# Title: Biohazard identification: Profiling microalgal cultures growing on municipal wastewater and fertilizer medium in raceway photobioreactors.

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# Abstract

Microalgae provide a promising way to combine wastewater treatment and biomass production. They are often used as pre-treatment to reduce pathogenic loads: however, a full characterisation of the risks is still needed to develop safety guidelines.

Two raceways were inoculated with *Scenedesmus*, one in a fertilizer medium and one in wastewater. Biohazards were then explored in three ways: NGS analysis, commercial qPCR array and plate counts.

Media and sampling locations contributed to shape bacterial and eukaryotic structures and pathogenic loads. Communities were variable across time with a higher diversity between inlets than between biomass and outlets. A lower presence of pathogens was seen in fertilizer, while wastewater showed a distinct reduction from inlet to outlet. The main pathogenic genera detected were *Arcobacter* and *Elizabethkingia* with an important presence of *Aeromonas*. The three analyses together identified the necessity of preventive and protection measures and of post-harvest treatments.

**Keywords:** Microalgae; Pathogens; Raceway photobioreactor; Waste management; Next generation sequencing.

# Introduction

Algal-based technologies have been around for the past 70 years. Microalgae (including Cyanobacteria) are known to have a high biodiversity and fast growth rates, with an ability to produce a wide array of molecules, and they are therefore considered a renewable, sustainable, and economical resource. With water shortages expected to affect 40% of the population by 2030, microalgae only lately emerged as a promising treatment technology for wastewater (WW) nutrient recovery that could lead to the reclamation of used water and to an increase in resources (Li et al., 2019). In the context of the circular economy and sustainability, microalgal treatments offer several advantages, as they enable the removal of pollutants and nutrients while producing biomass which can in turn be used as biogas, biofuels, fertilizers and feed, further reducing clean up, production costs and emissions (Hussain et al., 2021).

Unlike the direct use of WW for agricultural purposes, its use for microalgae cultivation is thought to come without the downstream complications to human health if environmental regulations and standards are in place (Sarker, 2021). The main risks previously identified and related to microalgal cultivation involve the integrity of photobioreactors (PBRs), the release of microalgae from the ponds (intentional and accidental), the dispersal by aerosols and wildlife, and catastrophic failure of containment systems (OECD, 2015). It was further highlighted that exposure will vary depending on the design of the reactors. Human focused risks on the other hand derive mainly from releases of microalgae into the environment (e.g. aerosols and phycotoxin production) and release of WW and waste biomass (e.g. production of biological materials, chemicals, nutrients, additives into the environment and bioaccumulation of heavy metals).

Wastewater is however known to be home to many microorganisms and pathogens mainly coming from faecal matter (Cai and Zhang, 2013) that, within WW treatment plants, may be subjected to aerosolization, producing airborne bacteria. WW generally requires limited contact with workers during treatment, therefore reducing health risks, which may however be increased in downstream uses and may affect public health. The potential of pathogens to lead to illness depends upon their stability in the environment and their infective dose, which varies according to the species considered (Ajonina et al., 2015). Although experts and experienced workers are aware of the possible impacts on health and might use protective devices, agricultural workers and people living in the surrounding areas from such plants are more exposed to risk, and a higher number of health problems has been reported for these categories (Sarker, 2021).

Reduction of pathogenic microorganisms can be achieved within algal WW treatment systems, thanks to alkaline pH and other factors (i.e., temperature, limiting substrates, predation, adsorption and sedimentation, sunlight, presence of allelochemicals, production of toxins) (Chambonniere et al., 2021). The microalgal biomass obtained therefore has the potential to be used as fertilizer after separation from the effluent (Mulbry et al., 2005) and the effluent is further suitable for agricultural reuse: however, due to the residual bacterial and pathogenic load, a further disinfection step may be necessary.

Within this perspective of recycling and the circular economy, understanding of the interactions between bacteria and microalgae and the effect on pathogens is still limited (Slompo et al., 2020). Although nutrient recovery is a necessity, the presence of pathogens is a serious threat to human and animal health. There are no truly universal indicators to measure pathogen removal, as at present, only faecal indicators are mainly used and often it is difficult to evaluate whether the pathogens have actually been killed or only removed (Chambonniere et al., 2021). Further research is necessary to fully characterise the biological risks, to develop a suite of correct management and safety guidelines and to put into place pre/post-treatments where needed, before the reuse of the WW or biomass. Within this paper two scenarios were explored to assess biohazards of raceway (RW) reactors. Two RWs were inoculated with the same *Scenedesmus* strain: one was fed with fertilizer, therefore with an expected low pathogenic load, while the second was fed with WW. Commonly, the assessment of waterborne human pathogens is not cost-intensive, therefore only few indicators are monitored (e.g. faecal coliforms) (Dar et al., 2019). Monitoring is still routine work, and several techniques have been developed (i.e. colony count, PCR and qPCR) that target specific organisms. However, to have a snapshot of the most abundant components of the communities and a more comprehensive analysis of the pathogens, techniques such as next generation sequencing (NGS) and shotgun sequencing are essential. Here, pathogen presence was explored in three ways. Firstly, the bacterial and eukaryotic community was characterised in terms of its most abundant components and of their dynamics, with a NGS analysis of both 16S and 18S rRNA genes. Secondly, a commercial qPCR array was used to further characterise and assess the potential presence of multiple targeted pathogens most commonly found in WW. Thirdly, the effectiveness of this array and the viability of the identified pathogens were assessed through plate counts. Aims were therefore: 1) the characterisation of the microbial community and its pathogenic potential through a multi-technique approach and 2) the identification of the possible microbiological risks for workers, environment quality and human health.

# Materials and Methods

## 2.1 Raceway reactors and culture conditions

Two RWs at demonstrative-scale were installed at the plant facilities of the University of Almería located at the IFAPA facility (Almería, Spain). Both RWs had an identical set- up with a total surface area of 80 m2 and a volume of 12,000 L. The reactors consist of a polypropylene algal pond of two 50 m length channels (0.46 m high and 1 m wide) connected by two 180° bends, with a 0.59 m3 sump located 1 m along one of the channels. A rotating paddlewheel moved by an electric motor was used to circulate the microalgal culture for which the height control setpoint was 0.15 m, while dilution rate was constant at 0.2 day -1 throughout the experiment.

A freshwater *Scenedesmus* sp. strain was used to inoculate the RWs. The inoculum was grown photo-autotrophically in a spherical flask (1 L capacity) and renewed weekly with freshly modified Allen and Arnon medium. The culture was continuously supplied with an air-1% CO2 mixture to maintain the pH at 8. The *Scenedesmus* sp. culture was maintained at 24 °C under artificial illumination on a 12:12 h light-dark cycle by using Philips PL-32W/840/4p white-light lamps. The inoculum was then used to grow *Scenedesmus* sp*.* in a 3 m3 capacity industrial tubular PBR with the modified medium prepared following industrial practices and using commercial-grade fertilizers instead of pure chemicals. This culture was then transferred to the RWs. One RW was maintained with the modified medium while the other was maintained with primary domestic WW from the University of Almeria (NO3- of 0.5±2.1 mg L-1, NH4+ of 80.2 ±41.5 mg L-1, PO43- of 27.3 ±7.6 mg L-1, COD of 209.1 ±85.0 mg L-1).

Environmental and operational conditions were monitored. Temperature and dissolved oxygen (DO) concentration were measured using an InPro 6050 probe (Mettler Toledo, Spain) (limit of detection (LOD): 0-80 °C and 0-400%, respectively) while pH was monitored with a 5342 probe (Crison Instruments, Spain). These sensors were connected to an MM44 control-transmitter unit (Crison Instruments, Spain) with a data acquisition system (SCADA) providing complete control of the systems. Irradiance was further measured through a CM11 pyranometer sensor (Kipp & Zonen, Netherlands) (LOD: 0-4000 Wm-2). Additionally, DO and pH were controlled on demand as air was supplied to remove excess DO (>200%) and pH was maintained at 8.0 by injection of pure CO2 within the sump. Reactors were operated in semi-continuous mode throughout the experiment with daily sampling collection (sampling volume was replaced with fresh media or WW) performed between 8:30 and 9:00 AM before harvesting and dilution.

## 2.2 Biomass concentration and analytical methods.

Biomass concentration (Cb) of the microalgal samples was measured daily by dry weight. Aliquots of the cultures (100 mL) were filtered through Macherey-Nagel MN 85/90 glass fibre filters then dried in an oven at 80 ºC for 24 h. The condition of the microalgae was monitored by checking the efficiency of the photosynthetic apparatus II (Fv/Fm) using an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic).

For the composition of the primary domestic WW and the microalgal samples, nitrate (NO3-) concentration was measured through optical density at 220 nm and 275 nm (Nitrate Standard for IC: 74246). Ammonium (NH4+) was quantified according to the Nessler method (Ammonium standard for IC: 59755). Phosphate (PO43-) was measured by visible spectrophotometry through the phospho-vanado-molybdate complex (Phosphate Standard for IC: 38364). The Chemical Oxygen Demand (COD) was determined by spectrophotometric measurement using Hach-Lange kits (LCl-400).

## 2.3 Samples collection and DNA extraction

Three different type of materials were collected from each system: inlet, biomass and outlet and sampling was carried out on three occasions during the course of the experiment: T1, after reaching the steady state (March 2021); T2: in the middle of the experimental period (May 2021); T3, at the end (June 2021). Biomass was harvested by centrifugation of the culture within the RWs and the remaining water after centrifugation constituted the outlet. After collection, samples were shipped refrigerated with next day delivery to the laboratory where they were stored at 4 °C and processed within two days.

Two additional samples, pig slurry and pig slurry after nanofiltration at 0.1 µm (nanofiltrate) were collected from a pig slurry treatment plant. Nanofiltrate and also PCR grade water were used as a negative control to test the technique while pig slurry was used as a “positive control”.

DNA extractions were performed in triplicate. Inlet and outlet samples (30-500 mL) were filtered through 0.2 nm filters (Ahlstrom-Munksjö, Germany) while biomass was used as it was. From each pellet and filter, DNA was extracted using the DNeasy Plant® Kit (Qiagen, Germany). Yield and purity of the DNA extractions was quantified on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) while eventual fragmentation was determined through gel electrophoresis on 1% (w/v) 1×TAE agarose gels. DNA was stored at -80 °C until analyses.

## 2.4 Community structure by next generation sequencing (NGS)

lllumina sequencing was performed in duplicate on all samples from T2 and T3 at Stab Vida Lda (Lisbon, Portugal). For bacteria, the V3-V4 hypervariable region on the 16S rRNA gene was selected and amplified with the primers Bakt\_341F and Bakt\_805R (Herlemann et al., 2011) while for eukaryotes the 18S rRNA gene V9 region was amplified with the primers 1389F and 1510R (Amaral-Zettler et al. 2009). DNA libraries were sequenced with MiSeq Reagent Kit Nano on the lllumina MiSeq platform. Nucleotide sequences generated are available at the NCBI SRA repository (BioProject: PRJNA837452). These sequences were quality checked through the FastQC software and analysed using DADA2 (Callahan et al., 2016). Reads were truncated at 220 (forward) and 200 (reverse) for 18S and at 280 and 245 for 16S to remove the low-quality section of the reads. The SILVA database was used for prokaryotic taxonomic assignment while eukaryotic classification was done using the Blastn algorithm against NCBI nucleotide database and taxonomy retrieved using Taxdump Repository.

## 2.5 Screening and vitality of pathogens

Samples were screened through the 96-well format Microbial DNA qPCR Array for Water Analysis (Qiagen, USA) on an Applied Biosystems 7300 Real-Time PCR System for the presence or absence of 45 bacterial pathogens or indicators of faecal contamination, targeting 16S rRNA and virulence genes (for a full list of targets and characteristics, see manufacturer’s manuals). Triplicates were then merged and DNA concentration of the samples was standardised at 2,500 ng following the manufacturer’s protocol for low-abundance microbial species. Negative, positive and inconclusive results were calculated following the manufacturer’s protocol. Results were reported semi-quantitatively using an inverse cycle threshold.

For T3, in order to assess the vitality of the pathogens that had shown up as positive with the array analyses, an aliquot of fresh samples was shipped to Istituto Zooprofilattico della Lombardia e dell’Emilia Romagna (Italy) for the cultivation of *Aeromonas* spp., *Citrobacter* *freundii*, *Clostridium* *perfringens* and *Vibrio* spp. and to Arcadia srl (Italy) for the cultivation of Intestinal *Enterococci*, *Escherichia* *coli*, *Legionella* *pneumophila* and faecal coliforms.

## 2.6 Statistical analysis

All statistical analyses were performed on R studio (version 4.1.2) mainly with packages vegan (Oksanen et al., 2020) and FactoMineR (Lê et al., 2008) while taxonomic summaries were performed using the phyloseq package (McMurdie and Holmes, 2013). Observed richness, Shannon diversity index and Pielou’s evenness were calculated and following a Shapiro-Wilk test to test normality, differences among samples of normally distributed data were tested by one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test while non-normal data were analysed through a non-parametric Kruskal-Wallis test followed by Dunn's Test for multiple comparisons. For pairwise comparisons, T-test and Wilcoxon signed rank test were used for normal and non-normal data respectively. Multivariate analyses were performed on amplicon sequence variants’ (ASVs) relative abundances. To test the effect of time and media on beta diversity, first, a nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances was applied and then results were confirmed through a PERMANOVA test. Furthermore, pairwise comparisons were performed with the package ‘pairwiseAdonis’ (Martinez, 2020). Significant PERMANOVAs were followed by a distance-based similarity percentage test (SIMPER) to discriminate genera between samples using Bray-Curtis dissimilarities and Linear Discriminant Analysis (LDA) Effect Size (LEfSe). Correlations between pathogens were tested by Spearman correlation though the ggcorrplot package (Kassambara, 2016).

NMDS was further used to detect differences in the pathogen presence among locations and across time while qPCR targets driving the ordination were analysed through an Envfit analysis. Statistical differences across media, locations and time in terms of total pathogens were assessed by Mann-Whitney rank sum tests and a Kruskal-Wallis test followed by Dunn’s test.

# 3. Results and discussion

## 3.1 Environmental conditions and reactor performance

To guarantee optimal *Scenedesmus sp.* growth, water level in the RW was maintained at an average level of 15 cm while DO was maintained at an average of 131% and pH at 7.8 (see e-supplementary materials) (Morillas-España et al., 2020). Across the whole experimental period, RWs registered an average temperature of 25 °C. Since microalgal growth rates are dependent on the intracellular concentration of nutrients, fertilizer and WW were supplied under non-limiting conditions of both N (N-NO3- in the F-RW: between 74 and 118 mg L-1; and N-NH4+ in the WW-RW: between 21 and 140 mg L-1) and P (P-PO43-: between 4 and 25 mg L-1) (data not shown). Concentration of total N in the range of 50-100 mg L-1 and of total P of 6-12 mg L-1 have been found to provide sufficient nutrients to maximize the growth rate and concentrations for high biomass production of *Scenedesmus* (Abu Hajar et al., 2017).

Growth performances were similar between both RWs and across time and were comparable to or slightly lower than the results from other studies. Here, productivity reached average values of 15.5 g m-2 day-1 and 17 g m-2 day-1 for the WW-RW and F-RW respectively (Table 1). In a previous study, productivity of *Scenedesmus* sp. in RWs (32 m2) reached maximum productivity at 24 g m-2 day-1, peaking during the summer months (Jul - Sep), while it ranged between 10 and 20 g m-2 day-1 from March to May (Morillas-España et al., 2020). In other *Scenedesmus* RWs run using fertilizers, production averaged at 20 g m-2 day-1 while at 25 g m-2 day-1 when working on 30% centrate which, similarly to WW, contains N mainly in the form of NH4+ (Morales-Amaral et al., 2015).

## 3.2 Eukaryotic community composition

Community composition and dynamics were investigated with NGS analyses of both reactors at T2 and T3 (T1 was excluded as reactors had only just reached the steady state).

For the eukaryotic population, input reads ranged between 37,752 and 127,084 while between 18,210 and 76,726 after DADA2 assignment.

At a phylum level, communities were characterised by a high abundance of unclassified Eukaryotes while the main microalgae were Ochrophyta (golden and brown algae) in the inlet and outlet of the F-RW at T3 and of the WW-RW inlet at T2 while Chlorophyta occurred across all WW-RW samples and biomass and outlets of F-RW (see e-supplementary materials).

A mix of multiple microalgal genera developed and alternated across time and reactors. Observed richness (number of different species in a given community) was similar between T2 and T3 while WW-RW showed a lower richness than F-RW (p<0.05) mainly due to the higher richness within the F-RW inlet (p<0.05) (see e-supplementary materials). Both Shannon diversity (indicating how diverse the species in a given community are) and Pielou's evenness (how close in numbers each species in a given community is) were similar between media and times, however a higher diversity was again seen within the inlet, especially in the F-RW samples (p<0.05).

In the inlets of both T2 and T3, the F-RW did not show any main microalgae while WW-RW was characterised by the dominance of *Ochromonas* at T2 (4.4%) and *Picochlorum* sp. (22.5%) at T3 (Fig. 1). Within F-RW biomass, again no main microalga was detected at T2, while T3 was characterised by the microalga *Oocystis* (8.9%). *Dictyosphaerium* appeared as the main microalga in the outlet of F-RW at T2 (3.8%) and joined *Oocystis* (4.2%) in the outlets at T3 (5.7%) together with *Poteriospumella* (5.7%). On the other hand, *Ochromonas* was replaced by *Micractinium* sp. as main microalga in the WW-RW biomass at T2 (3.1%) and by *Dictyosphaerium* in its outlet (3.8%) while no main microalgae were detected in the biomass and outlet of T3. At a lower abundance (<3%), a wide variety of other microalgae was detected, such as a few red algae: *Audouinella* *macrospora*, *Cyanidioschyzon* sp. Y16 and *Madagascaria* *erythrocladioides*, all present in the F-RW, the first two in the outlet and biomass respectively at T3 while the latter in the inlet at T2; and two golden microalgae: *Mallomonas* *annulata* and *Spumella* spp. in WW-RW, the first in the inlet at T2 while the latter was found at both sampling times. Numerous (23) green microalgae were further found (<3%) including *Chlorella* spp. and *Tetradesmus* *obliquus* (see e-supplementary materials).

Although all reactors were inoculated with *Scenedesmus* sp., the absence of the inoculum might come as a surprise, despite the primers used have previously shown to amplify *Scenedesmus* sp. and other genera commonly used in PBRs(Su et al., 2022). Although DNA barcoding enable a rapid and reliable identification of organisms, it has the disadvantage of being a PCR-based approach and as such it is inherently biased by prior knowledge. Unfortunately, it is not possible to design primers that will amplify all targets universally and, especially for microalgae, there is still a need for a methodology that can lead to a more complete identification process (Khaw et al., 2020). Within the context of this study, however, the identification of a wide number of low abundance microalgae and eukaryotes in general is still useful to create a snapshot of the communities.

When looking at the general eukaryotic composition, it is noticeable that F-RW inlets were characterised at both sampling dates by a high abundance of *Tropocyclops* (34.5% and 14.3% at T2 and T3 respectively), copepod crustaceans, and *Adineta* (6.3% and 7.3%). T2 was further characterised by the bacterial grazer *Cyclidium* (13.9%) while T3 by the flatworm *Stenostomum* (5.4%) and *Paramonas* (4.6%). *Brachionus* *calyciflorus* was further found abundant in all biomass and outlet samples of the F-RW (T2 biomass: 49.5%, and outlet: 8.4%; T3 biomass: 9.8%, and outlet: 4.4%), it is mainly known to be a grazer often used as an indicator of bad water quality and sewage (Gao et al., 2021). F-RW biomass of T2 was further dominated by *Filinia* (24.4%), and at T3 by *Amoeboaphelidium* (23.0%) and *Adineta* (8.0%) in the biomass, while only by *Amoeboaphelidium* (5.4%) in the outlet.

WW-RW inlets on the other hand, were characterised by the presence of *Paratrimastix* (T2: 50.7% and T3: 29.1%) and *Rictus* (T2: 7.7% and T3: 8.9%) at both sampling times. T2 further showed the presence of *Trepomonas* (3.0%). As with the F-RW, WW-RW biomasses were characterised by *Brachionus* and *Filinia* at both T2 (39.5% and 6.9%, respectively) and T3 (30.4% and 31.6%) with the addition of *Amoeboaphelidium* (61.1%) at T3. WW-RW outlet at T3 showed again *Amoeboaphelidium* (46.4%) as the dominant genus, followed by *Rhizophydium* (4.8%) and *Brachionus* (4.5%). *Paratrimastix* are bacterivores often found inside the tissues of dead and decaying marine vegetation (Brugerolle and Patterson, 1997). *Adineta,* and *Brachionus* are known microalgal grazers (Day et al., 2017) while *Amoeboaphelidium,* here mainly *A. occidentale*, is analgal parasitoid, and *Rhizophydium* are fungi acting as parasites and decomposers. The presence of these predators in both reactors could negatively affect the RWs biomass yield by reducing microalgal growth and modifying the community structure, thereby leading to the collapse of the system (Molina-Grima et al., 2022). In open systems and especially in the F-RW, their presence might point to aerosolization, contamination through carrier animals or human operations (e.g. cleaning, sanitization, preparation of inocula and media, water supply and aeration gases) (Molina-Grima et al., 2022).

## 3.3 Bacterial community composition

A second NGS analysis was carried out simultaneously to take a snapshot of the bacterial community in its complexity and to capture a first screening of the genera that could represent a health risk by possibly containing pathogenic species. Between 38,623 and 76,132 bacterial reads were retrieved (between 16,332 and 39,840 after DADA2).

Bacteroidota and Proteobacteria (max. and min. values across all samples: 18.2-35.5% and 7.0-53.6% respectively) are known to be globally distributed and together with Planctomycetes (1.5-16.6%), they are ecologically important, being involved in carbon, sulphur and nitrogen cycles (Fuerst et al., 2011) (see e-supplementary materials). Unlike the F-RW inlets, WW-RW inlets were characterised by the presence of Campylobacterota (4.7% and 11.7%) which show, together with Firmicutes (15.8% and 18.7%), a high number of genera which are inhabitants of the digestive tract mainly as symbionts but also as pathogens (van der Stel and Wösten, 2019). Cyanobacteria were only found below 3% in the outlets of both RW at T3.

Observed richness was similar between times and between media while the inlet samples generally showed a higher species richness than biomass samples (p<0.01) (see e-supplementary materials). Shannon diversity again showed similarity between media but there was a higher diversity at T2 and again a higher diversity in the inlets than in the biomasses (p<0.01). Pielou's evenness was higher in wastewater samples (p<0.01) and at T2 (p<0.05) and again in the inlets (p<0.05). The trend of higher richness and diversity in the inlet samples points to the possibility to encounter a higher variety of pathogens during early-stage management operations and environments where inlet water is present.

At genus level, communities were very variable among RWs and times, with a high percentage of low abundance genera (Fig. 1). Inlets of both RWs were characterised by the presence of multiple degraders with WW-RWs showing additional indications of possible faecal contamination and pathogenic risk. F-RW inlet at T2 was characterised by genera such as *Schlesneria* (5.4%) and *Nitrospira* (4.4%), Both are involved in the N cycle with *Schlesneria* further able to degrade biopolymers (Kulichevskaya et al., 2007; Daims et al., 2015). At T3, *Schlesneria* (3.0%) was accompanied by *Sediminibacterium* (6.1%), a genus stimulated by algal growth in WW environments. On the other hand, WW-RW inlets were characterised by *Arcobacter* and *Bacteroides* (T2: 10.3% and 5.0%; and T3: 3.3% and 3.1%, respectively, accompanied by *Thiovirga* (3.4%) and *Anaerovorax* (3.1%). The *Arcobacter* genus incorporates emergent enteropathogens and potential zoonotic agents often associated with water outbreaks (Collado and Figueras, 2011), while *Bacteroides*, although commonly found in the gut microflora, are also significant clinical pathogens (Wexler et al., 2007). On the other hand, *Thiovirga* contributes to sulfide removal (Lan et al., 2019) and *Anaerovorax*, to the degradation of protein-related molecules (Kong et al., 2022).

Looking at the biomasses and outlets, samples showed a high difference of genera when compared to the inlets. However, some genera were common to both RWs, possibly indicating an external environmental influence in shaping the communities of both RWs due to their open set-up (Bani et al., 2021) which could lead to pathogens’ presence even when their absence is assumed (Molina-Grima et al., 2022).

Within biomass and outlet samples, a high proportion of bacteria were characterised by genera that harbor pathogenic species (e.g. *Chryseobacterium*, *Aeromonas*, *Brevundimonas*, *Roseomonas* and *Elizabethkingia* (see section 3.5)). However, as expected, multiple genera were also involved in biodegradation and bioremediation activities (e.g. *Arenimonas, Phenylibacterium, Porphyrobacter, Gemmatimonas, Leptothrix* and *Polymorphobacter*)*.* At T2, the F-RW biomass was characterised by *Arenimonas*, *Phenylobacterium*, *Sandaracinobacter*, *Chryseobacterium* and *Polymorphobacter*; many of these genera were retrieved again in the outlet together with *Aeromonas*, *Brevundimonas* and *Flavobacterium*. At T3, *Sandaracinobacter*, *Polymorphobacter* and *Flavobacterium* were again present in the biomass together with a new set of most abundant genera: *Ahniella*, the cyanobacteria *Mariniradius*, *Roseomonas*, *Porphyrobacter* and *Gemmatimonas*. Similarly, in the T3 outlet, *Flavobacterium* and *Porphyrobacter* were again present with *Elizabethkingia* and the cyanobacteria *Geminocystis*.

Similarly to the F-RW, the WW-RW biomass at T2 was characterised by *Sandaracinobacter, Arenimonas, Ahniella* and *Elizabethkingia,* while the outlet contained *Flavobacterium, Phenylobacterium* and *Aeromonas*. At T3, the biomass of WW-RW again showed *Sandaracinobacter, Rubribacterium* and *Ahniella* with the addition of *Leptothrix* and the Cyanobacteria *Mariniradius,* while theoutlet of T3 was characterised by *Elizabethkingia, Flavobacterium, Ahniella, Sandaracinobacter* with both *Mariniradius* and *Geminocystis.*

## 3.4 Community structure and interaction

Looking at the beta diversity (PERMANOVA) as dissimilarity among communities, all factors (time, media and sampling point) and their interaction seemed to have an effect in shaping both eukaryotic and bacterial community composition (p<0.001). From the NMDS and the pairwise analyses, evaluating the extent of change in composition in relation to the environments, we found that inlets’ communities, in general, showed a higher dissimilarity from biomass and outlets for both eukaryotic (p=0.003 and p=0.006 respectively) and bacterial (p=0.001 and p=0.03 respectively) communities, while biomass and outlets were more similar (Fig. 1) between the two RWs. The general eukaryotic community of the F-RW at T2 further showed dissimilarity from WW-RW at both times (p<0.05) which were also dissimilar between each other (p<0.05).

Significant PERMANOVAs were followed by a distance-based similarity percentage test (SIMPER) to identify specific genera that cumulatively contributed to 50% of dissimilarity between each group. In general, difference among WW-RW and F-RW eukaryotic communities was led mainly by uncultured eukaryotes (17%), *Amoeboaphelidium* (15%), *Brachionus* (10%), *Paratrimastix* (7%), *Tropocyclops* (6%). These genera were again the main drivers of the difference between F-RW and WW-RW inlets (i.e. *Paratrimastix* (17%), *Tropocyclops* (17%), uncultured *Eukaryotes* (8%)) together with an uncultured *Chrysophyceae* (5%) and *Adineta* (4%). No human parasitic genera were highlighted as drivers of main difference among all samples. A LEfSe analysis revealed both statistical significance and biological relevance highlighting differences in (overrepresented) genera between the six points of sampling (biomarker discovery) (see e-supplementary materials). Here the inlets showed an enrichment in *Tetradesmus* and golden algae *Ochromonas* for WW-RW, while in FR-RW, of *Adineta* and *Chlamydaster*. WW-RW biomass showed a further enrichment in *Amoeboaphelidium*, *Desmodesmus*, *Cymbella* and *Micractinium* while outlets were enriched in uncultured fungus and Nuclearidae. On the other hand, F-RW biomass was characterised by an enrichment in *Brachionus*, *Nucelaria* and *Acricotopus* while by uncultured eukaryotes, *Dictyosphaerium* and *Poteriospumella* in the outlet. The LEfSe of the bacterial communities for both WW-RW and F-RW biomasses and for F-RW inlet did not show an enrichment in genera containing possible pathogens, while WW-RW inlet could be differentiated by an enrichment in *Bacteroidetes*, *Desulfomicrobium* and *Pseudomonas*, and the WW-RW outlet by *Elisabethkingia*, *Aeromonas* and *Cytophaga* and the F-RW outlet by *Flavobacterium* and *Chryseobacterium*.

## 3.5 Identification of biohazards

According to the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Lundholm et al., 2022) no toxic eukaryotic microalgae were encountered except for traces of *Fibrocapsa* *japonica*, a Raphidophyceanwith toxins that act as hemolysins (de Boer et al., 2012), (0.2% in the WW-RW inlet at T3). However, the replacement of the selected strain of the inoculum with spontaneous microalgae together with the change of the community over time and the high abundance of unclassified reads might point to the risk of casual development of toxin producing microalgae.

A few possible human and animal parasites (i.e. *Acanthamoeba* sp., *Balamuthia* *mandrillaris*, *Basidiobolus* *microspores*, *Blastocystis* sp., *Cryptosporidium* *parvum*, *Iodamoeba* spp., *Naegleria* *neojejuensis*, *Paralagenidium* *karlingii*, *Pythium* *aphanidermatum*. *Rhynchomonas* *nasuta,* *Tetratrichomonas*, *Trepomonas*, *Vermamoeba* *vermiformis* and *Vexillifera* spp.) or carriers of pathogens (i.e. *Echinamoeba* *exundans, Vannella* spp., *Paramecium* spp., and *Tetrahymena*) were encountered at low abundances (<3%) (see e-supplementary materials). These microorganisms all together accounted for 0.1-3.3% of the total community with highest peaks in the WW-RW inlets.

When considering the bacterial communities, the abundance of non-Cyanobacterial bacteria containing pathogenic species at genus levels indicated a total of 58 genera (see e-supplementary materials). These amounted to 2-3% and 13-22% for the F-RW and WW-RW inlet respectively. In the biomass, pathogen-containing genera accounted for the 16-29% and the 8-17% respectively and in the outlet for 28-31% and 19-25%. In a high-throughput shotgun sequencing analysis on samples collected from a WW treatment plant, Cai and Zhang (2013) revealed a pathogen abundance that averaged at 4.3% for the inlet, 1.7% for the sludge and 7.6% for the outlet with most abundant genera identified as human gut bacteria. Here, the highest number of genera were detected in WW-RW inlet (27 and 32), while generally lowest values were detected in the biomasses (WW-RW: 10 and 15; F-RW 9 and 17). Genera that have been recognised as containing opportunistic species were for example *Chryseobacterium*, *Aeromonas* known to cause gastrointestinal system infections and the genera *Brevundimonas* and Elizabethkingia containing emerging opportunistic pathogens (Ryan and Pembroke, 2018; Lin et al., 2019). *Roseomonas* has been further been recognized to cause infections (Ioannou et al., 2020) and although *Flavobacterium* are mostly harmless, some species have been identified as opportunistic or pathogenic (Waśkiewicz and Irzykowska, 2014).

In terms of toxic cyanobacteria, three genera, *Cyanobium* (0.03-0.90%), *Limnothrix* (0.03-0.05%) and *Synechocystis* (0.01-0.03%) were detected. *Cyanobium* was mainly found in the F-RW biomass at T2 while it was present in almost all samples at T3. On the other hand, *Limnothrix* was found in the outlet of both RWs at T2 and *Synechocystis* in the F-RW inlet and WW-RW outlet at T3*.* A few species of these three genera are known to produce hepatotoxic microcystins (Kubickova et al., 2019), while a few strains of *Cyanobium* are able to produce the neurotoxic saxitoxin (Testai et al., 2016).

Although the relationship between bacteria and microalgae is still obscure (Cooper and Smith, 2015) since these communities developed together within the same reactors, the study of their interactions, especially those including genera not routinely considered pathogens, could provide ecological information on removal capacity and connections. Co-occurrences were analysed between microalgae against supposed eukaryotic parasites and bacterial pathogens (see e-supplementary materials). The eukaryotic parasite that showed the highest number of interactions was *Echinamoeba* *exundans* (10 positive and 4 negative), an *L. pneumophila* carrier, followed by *Cryptosporidium parvum* (10 positive and 4 negative), a leading cause of diarrheal-related deaths in children and a major cause of cattle economic loss (Crawford and Kol, 2021). Most interestingly, both eukaryotic parasites were strongly negatively correlated with the main microalgae of these systems such as *Micractinium* and *Dictyisphaeridium*. Among bacterial genera that harbour some pathogenic exponents, highest number of interactions and positive interactions was shown by *Bacillus* (12; positive: 9), *Legionella* and *Leptospira* (both 11; positive 8). Although the *Bacillus* genera might contain pathogens, several species can enhance microalgal growth by fixing atmospheric N or producing phytohormones (Palacios et al., 2022) which can possibly be the reason for its high number of positive correlations. The highest numbers of negative interactions were detected for *Enterobacter*, *Leptothrix* and *Roseomonas* (4 negatives).

The characterisation of possible pathogens highlighted the usefulness of post-treatments and the necessity for the adoption of preventive and protective measures in the workplace to further reduce downstream infection risks according to the control hierarchy: 1) Design work processes and controls, and use adequate equipment to reduce the release of biological agents when working with RWs, even when using freshwater with fertilizer as medium and especially with prolonged exposure; 2) Apply collective protection measures at the source of the risk (e.g. ventilation in closed spaces and appropriate organisational measures); 3) Adhere strictly to practices of personal hygiene and apply individual protection measures including personal protective equipment where exposure cannot be prevented by other means (OJEC, 2000). Several operations have been pinpointed that might lead to the contact of the operator with the hazardous biological material through skin contact or inhalation of aerosols: 1) Media preparation as the operator handles inlet water and WW; 2) Monitoring of cultures and inlets as in this phase the operator samples material from the RWs to check the vitality and growth parameters; 3) Collection of biomass as the operator might intervene to manually facilitate operations; and 4) manipulation of biomass for drying or storage operations.

## 3.6 Screening of putative pathogens

A screening of the pathogens was further carried out to evaluate the infectivity potential of these systems, narrowing it down to a species level while using an easily retrieved and interpretable technique that could potentially be used as a routine monitoring step with a high number of species detected in a short time. Previous studies on WW treating plants have highlighted a set of pathogenic bacteria commonly present in WW (e.g. Cai and Zhang, 2013) most of which were included in the array used.

Enteric and pathogenic bacterial species and virulence markers were detected by qPCR in 17 out of 18 samples with WW-RW showing a number of pathogens than F-RW (Fig. 2). The most abundant pathogens retrieved were *Aeromonas spp.* (15 positives) followed by *Desulfovibrio desulfuricans* and *vulgaris* (9 positives for both), *Arcobacter butzleri* (8 positives) and *C. freundii* (7 positives). *Aeromonas* areintestinal symbionts known to cause a wide range of infections and gastrointestinal diseases; *D. desulfuricans* and *vulgaris* are considered ubiquitous opportunistic pathogens implicated in a variety of human bacterial infections (Goldstein et al., 2003); *A. butzleri* is an opportunistic pathogen associated with diarrhoea (Soelberg et al., 2020) while *C. freundii* is an important emerging human pathogen. Additional bacteria which were positive and have been highlighted as significant risks for health and of important relative infectivity (EPA, 2021) were: the enterohaemorrhagic *E. coli* (its virulence factor *eae*, 1 positive), *L. pneumophila* (1 positive), *Salmonella enterica* (1 positive) and *Vibrio* *cholerae* (5 positives).

As expected, the WW-RW was characterised by a higher pathogenic load, a higher number of both positives and inconclusives (p<0.05), when compared to the F-RW. When considered separately, both WW-RW and F-RW samples showed no differences across time, while, in terms of sampling points, WW-RW samples showed a higher number of positives and inconclusives in the inlet when compared to the biomass (p=0.02). The number of positives showed different trends within the two media, the F-RW showed an average of 1.5 positive targets in the inlet, 2.3 in the biomass and 3.0 in the outlet therefore showing an increase in pathogens species from the inlet to the outlet, possibly as the result of bacterial growth and/or external contamination due to the open set up. On the other hand, the WW-RW showed a count of 18 positive species in the inlet, 3 in the biomass and 9 in the outlet with a decrease of 81% in positive pathogens species from the inlet to the biomass and 51% to the outlet. Therefore, RWs showed potential as an effective WW biotreatment method against pathogens, with most of the retained pathogens released in the outlet and only a minor part settled with the biomass.

Similarly to the NGS, the NMDS analyses pointed to a higher diversity when considering the two inlets while this difference decreased for biomasses and outlets. The Enfit analyses highlighted species driving samples distribution pattern (intrinsic variables) (Fig. 3). These species were *Actinomyces gerencseriae* and *israelii*, *D. vulgaris*, *Enterococcus faecalis* and *faecium*, *Ruminococcus bromii* and *S. enterica* at p<0.05, while *Bifidobacterium longum*, *Enterococcus gallinarum* and *casseliflavus*, *Ruminococcus obeum* and *Yersinia enterocolitica* at p<0.01 and *Aeromonas*, *Akkermansia* *muciniphila*, *Bacteroides* *vulgatus*, *Bifidobacterium* *adolescentis*, *Lactobacillus* *gasseri* and *Morganella* *morganii* at p<0.001.

Of the pathogens highlighted as positive at T3, the eight most common targets were selected for cultivation to check the vitality of these species and the real risk of infectivity. Three targets (*Aeromonas* spp., *C. freundii*, and *Vibrio* spp.) were not detected and were classed as negative, while *L. pneumophila*, *E. coli*, *C. perfringens*, faecal coliforms and intestinal enterococci were found in the living form (Fig. 2). Of the five targets that were detected, faecal coliforms(e.g. the genera *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter*) were absent in one (WW-RW biomass) out of six samples (data not shown).

Of the species detected with the array and following plate counts, few of the relative genera were found also by the NGS analyses i.e. *Aeromonas* (0.01-5.20%) in all samples, *Akkermansia* (0.09-0.19%) only in the WW-RW inlets, *Arcobacter* (0.04-10.64%) again in the WW-RW inlets and all outlets; *Bacteroides* (0.01-4.99%) was found in all WW-RW samples (except in the T3 biomass) and in biomass and outlet of F-RW at T2; *Bifidobacterium* (0.03-0.12%) in WW-RW inlets; *Clostridium* (0.01-1.53%) was present in all WW-RW samples and in the F-RW inlets (only T2) and outlets; *Desulfovibrio* (0.02-0.82%) in all WW-RW inlets and T2 outlets; Faecal coliforms (*Enterobacter* (0.06-1.91%) in all outlets and biomasses; and *Escherichia*-*Shigella* (0.02%) in the WW-RW T2 inlet), *Legionella* (0.03-0.17%) in the T3 F-RW inlet and outlet, *Mycobacterium* (0.05-0.80%) in all outlets and inlets, *Ruminococcus* (0.15-0.30%) in WW-RW outlets, *Streptococcus* (0.01-0.08%) in all inlets and *Vibrio* (0.1%) in the T3 WW-RW outlet.

# Conclusion

Media types and sampling locations had obvious effects on community structures and pathogenic loads. Communities were variable over time with a more pronounced diversity between inlets than between biomass and outlets. The F-RW showed a significantly lower presence of pathogens than the WW-RW, which however showed a reduction from inlet to outlet. Main pathogenic genera detected were *Arcobacter* and *Elizabethkingia* while the array highlighted the importance of *Aeromonas*. The NGS and the array analyses were found to be economic and useful techniques to identify possible risks. However, plate counts, although with culturable limitations, confirmed that pathogens are not always viable.

# E-supplementary data

E-supplementary data for this work can be found in the e-version of this paper online.

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# Figure captions

**Fig. 1.** Eukaryotic (A) and bacterial (B) communities composition at genus level. Relative abundance of the average values of two replicates are shown for each bar with a cut-off >3%. Points are shown as the average values of two replicates. Non-metric multidimensional scaling (NMDS) ordination plots based on Bray-Curtis distances between samples of eukaryotic (C) and bacterial (D) communities composition. Points are shown as the average values of two replicates.

**Fig. 2.** Enteric and pathogenic bacterial species and virulence markers screening. Positive indicators in at least one sample are indicated in bold. a Highly important and emerging pathogens in drinking water. *Aeromonas* spp. includes the species *A. enteropelogenes*, *A. hydrophila*, *A. punctata*, *A. media*, *Campylobacter* spp. includes *C. coli*, *C. subantarcticus*, *C. lari*, *C. jejuni*: *Enterococcus* spp. includes *E. gallinarum*, *E. casseliflavus*, *Yersinia* spp. includes *Y. pestis*, *Y. pseudotuberculosis*. b Strain tested for cultivation at T3, *Enterococci* were tested as intestinal enterococci*.*

**Fig. 3.** Non-metric multidimensional scaling (NMDS) ordination plots of the sites based on semi-quantitative detection of enteric and pathogenic bacterial species and virulence markers with significant species driving the distribution (p<0.05).

# Tables

**Table 1.** Performance of the two raceways across time (average (± standard deviation).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |
|  | **Dilution rate** | **Biomass Concentration** | **Biomass productivity** | **Biomass productivity** | **Fv/Fm** |
|  | d-1 | g L-1 | g L-1 day-1 | g m-2 day-1 |  |
| **F-RW** |  |  |  |  |  |
|  **T1** |   | 0.48 (±0.09) | 0.09 (±0.01) | 17.40 (±3.40) | 0.61 (±0.07) |
|  **T2** | 0.14 (±0.09) | 0.45 (±0.13) | 0.09 (±0.02) | 16.23 (±4.99) | 0.30 (±0.07) |
|  **T3** | 0.14 (±0.09) | 0.51 (±0.11) | 0.10 (±0.02) | 18.39 (±4.19) | 0.27 (±0.05) |
| **WW-RW** |  |  |  |  |  |
|  **T1** | 0.15 (±0.08) | 0.43 (±0.08) | 0.08 (±0.01) | 15.74 (±3.02) | 0.49 (±0.05) |
|  **T2** | 0.14 (±0.09) | 0.43 (±0.24) | 0.08 (±0.05) | 15.20 (±9.24) | 0.41 (±0.10) |
|  **T3** | 0.14 (±0.09) | 0.43 (±0.14) | 0.08 (±0.02) | 15.76 (±5.24) | 0.42 (±0.09) |

# Figures

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**Fig. 1.**



**Fig. 2.**



**Fig. 3.**