition of Dishwasher Wastewater Colonisers and Consortium Components

DNA metabarcoding (by Next Generation Sequencing) allowed us to taxonomically identify the microbial colonisers of three dishwasher wastewater samples, collected at different times (8, 16 and 28 October 2021). The Power Soil® DNA isolation kit (Mo Bio, Carlsbad, CA, USA) was used to extract metagenomic DNA from the pellet after centrifuging 0.5 mL of each sample (in triplicate). The primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) were used to amplify the 16S rDNA gene, encompassing the V3–V4 region. These primers allowed us to taxonomically identify bacteria and archaea [18]. Amplicon sequencing was carried out by BMR Genomics (Padua, Italy) using an Illumina MiSeq platform. Quantitative Insights Into Bacterial Ecology software (QIIME 2.10) was used to analyse the raw paired-end sequences obtained [19]. Initially, the sequences were demultiplexed and then processed by the DADA2 plugin (quality filtering, chimaera checking and pair matching) [20]. Taxonomic identification of the 16S rRNA gene sequences was performed as in Alabiso et al. [14,21].

The Targeted Locus Study project's raw data has been uploaded to the NCBI Sequence Read Archive (SRA), under the BioProject number PRJNA942250.

2.7. Testing the Consortium with Three Dilutions of Dishwasher Wastewater

To evaluate the consortium survival and growth, a set of static experiments (batch tests) were performed by exposing the consortium to wastewater diluted with 50%, 25% and 0% $BG11_0$ medium to obtain 50–75–100% DWW. All experiments were performed in triplicate.

The consortium was assembled as already reported (Section 2.2), in 100 mL of the three DWW dilutions (50–75–100%) and $BG11_0$ as a control, and maintained at 25 °C, under an L:D cycle of 12:12 h (130 µmol photons m^{-2} s^{-1}) for 42 days.

The consortium's growth was assessed by measuring in vivo chlorophyll *a* absorbance and culture turbidity, as reported in Section 2.2.

2.8. Statistical Analysis

In the lighting tests, Student's *t* test was utilised to compare consortium biomass and surface area data. One-way ANOVA and post hoc Tukey's have been used to compare biomass and surface area data in pH and organic load tests.

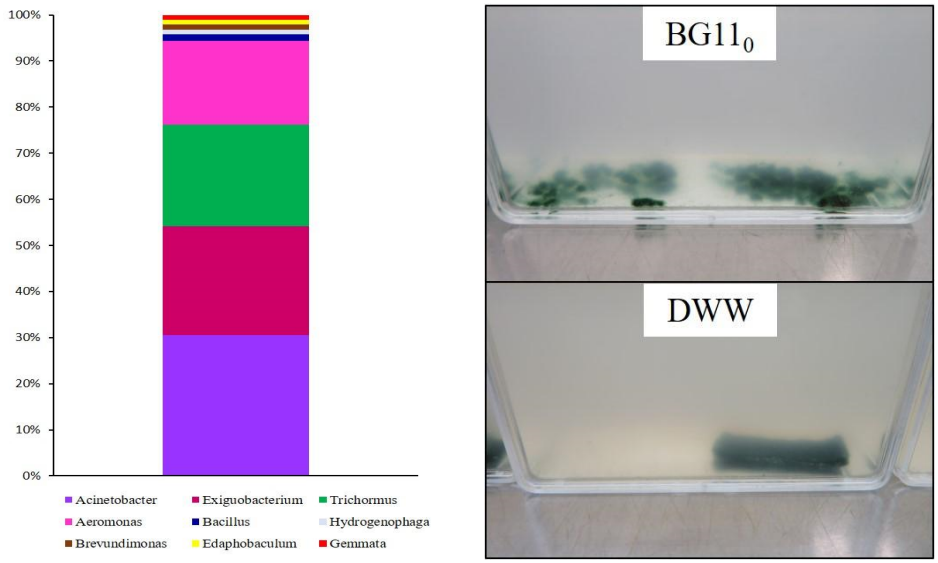
In the batch tests, the Shannon index was calculated and analysed through ANOVA one-way post hoc Tukey's test, to obtain the alpha diversity of dishwasher wastewater colonisers; two-way ANOVA and Sidak's multiple comparisons test were used to compare consortium growth in DWW and $BG11_0$.

These analyses were carried out by using GraphPad Prism 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA) and PAST version 4.06b software (Øyvind Hammer, Oslo, Norway).

3. Results and Discussion

3.1. Consortium Assemblage

Consortia were assembled by combining the cyanobacterium *T. variabilis* and three heterotrophic bacterial strains, isolated in 2019 from a DWW (from Milan Polytechnic dishwasher), as described by Congestri et al. [8]. As the cyanobacterium *T. variabilis* is not grown as an axenic culture, the taxonomic composition of the microbial consortium in $BG11_0$ has been evaluated by 16S rDNA metabarcoding before the tests. A total of 6102 sequences, clustered in 10 OTUs assigned to the consortium, were found (Figure 2a); a large part of sequences belonged to the four bacteria included for the consortium (*T. variabilis*, *Aeromonas* sp., *Acinetobacter* sp. and *Exiguobacterium* sp.), found almost at the same percentage as it is assembled; the other bacterial strains (about 6%) are the microbes associated with *T. variabilis*.



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

Figure 2. (**a**) Distribution of the bacterial genera (as OTU frequency) in the microbial consortium just assembled in $BG11_0$; (**b**) 3D structured consortium in $BG11_0$ and dishwasher wastewater (DWW; the DWW picture is from Alabiso et al. [14]).

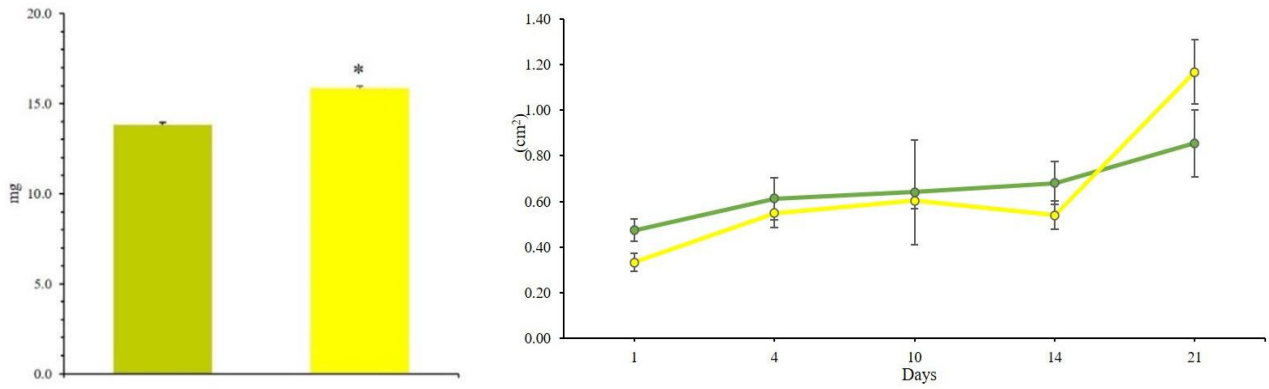
Important for the Zero Mile system is the peculiar characteristic of this consortium to form a three-dimensional structure, visible to the naked eye, that can be disaggregated by manual shaking, as is reported by Alabiso et al. [14]. Although using a different wastewater (Rome, household dishwasher), as already observed by using the Milan Polytechnic dishwasher, within a short time after bacterial assembly (6–12 h), the consortium aggregates in a reversible 3D structure that formed in both $BG11_0$ and DWW. In the wastewater, it appears more tightly aggregated than that in $BG11_0$ (Figure 2b) and requires stronger shaking to be disaggregated. This tight floating aggregation of the consortium makes its use easier in the Zero Mile system, as it does not flocculate or stick to the surfaces, hampering the system piping.

3.2. Setting the Operational Conditions of the Zero Mile for Consortium Thriving

Microbial consortium growth is known to be influenced by several abiotic factors including light (quality and quantity), temperature, pH, salinity, nutrient availability, dissolved-oxygen concentration and the presence of toxic compounds [16,22,23]. The tests here reported aimed to better understand the eco-physiological characteristics of the consortium, focusing on the effects of irradiance, pH and organic load, the most important parameters to be set with the aim of optimising the culture conditions to promote consortium growth. Although the importance of these conditions for industrially used strains/consortia is well understood, quantitative relationships between growth rate and environmental factors such as illumination intensity, temperature and pH are rarely reported [22].

The most important consortium feature for the Zero Mile biofilter is the maintenance of aerobic conditions in the growth medium; to this end, one partner of the consortium is the photosynthetic cyanobacterium *T. variabilis*, entrusted with oxygen production. Hence, the first parameter to be set is the lighting regime, which allows the consortium to thrive. Therefore, the effects of two light intensities (white artificial light) have been analysed. The irradiance values chosen were 60 and 130 µmol of photons m^{-2} s^{-1}, with a temperature of 25 °C. These values allow the consortium to thrive well, since *T. variabilis* exhibits photosynthetic activity in a broad light intensity (42–562 µmol of photons m^{-2} s^{-1}) and temperature range (10–35 °C; [24]).

As expected, both the biomass at the end of the experiment (Figure 3a) and the surface of the consortia during the three weeks of the experiment (Figure 3b) showed a significant increase (Student *t*, $p < 0.05$) under the highest light intensity, evident since the second week of the experiment. Indeed, it is well known that photoautotrophic growth is driven by light supply, as this is the energy source that is used to convert CO_2 into organic carbon [23]. However, the increasing biomass and the consequent increased thickness seemed not to affect light penetration and cyanobacterial activity in the consortium deep layers, even if thickness could determine a light gradient (quality and quantity) and a consequent spatial organisation of different species as a response to different photosynthetic activity [25].



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

Figure 3. (**a**) Consortium biomass after 21 days under irradiance of 60 (green) and 130 µmol photons m^{-2} s^{-1} (yellow); asterisk indicates the significance ($p < 0.05$) of the Student's *t* test; (**b**) consortium surface growth in 21 days test under light intensity of 60 (green) and 130 (yellow) µmol of photons m^{-2} s^{-1}.

In terms of the Zero Mile system, this experiment answers a crucial point in the design of the biofilter container: it will be necessary to design it giving the highest importance to light (as irradiance, light–dark cycle and distance from the consortium).

Many cellular processes rely on pH, including energy metabolism, organelle structure and function, enzymes and proteins [26]. Since it is well known that different pH values in the culture medium may determine conformational changes of enzymes, potentially altering their activity, then pH value in the media may affect the biochemical performance [22]. In the case of our consortium, the easy and fast reduction in organic load under basic pH is another pivotal point, as the pH of dishwasher detergents is usually quite high [27–29]. For these reasons, pH tests were performed on both a newly assembled consortium in $BG11_0$ and on a 30-day-old consortium assembled and grown in $BG11_0$, resuspended to be utilised in the experiment at three different pHs.

As shown in Figure 4a, pH strongly affects the consortium growth in terms of dry biomass: both the newly assembled and the 30-day-old consortia grew less under acidic pH than neutral pH, although the highest growth was found at basic pH (Figure 4a, one-way ANOVA $p < 0.05$). The growth differences between the three pHs are statistically significant in both the newly assembled and 30-day-old consortia (one-way ANOVA $p < 0.05$). Furthermore the 30-day-old consortia grew less than the newly assembled consortia, even if this difference is significantly lower only at pH 7.5 (one-way ANOVA, $p < 0.05$, asterisk in Figure 4a).

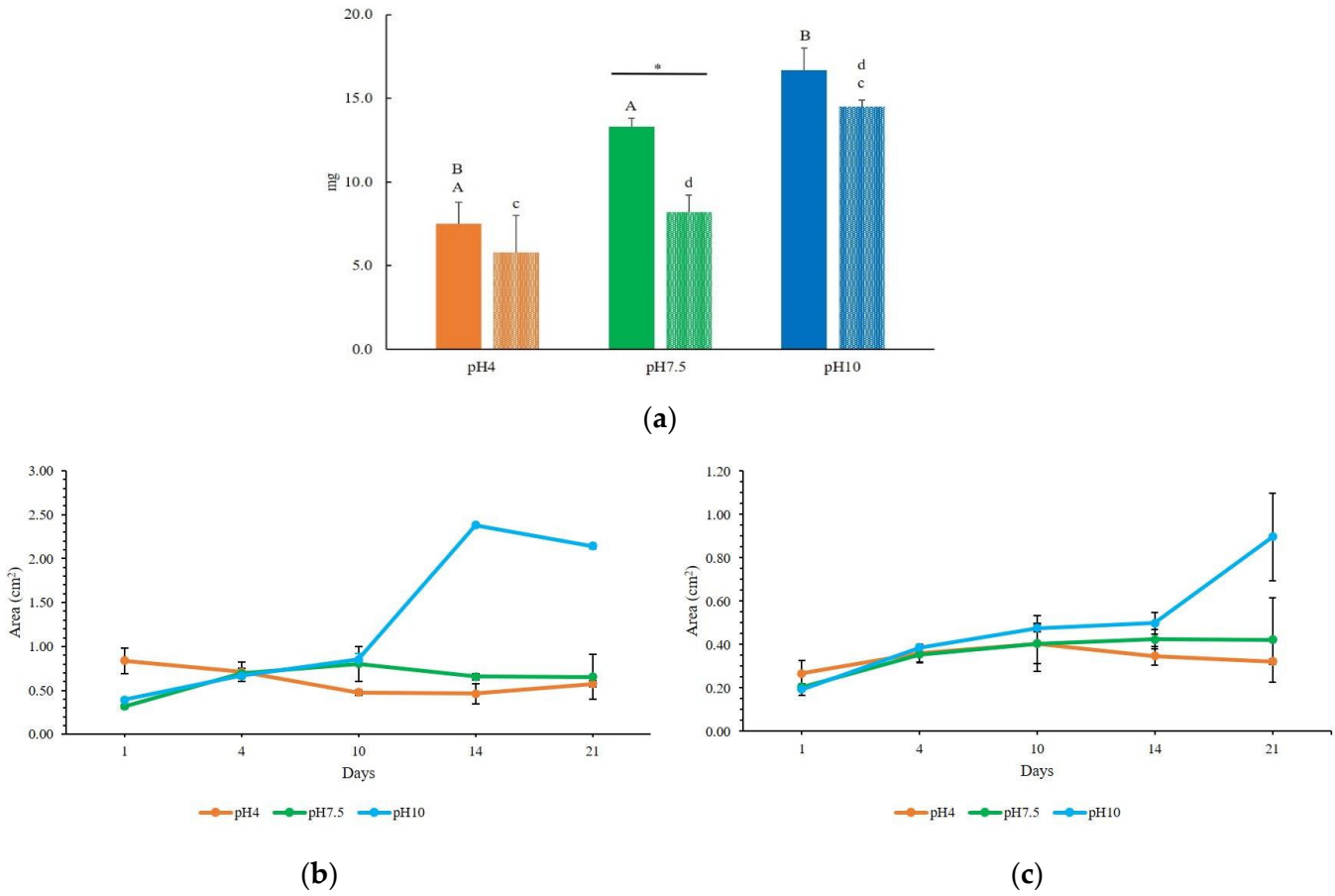


Figure 4. (**a**) Consortium biomass growth in $BG11_0$ at different pH; solid bars indicate consortia assembled at the start of the experiment, while textured bars indicate 30-day-old consortia. Capital letters refer to the statistical comparison among consortia assembled at the start of the experiment; lowercase letters refer to the 30-day-old consortia at the start of the experiment; asterisk refers to a significant difference ($p < 0.05$). Growth of the consortium (as area of the 3D structure) during the 21-day experiment at different pH: (**b**) consortia assembled at the start of the experiment and (**c**) 30-day-old consortia at the beginning of the experiment.

The reduced growth observed at acidic pH most likely depends on the consortium photosynthetic partner, as cyanobacterial growth may be inhibited in habitats with pH values below 4–5, which hampers the maintenance of their intracellular alkaline pH; in fact, the cytoplasm acidification in cyanobacteria has been associated with reduced growth rate [30]. Also, surface measurement confirmed the different growth results (Figure 4b,c), and from these data, the most interesting point is the dramatic increase at basic pH from the 10th day onwards, shown by the newly assembled consortium, which may also be due to an expansion and/or relaxation of the three-dimensional structure of the consortium.

In terms of the Zero Mile system, the microbial consortium showed the ability to grow well in alkaline media; this is a very promising result since, as already stated, the DWW pH is mainly basic, due to the composition of the detergents. Moreover, these tests showed that the newly assembled consortium is able to face changing pH, without the need to pre-assemble or pre-condition the consortium, as it can efficiently thrive even assembled when the system is put into operation. A last consideration can be added about the consortium acidic tolerance, as calcium (for instance, present in high amounts in Rome tap water, and consequently in the dishwasher wastewater composition) may increase the acidic environmental tolerance, allowing the consortium to grow even in limiting conditions [30].

Another highly variable parameter to be considered in a system devoted to food waste attack and recycling is the organic load; to this end, the microbial consortium was exposed to different concentrations of an easily available source of nutrients, as TSB medium. This allowed us to evaluate the response of the consortium to different loads of organic matter, as can happen in dishwasher wastewater.

In the first 10 days, the absorbance data at $\lambda = 665$ nm showed a notable consortium growth under high organic load (Figure 5a), but the high turbidity values, measured at the wavelength of 730 nm, revealed that there was an overgrowth of heterotrophic bacteria (Figure 5b). This overgrowth damages the photosynthetic partner of the consortium, as shown by naked-eye observation (Figure 5c). Indeed, with higher TSB amounts, the culture turned brown and opaque in a short time, due to the proliferation of heterotrophic bacteria and degradation of cyanobacteria (Figure 5c: 0.27 g/L TSB at T_{11} and 0.54 g/L TSB at T_9). This experiment shows that the consortium does not survive the increasing—and oxygen-consuming—degradative activity of the aerobic heterotrophic bacterial overgrowth. These organic loads are hardly reached in a dishwasher cycle, as the food leftovers usually consist of a few grams wet weight (foods contain up to 70% water by weight or greater, and fruits and vegetables contain up to 95% water or greater; [31]), while the tested concentrations consist of easily available concentrated powdered organic matter.

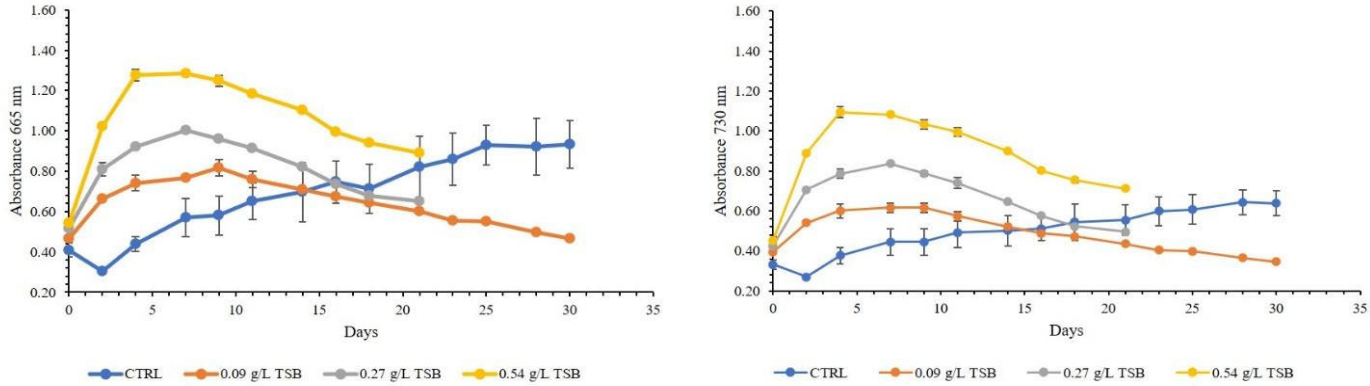
3.3. Dishwasher Wastewater Microbial Colonisers

In order to characterise the DWW's bacterial community, the wastewater produced after the 'eco' programme was collected and analysed using both the cultivation-dependent and metagenomic approach.

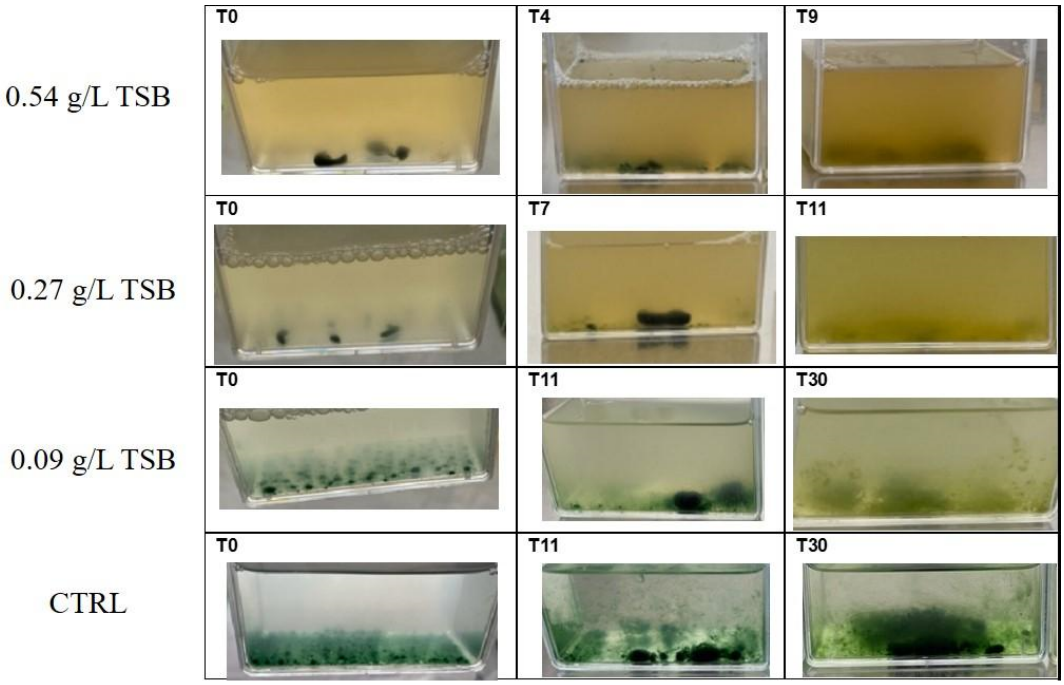
One sample was analysed by standard microbiology techniques to gain the culturable microorganisms; their taxonomic identification was obtained by Sanger sequencing. Three samples were analysed by DNA metabarcoding (in Next Generation Sequencing) to obtain information about the entire community colonising the wastewater, including possible non-culturable microorganisms.

To obtain a fairly complete picture of the cultivable aerobic heterotrophic microbial community of the wastewater, samples were plated on two solid media (TSA and PSA). On the two solid media, the microbial load ranged from 10^6 to 10^4 cells/mL; from these cultures, 20 isolates were collected on the basis of their morphology, and 16 were taxonomically identified by Sanger sequencing (Figure 6, Isolated_Sanger sequencing bar). The main colonisers were *Firmicutes* (*Bacilli* class; n = 9 isolates), followed by *Proteobacteria* (*Gamma-Proteobacteria*; n = 7 isolates). All the *Firmicutes* belonged to the order *Bacillales* (two *Bacillus*, four *Exiguobacterium*, two *Staphylococcus* and one *Lysinibacillus*), the *Proteobacteria* belonged

to *Enterobacterales* (three *Citrobacter* and two *Klebsiella*), *Pseudomonadales* (one *Pseudomonas*) and *Xanthomonadales* (one *Stenotrophomonas*).



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



(**c**)

Figure 5. Effect of different organic loads (0.09, 0.27 and 0.54 g/L TBS powder, dry weight, in $BG11_0$) on the growth of the consortium. (**a**) In vivo chlorophyll *a* content (OD λ = 665 nm), proxy for the cyanobacterial growth curve, and (**b**) culture turbidity (OD λ = 730 nm), proxy of the cultures' overall growth. (**c**) Consortium aspect at the different TSB concentrations over time; the first two concentrations caused a very early decay of the consortium. T# indicates time and the number of days from the start of the experiment.

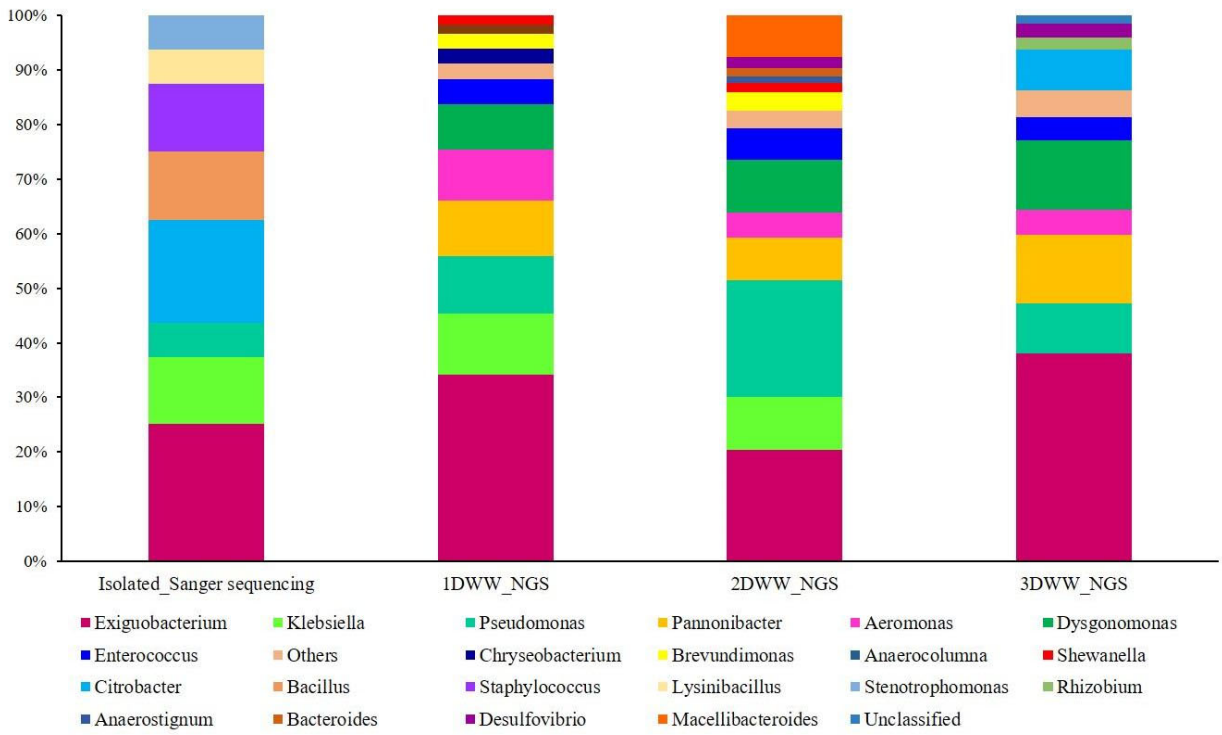


Figure 6. Bacterial genera found in dishwasher wastewater samples, analysed by Sanger sequencing. Live isolates grown on culture media plates (1st bar on the left) or by DNA metabarcoding on dishwasher wastewater samples (the other three bars, #DWW bars).

As already found, the microbial colonisers of dishwashers are heterotrophic aerobic generalists, able to survive in the limiting environmental conditions of the dishwasher (in wastewater [8] and on the rubber seal [32,33]). The identified strains are environmental ubiquitous bacteria, as well as common commensal organisms found as colonisers of humans and animal skin (*Staphylococcus epidermidis* and *Staphylococcus warneri*).

To gain further insight into the diversity of the non-cultivated/rare components of the bacterial communities inhabiting DWW, DNA metabarcoding was applied on three different dishwasher wastewaters. The taxonomic identification of the bacterial DWW community showed comparable results to those obtained by the cultivation-based approach (Figure 6). Analysis of DNA metabarcoding resulted in 21,449 sequences clustered in 51 OTUs (97% similarity). To assess differences and efficiency in the sampling effort, rarefaction curves were created for each sample in each experimental batch; they confirmed that the sequencing depth was suitable.

The most abundant genus found in all DWW samples was *Exiguobacterium*, also found by a cultivation-based approach; it was represented by the highest number of reads, followed by the *Pseudomonas*, *Panninobacter*, *Klebsiella*, *Dysgonomonas*, *Aeromonas* and *Enterococcus* genera. Indeed, the *Exiguobacterium* dominance may depend on the well-known tolerance to a wide range of temperatures (−12 to +55 °C), salinity (up to 13%) and pH (5–11) [34,35]. The composition of the DWW bacterial community was significantly different from the dishwasher rubber seal biofilms [32,33], although it has comparable components, if analysed at the genus level. Unfortunately, to the best of our knowledge, at the moment, there is no other information in the literature on the taxonomic composition of DWW colonisers to be compared.

It is worth noting that in the samples analysed in this study (Rome household dishwashers), two of the three bacterial genera of the consortium, *Exiguobacterium* and *Aeromonas*, were always present (above 20% and less than 10%, respectively), in particular *Exiguobacterium* was the most abundant component. Conversely, no isolates belonging to the genus *Acinetobacter* were found. The three heterotrophic components of the consortium

were isolated by Milan Polytechnic dishwasher wastewater; they were chosen among the cultivated fraction of the wastewater, according to a dominance/rarity criterion: *Acinetobacter* and *Aeromonas* being part the dominant component and *Exiguobacterium* being quite rare [8]. The difference between the two wastewaters may depend on both water quality and the use of the dishwasher: frugal lunch in a workplace, for the Milan Polytechnic dishwasher vs. complete and rich meals, for the Rome household dishwasher. In any case, the similarity in the DWW bacterial colonisers can be imputed to the dishwasher environment, which is extremely selective, while the variations may be due to the differences in food leftovers.

The presence of bacteria on breakfast cups, dishes and cutlery soon after meals and immediately after the end of the washing cycle was also investigated. While a quite high bacterial load is present on dirty cups, plates and cutlery (colony-forming unit, CFU, not countable), it is almost completely eliminated on washed items (cup—6 CFU; dishes—8 CFU; cutlery—22 CFU), probably as a consequence of the rinsing with tap water (almost sterile) and the high drying temperature at the end of the washing cycle, significantly reducing the microbial load.

The consortium survival and growth were evaluated in batch tests at different wastewater dilutions; in these tests, the amount of nutrients was limited to the initial filling. The growth curves, indicating both the cyanobacterial and the total bacterial dynamics, showed that in 75% and 100% DWW, the consortium died in a few weeks, while consortia thrive well in 50% DWW (Figure 7a,b, left). The consortium suffered at the lowest DWW dilution (Figure 7a,b, right, 75% and 100% DWW): despite an initial significantly high growth (two-way ANOVA Sidak's multiple comparisons test; $p < 0.0001$), the growth rate sharply decreases after the ninth day, and the consortium dissolves (as in Figure 5, at 0.18 and 0.36 mg/L TSB). A significant increase in consortium chlorophyll *a* and turbidity values, if compared to control batches, was found from day 18 onwards only in the 50% DWW test (two-way ANOVA Sidak's multiple comparisons test; $p < 0.0001$).

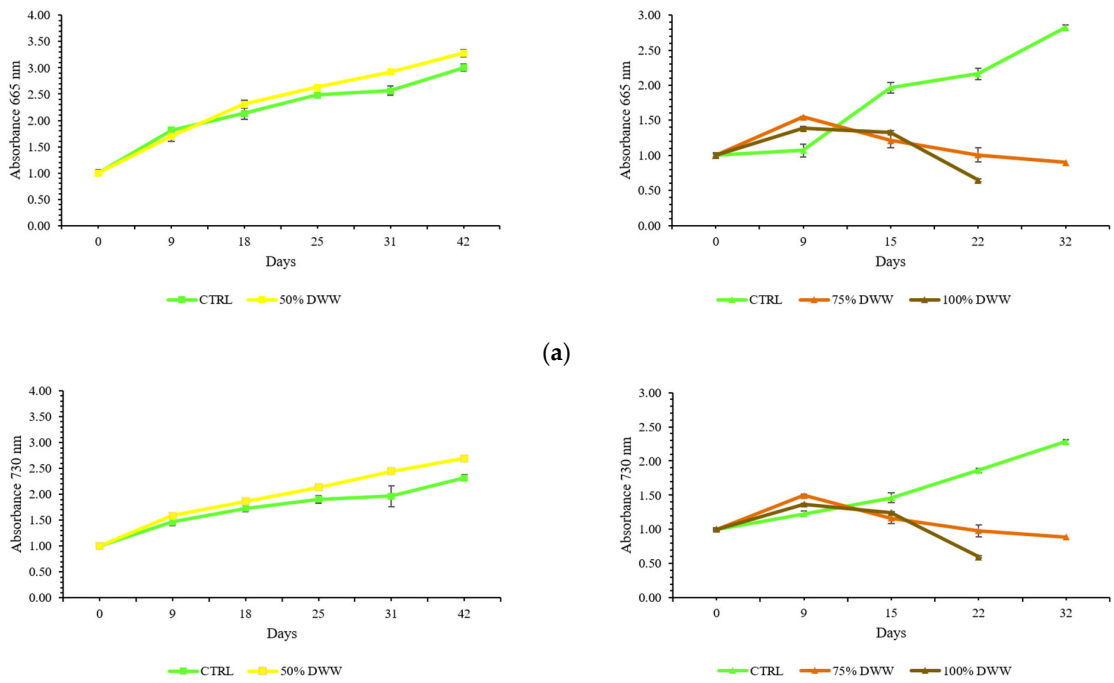


Figure 7. Growth curves of consortia in batch test, at different DWW dilutions, evaluated as absorbance of: (**a**) in vivo chlorophyll *a* (OD λ = 665 nm), proxy for the cyanobacterial growth, and (**b**) culture turbidity (OD λ = 730 nm), proxy of the cultures' overall growth. Data were normalised.

The consortium's capability to thrive in wastewater depends on the mutualistic interaction of autotrophic and heterotrophic microorganisms: heterotrophic bacteria consume the organic matter, mineralising nutrients and releasing CO_2; cyanobacteria utilise nutrients and CO_2, convert them into biomass and other valuable products and, more important, supply oxygen for the catalytic processes of the heterotrophic partners [36,37]. This is a dynamic interplay which means a continuous 'stoichiometric' exchange between the two partners of the consortium. In the case of the batch test, the consortia thriving in 100 or 75% wastewater showed a significant increase in both partners in the initial phase (until day 9); then, when the nutrients were depleted, a significant decrease ($p < 0.0001$) in both the cyanobacterial and heterotrophic bacteria growth curve began, leading to the disaggregation of the consortium. In the 50% DWW test, the reduced amount of nutrients determines a low growth rate, maintained since the beginning of the test; this allowed a longer life-span of the consortium. These data agree with the results of other our studies, showing that a partial (one-quarter) or complete substitution of the dishwasher wastewaters allows the consortium to survive over a much longer time span [14].

It is evident that under batch conditions, the consortium is unable to survive: in terms of the Zero Mile system, these tests make clear the assumption that a further and constant nutrient supply is necessary for the consortium's survival and growth, and in turn, they strengthen the design of the system, based on a regular refill of DWW to the consortium, i.e., the biofilter of the Zero Mile system [7,14].

3.4. Design Implications

The impacts of abiotic conditions on the microbial consortium growth and viability indicate the needs of illuminating the biofilter, the good growth in basic pH (those found in detergents) and the response to organic loads in batch tests.

In more detail, this study highlights the importance of providing light to the consortium, which supports cyanobacterial growth, giving insight into structural aspects of the biofilter container: it must be made of transparent material which allows uniform light supply in the consortium container. An additional requirement, beyond the ones resulting from the experiments, in the development of the biofilter container is its capacity, which should be able to to contain the whole wastewater production of a standard dishwasher washing cycle, e.g., 12 L.

As regards pH and nutrient content, alkaline dishwasher wastewater pH does not damage the consortium growth: hence, the container must be made of an inert material, tolerant to basic pHs. A constant nutrient supply is necessary to maintain the consortium's survival and growth with time; otherwise, in a batch condition, the consortium will die. A regular nutrient refill, as already demonstrated by Alabiso et al. [14], maintains the consortium capability to break down a high organic load with a four-day rate of refill. Hence, the container must allow an incoming flow of freshly produced wastewater and an outgoing flow of remediated wastewater without damaging the consortium's 3D structure.

The observed growth of the heterotrophic bacteria implies the necessity of a comfortable system to retrieve the biomass that can be used as fertiliser, while maintaining the container generally sealed as in the experimental setting.

As a last point, operating conditions between 10 and 35 °C imply the need for insulation, heating and refrigeration in case of outdoor applications entailing the placement of the biofilter in open air.

The outcomes that emerged from the experiments involving the microbial consortium produced results that significantly impacted the design framework initially assumed in the research [7]. Hence, there is a need for a framework review with the implementation of new aspects related to the environmental requirements of the system.

Among them, a prominent one is the light factor, which assumes a central role beyond the plant and consortium growth. The light required for the survival of the microbial consortium directs the design of the biofilter container, implying an investigation on alternative construction materials and on the degree of independence of the container

itself in the overall Zero Mile system. A key factor in the requirement framework will be the maintenance of illumination values in a range to be updated by forthcoming steps of the research. In terms of performance, the need to control environmental factors, such as temperature and exposure to direct sunlight, through the adoption of surface shielding strategies (e.g., opacity vs. transparency, photochromic films) emerges. In terms of structure, the independence of the biofilter will allow a placement flexibility useful to take advantage of favourable natural or artificial light conditions. Thus, the new requirement framework guiding the design review in the advancement of the research has to consider the design implications that prefigure Zero Mile no longer as a unitary system, but as the result of the interaction between different (bio)technological components.

The reviewed requirements of the design framework result in:

- Modularity and independence of the biofiltering, watering and cultivation sub-systems;
- Control of the biofilter light conditions, both through passive strategies (positioning and photo-adaptive materials) and active strategies (direct light from artificial sources);
- Separation of the plant cultivation sub-system in building structure and vegetable support;
- Flexibility of the plant cultivation sub-system in order to host different cultivation typologies;
- Accessibility, maintainability and mobility of all components and subsystems;
- Up-scaling of the system focusing on the mesoscale and urban scale, also looking at the key issues of socialisation and living space regeneration.

4. Conclusions

In conclusion, a necessary step for the Zero Mile industrial application is the design of the biofilter structure, which is strictly linked to the microbial consortium characteristics and its growth demands.

From this last point of view, the response to the first two questions we pose in this study is clear. As regard point (i), the experimental results showed the importance of evaluating different abiotic conditions on the microbial consortium growth and viability, which guarantee the bioremediation of wastewater by reducing N and P concentrations [8,14]. The results indicated the need to provide correct light/illuminating and good growth conditions, as basic pH (those found in detergents). As regards point (ii), it has been shown that a constant nutrient supply is necessary to maintain the consortium survival and growth with time, as already demonstrated by Alabiso et al. [14]; otherwise, in a batch condition, the consortium will die. These data will guide the planning of the Zero Mile system prototype.

From a design point of view, the results highlighted the importance of the biofilter container characteristics, point (iii), showing the increasing independence of the biofilter container from the dishwasher and the plant cultivation structure. This foreshadowed new morphological synergies with interesting spatial outcomes for the system in its entirety. Regarding future development, those spatial outcomes can address, for instance, the design of living labs and living artefacts [38] enabled by biomaterials and digital technologies, as in point (iv). The application of the proposed technology is promising to allow a design transition towards more resilient indoor and outdoor (bio)artefacts integrated into buildings as a whole living environmental system.

The Zero Mile system, dealing with the reuse of dishwasher wastewater in vegetable cultivation, produces several benefits in terms of sustainability. The first is a reduction in both the amount of freshwater consumed and the amount of wastewater discharged. The second is the energy performance, since the onsite biological treatment applied to reclaim the dishwasher wastewater for food production is a strategy to achieve a lower energy consumption in comparison to centralised systems. As stated by Garrido Baserba [39–41], "when the cost offsets associated with food and energy production are considered, the distributed system is about half the cost of the centralized system". Further benefits are linked to the Zero Mile production of healthy edible plants, which contributes to a more sustainable diet and reduces the environmental impact of food packaging and

transportation. The last benefit deals with the conversion of waste to commodity, since the consortium biomass resulting from the wastewater treatment process could be directly valorised in plant fertilisation.

Overall, the reported research highlights the possibility of applying the benefits of sustainability, by shifting from a linear to a more regenerative circular economy, even in the management of household wastewater.

Author Contributions: A.A., R.C., F.C. and L.M. devised the research; A.A. and M.B. carried out the tests; A.A., M.M.D. and S.F. conducted the Next Generation Sequencing data collection and analysis; L.M. and M.M.D. oversaw the statistical analyses; G.B., F.C., M.M. and A.N. developed the Zero Mile system's design framework and the scenario applications; A.A. and L.M. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: A three-year fellowship from the Ph.D. School of Evolutionary Biology and Ecology at Tor Vergata Rome University funds Annamaria Alabiso and Sara Frasca (XXXVI and XXXVII cycles, respectively). Annamaria Alabiso obtained an industrial doctorate in partnership with AlgaRes s.r.l., while Sara Frasca holds a PON doctorate in collaboration with GT50 s.r.l. The ISCRA CINECA Project Class C on the Galileo100 server, titled "The JETSONS' Kitchen" and granted to A.A. (HP10CF8YO project), enabled the data elaboration.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences have been deposited in GenBank as Targeted Locus Sequences under the BioProject ID PRJNA942250.

Conflicts of Interest: No conflicts of interest are disclosed by the writers. The study's design, data collection, analysis, interpretation, paper writing and the decision to publish the findings were all carried out without the funders' input.

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