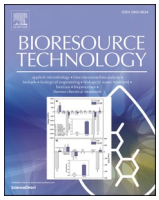
Bioresource Technology 401 (2024) 130735

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Conventional activated sludge vs. photo-sequencing batch reactor for enhanced nitrogen removal in municipal wastewater: Microalgal-bacterial consortium and pathogenic load insights

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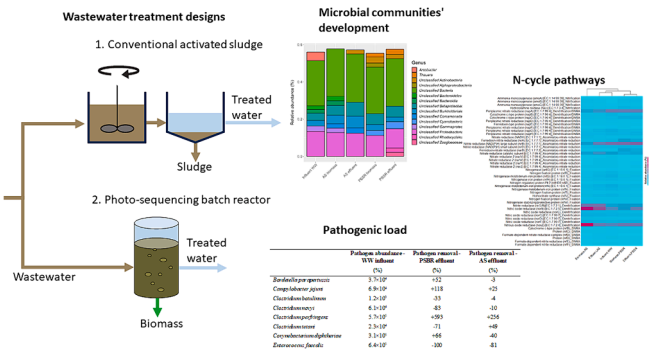
HIGHLIGHTS

- The PSBR setup enhanced denitrification due to larger microalgal-bacterial flocs.
- PSBR was enriched in photosynthetic, anammox and nitrifying microorganisms.
- The AS set up point towards enhanced GHGs production under suboptimal conditions.
- The AS setup showed a higher pathogenic load and need for protection measures.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Wastewater
Nitrogen removal
Conventional biological treatment
Microalgae
Photobioreactors
Shotgun metagenomic analysis

ABSTRACT

Municipal wastewater treatment plants are mostly based on traditional activated sludge (AS) processes. These systems are characterised by major drawbacks: high energy consumption, large amount of excess sludge and high greenhouse gases emissions. Treatment through microalgal-bacterial consortia (MBC) is an alternative and promising solution thanks to lower energy consumption and emissions, biomass production and water sanitation. Here, microbial difference between a traditional anaerobic sludge (AS) and a consortium-based system (photo-sequencing batch reactor (PSBR)) with the same wastewater inlet were characterised through shotgun metagenomics. Stable nitrification was achieved in the PSBR ensuring ammonium removal > 95 % and significant total nitrogen removal thanks to larger flocs enhancing denitrification. The new system showed enhanced

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https://doi.org/10.1016/j.biortech.2024.130735
Received 1 February 2024; Received in revised form 2 April 2024; Accepted 23 April 2024
Available online 24 April 2024
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pathogen removal, a higher abundance of photosynthetic and denitrifying microorganisms with a reduced emissions potential identifying this novel PSBR as an effective alternative to AS.

1. Introduction

The growth of global population led to an increased production of wastewater (WW). In the context of circular economy, there is a need for the sustainable treatment of WW, nutrient recovery, contaminants bioremediation and reduction of energy demand and carbon emissions (Kadam et al., 2023). The removal of organic matter and nutrients, such as nitrogen (N) and phosphorus (P), from WW is a priority to avoid eutrophication and the worsening of the receiving water bodies' quality (Kennish and de Jonge, 2011).

Worldwide, municipal WW treatment plants (MWWTPs) mostly rely on conventional biological treatments based on activated sludge (AS), thanks to the high removal efficiency, the robustness of the process and the relative simplicity of design and building (Kadam et al., 2023). The AS process is based on the ability of heterotrophic and nitrifying bacteria of oxidising biodegradable organic compounds and ammonium (NH_4^+), respectively, by using WW as a growth medium (Forster-Carneiro et al., 2010). Furthermore, N removal from WW can be obtained by coupling nitrification and denitrification; briefly, nitrifiers oxidise NH_4^+ to nitrate (NO_3^-) under aerobic conditions, while heterotrophic denitrifiers reduce NO_3^- to dinitrogen (N_2) under anoxic conditions. Although widely applied, MWWTPs based on AS processes have drawbacks that now appear as problems of upmost importance: (i) high energy consumption for mixing and mechanical aeration as the biological oxidation of organic matter and NH_4^+ is supported by external oxygen supply, (ii) large amount of excess sludge that needs to be disposed at high costs and with environmental problems (Yang et al., 2015); (iii) greenhouse gases emissions such as carbon dioxide, nitrous oxide and methane (Kadam et al., 2023).

In the last decade, WW treatment based on microalgal-bacterial consortia has been considered as a promising solution to overcome the drawbacks of MWWTPs based on AS processes (Zhang et al., 2020). Microalgae is a wide term encompassing all unicellular and simple multicellular photosynthetic micro-organisms, both prokaryotic (cyanobacteria) and eukaryotic (microalgae in a narrower definition). The benefits of these mixed consortia include: (i) photosynthetic oxygenation from microalgae to sustain bacterial oxidation instead of mechanical aeration (lower energy consumption); (ii) reduction of greenhouse gases emissions due to the capture of carbon dioxide by microalgae; (iii) excess biomass as a value-added product to produce sustainable biofuels or nutrient recovery; (iv) water sanitation (Zhang et al., 2021).

To date, many studies aimed at improving WW treatments using microalgal consortia have been performed in bench-scale photobioreactors (PBRs) (Molinuevo-Salces et al., 2019). However, the literature focuses often on the use of pure and selected microalgae strains and pre-treated WW to limit the development of spontaneous microorganisms therefore maintaining the inoculum pure as much as possible (Kang et al., 2018). Conversely, when looking towards full-scale applications of PBRs, it is essential to consider the interaction of microalgal-bacterial consortia inside the reactors with the microbial communities naturally present in the influent untreated WW that develop selective pressure on the growing consortia (Foladori et al., 2020; Clagnan et al., 2022).

Studies exploring the features of WW-borne microalgal-bacterial consortia are limited (Li et al., 2023), and thanks to the use of molecular biology methods, such as amplicon or whole genome sequencing, more information can be retrieved on population dynamics and metabolic pathways (Nagarajan et al., 2022; Clagnan et al., 2022). Due to different operational conditions, biochemical processes and composition of the consortium, microalgal-bacterial consortia could hide unexplored aspects when compared to the common knowledge acquired on conventional AS systems.

The aim of this study was to provide insights into the enhanced nitrogen removal of microalgal (mainly Cyanobacterial)-bacterial consortium (MBC) compared to conventional activated sludge, which is of practical significance to improve the knowledge on WW treatments with MBC and develop future applications with high removal efficiency, sustainability, and resource recovery. Within this study, two systems (AS and MBC) were operated in parallel and differed for the operational conditions as each system requires specific and optimised conditions, such as sludge retention time (SRT), hydraulic retention time (HRT), oxygen concentration or influent flow rates. The objective of this research was to investigate in depth the development of a specific MBC and to highlight the difference with respect to a conventional and AS system. Shotgun metagenomic analysis was performed to examine microbial difference (i) between the AS collected from a full-scale MWWTP and the microalgal-bacterial consortium developed in a lab-scale PBR treating the same influent WW; and (ii) between the influent WW and the effluents from the two systems. Dynamic changes in community and environmental variables from the influent WW to the effluents within two different biological treatments were characterised to better understand their significant microbiological differences and to create a scientific basis for future developments of design and modelling of MBC systems.

2. Materials and methods

2.1. Conventional activated sludge

The MWWTP of Trento Nord (Trento, Italy) is a full-scale plant that serves a population equivalent (PE) of 120,000. The MWWTP treats an average daily flow rate of 21,000 m^3/d and an average daily organic load of 11,000 kg COD d^{-1} (COD, Chemical Oxygen Demand).

The layout of the MWWTP includes the mechanical pre-treatments (fine screening and aerated grit chamber) followed by the primary settling with two settlers (total volume of 2,478 m^3) (Fig. 1a). Then, the pre-settled WW enters the biological treatment made up of an AS stage divided into 3 lines (total volume of 4,200 m^3) followed by secondary settlers (total volume of 5,648 m^3). The AS lines work under intermittent aeration to implement simultaneous nitrification–denitrification (SND) process. The HRT in the AS stages and secondary settlers was 4.8 h and 6.5 h, respectively (total HRT in AS was 11.3 h). The concentration of total suspended solids (TSS) in the AS stages was around 4 g TSS L^{-1} by maintaining the SRT at approximately 12 d. Effluent WW after biological treatment and secondary clarifiers is discharged in a receiving river.

2.2. Photo-sequencing batch reactor

The lab-scale PBR was operated in batch mode obtaining a configuration called photo-sequencing batch reactor (PSBR) (Fig. 1b); configuration and equipment were previously described by Petrini et al. (2018). The PSBR was operated for two years prior to this study and therefore the microalgal-bacterial consortium investigated here can be considered acclimatised to treat the influent WW, as demonstrated by the removal efficiency of COD, TSS and NH_4^+ in accordance with the regulation. In particular, the MBC spontaneously developed within the PSBR in approximately two weeks of acclimation using only pre-settled WW (from the municipality) and without the need of an inoculum of pure strains. Details about the MBC used in this study were reported previously by Petrini et al. (2020). The typical cycle of the PSBR, that includes filling-reaction-settling-discharge, lasted 48 h. The working volume was 2 L and the PSBR was managed with a photoperiod of 16 h

of light and 8 h of dark and HRT of 5.6 days (0.7 L of feeding per cycle). Although these alternating light and dark conditions produced differences in removal efficiency during the 24 h of the day (see Petrini et al., 2018), effluents from the PSBR were always discharged 48 h after the filling (48-h cycle), so that the effluents from the various cycles could be compared.

The reactor was equipped with a magnetic stirrer to mix the biomass and to maintain the flocs in suspension avoiding undesired settling. No external aeration was supplied in the reactor, and the oxygen was only produced by the photosynthetic activity of the microalgae. The PSBR was fed with real municipal WW collected after the primary settling of the full-scale MWWTP and maintained temporarily in a tank where a pump fed the pilot PSBR (see Fig. 1). The biomass concentration maintained in the PBSR during this experimental period was around 2 g TSS L^{-1}. Settling of biomass lasted 30 min at the end of each cycle ensuring an up to standards quality of the effluents with low solids (in agreement with the requirement of 35 mg TSS L^{-1} for effluent discharge). Oxidation-reduction potential (ORP), dissolved oxygen (DO) and pH were continuously monitored in the PSBR through on-line devices and real-time data acquisition. Data were used for the daily management of the reactor (data not shown).

2.3. Sampling plan

WW, biomass and effluent samples were collected at the sampling points indicated in Fig. 1.

Sampling at the full-scale MWWTP was carried out by operators as part of their weekly monitoring routine. In particular: 1) the influent pre-settled WW was collected after primary settler, 2) AS samples were taken from the combiner collecting all AS lines and 3) the effluent was collected after secondary settling but before disinfection.

For the PSBR, the influent WW was collected from the feeding tank of the pilot plant, samples of the MBC were collected from the PSBR and the effluent was taken from the tank collecting the whole volume discharged during a cycle.

2.4. Physico-chemical analyses

Influent and effluent WW were analysed for total COD, soluble COD (sCOD, measured after filtration on a 0.45-μm membrane), TSS, total Kjeldahl nitrogen (TKN), NH_4^+-N, NO_2^--N, NO_3^--N, Total N (TN), PO_4^{3-}-P and Total P (TP), according to standard methods (APHA/AWWA/WEF, 2012). The concentration of the MBC in the PSBR was measured as TSS (as per Petrini et al., 2018). Physico-chemical analyses were performed approximately once per week over a period of three months.

Statistical comparison of physico-chemical parameters in AS and PSBR systems was done by the one-way ANOVA test (data analysis tool in MS-Excel, Microsoft).

2.5. Shotgun metagenomic sequencing and bioinformatic analysis

Samples were collected in duplicate for DNA extraction. Samples (2 mL for WW and biomass in AS lines and PSBR, 200 mL for the effluents) were centrifuged for 25 min at 13,000 rpm, pellets were recovered, and the total DNA was extracted using the DNeasy PowerSoil kit (QIAGEN, Germany) according to manufacturer's instructions. The recommended initial step of "vortexing" was carried out in an Eppendorf ThermoMixer Comfort (Germany) at 1,400 rpm for 10 min. The extracted DNA was quantified using Qubit™ (Thermo Fisher Scientific, USA) and quality was checked through gel electrophoresis on 1 % (w/v) 1 × TAE agarose gels. Prior to shotgun analysis, DNA was stored at −80 °C.

Shotgun analysis was performed at FISABIO (Valencia, Spain). Samples were sequenced through the Illumina NexSeq500 platform with 150 bp paired-end chemistry. The generated sequences can be retrieved on the NCBI repository (Accession number: PRJNA1000295). The FastQC software was used to check the quality of the sequencing. Sequences were trimmed and adaptor-related sequences were eliminated using Trimmomatic v0.36 (Bolger et al., 2014). Forward and reverse sequences were joined with the FLASH program (Magoc and Salzberg, 2011), and default parameters. Kraken2 with default settings and standard RefSeq database followed by Bracken (Breitweiser et al., 2017) was used for the taxonomy assignment followed by Pavian to explore metagenomics classification results (Breitwieser & Salzberg, 2020). Functional contribution of genes from the microbiota was predicted using MG-RAST (https://www.mg-rast.org/). Co-occurrence networks were performed using the CoNet plugin for Cytoscape (http://www.cytoscape.org).

Statistical analyses for the microbiomes were performed on R (version 4.1.2) through the vegan package.

The reduction in pathogens content was calculated with the

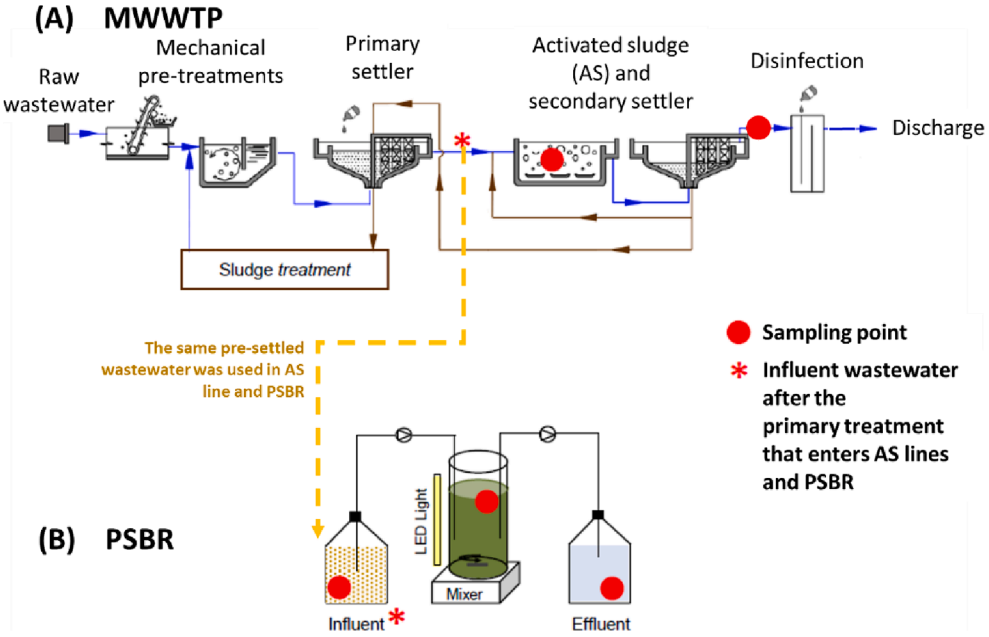

Fig. 1. Flow sheets of (A) full-scale MWWTP and AS lines and (B) lab-scale PSBR. Red dots indicate sampling points.

following equation: $((A_e - A_i) / A_i) \times 100$, were Ai and Ae are the abundance of a specific pathogen in the influent and in the effluent, respectively.

3. Results and discussion

3.1. Removal of physico-chemical parameters in activated sludge and photo-sequencing batch reactor systems

The concentrations of the main physico-chemical parameters in WW influent, AS and PSBR effluents during the monitoring period are summarised in Table 1. Both AS and PSBR were fed with pre-settled wastewater and the concentrations indicated in Table 1 are typical of municipal WW after primary settling, at this stage the removal of COD and TSS is around 40 % and 60 %, respectively. The total COD in the influent (306 ± 102 mg COD L^{-1}) was reduced to 67 ± 8 mg COD L^{-1} and to 33 ± 7 mg COD L^{-1} in the effluents from the PSBR and AS systems, respectively, leading to an average removal efficiency of 78 % in the PSBR and of 89 % in the AS system (performances statistically different, ANOVA $p > 0.05$). The sCOD, largely made up of biodegradable compounds that are readily metabolised by the biomass, was removed with an efficiency of 83 % in PSBR and of 82 % in AS system, which are statistically comparable ($p > 0.05$).

In the WW influent, the difference between total COD and sCOD is associated to the particulate organic matter (or suspended solids) that constitutes most COD. The removal of TSS was 70 % and 90 % in PSBR and AS, respectively (statistically different, $p < 0.05$). This indicates a higher capacity for physical separation of solids in the AS system compared to the PSBR and therefore suggest that the MBC should preferably be fed at not excessive concentration of particulate COD.

Influent N was made up mostly of NH_4^+-N and organic N, both included in the global parameter of TKN, while the concentrations of NO_2^--N and NO_3^--N were negligible in the influent (Table 1). With an average influent TKN of 59.9 ± 14.8 mg L^{-1}, the effluent concentrations were 0.2 ± 0.2 mg NH_4^+-N L^{-1} and 3.3 ± 0.2 mg TKN L^{-1} from PSBR, and 13.5 ± 5.4 mg NH_4^+-N L^{-1} L and 15.3 ± 5.6 mg TKN L^{-1} from AS lines. Therefore, the TKN and NH_4^+-N in the two effluents were statistically different, with a better efficiency of nitrification in the PSBR (approximately 95 %).

The concentration of TN averaged at 60.7 ± 14.7 mg TN L^{-1} in the influent which was reduced to 16.1 ± 2.8 mg TN L^{-1} and 22.8 ± 4.0 mg TN L^{-1} in PBSR and AS, respectively ($p < 0.05$). TN removal efficiency resulted thus higher in PSBR (73 %) compared to AS lines (62 %). The higher TN removal in the PSBR can be attributed to an enhanced denitrification process, as highlighted by the nitrogen mass balance considering the use of N for surplus sludge. In particular, the large flocs formed in the MBC were responsible for the improved denitrification, together with the limited availability of oxygen that is produced only by microalgae (not provided by forced mechanical aeration as in AS tanks). In the PSBR, the large aggregates were formed by filamentous microalgae and cyanobacteria (examples retrieved in this study: Synechococcales, Oscillatoriales, Nostocales) able to develop a complex and dense structure where heterotrophic bacteria find a low-oxygen environment suitable for denitrification.

Surprisingly, despite the presence of microalgae and cyanobacteria, the removal of TP and phosphate in the PSBR was remarkably lower than in the AS system (Table 1), equalling 27 % and 83 % on average, respectively.

Summarizing, the two systems appeared equally efficient when considering the removal of sCOD; the PSBR showed a higher efficiency in nitrification and TN removal due to the enhancement of denitrification, while the removal of TP in the PSBR remained scarce.

Table 1
Characterization (Av. ± St. Dev., n = 2) of the influent WW collected after primary settling and effluents from PSBR and AS systems.

Parameter	Influent WW (mg L^{-1})	PSBR effluent (mg L^{-1})	AS effluent (mg L^{-1})
Total COD	306 ± 102	67 ± 8	33 ± 7
sCOD	138 ± 49	23 ± 8	24 ± 3
TSS	133 ± 53	40 ± 8	8 ± 6
TKN	59.9 ± 14.8	3.3 ± 0.2	15.3 ± 5.6
NH_4^+-N	56.1 ± 12.0	0.2 ± 0.2	13.5 ± 5.4
NO_3^--N	0.7 ± 0.4	12.7 ± 2.9	7.2 ± 3.9
NO_2^--N	0.2 ± 0.2	0.1 ± 0.1	0.4 ± 0.4
TN	60.7 ± 14.7	16.1 ± 2.8	22.8 ± 4.0
TP	5.9 ± 1.6	4.3 ± 1.0	1.0 ± 0.2
PO_4^{3-}-P	3.8 ± 0.8	3.4 ± 1.0	0.8 ± 0.2

3.2. Bacterial community structure and composition

Bacteria accounted for 98 % of the totality of the microbial cells forming the MBC, while microalgae accounted for 2 % (approach based on flow cytometry according to Foladori et al. (2020)). Despite rare studies about direct quantifications, there is an agreement that bacteria predominate these consortia (Posadas et al., 2017). Although at low concentration, microalgae are characterized by a remarkable larger biovolume (approximately 2 orders of magnitude, Foladori et al. (2020)) than bacteria yielding a comparable photosynthetic biomass.

At phylum level, considering only the bacterial community, Proteobacteria were the most dominant phyla (56–47 %) across both systems and all sampling points followed by Actinobacteria (9–3 %) (see supplementary materials). Proteobacteria, which are involved in organic matter degradation and nutrient removal (Kersters et al., 2006), have been usually reported as the most abundant phylum in WW and activated sludge together with Bacteroidetes (Xia et al., 2018). Bacteroidetes and Firmicutes (now Bacillota) followed Proteobacteria in higher abundance in influent WW, AS biomass and AS effluent (10 %, 12 % and 10 %, for Bacteroidetes respectively and 5 %, 4 % and 2 % for Firmicutes) while Cyanobacteria, the largest group of free-living photosynthetic bacteria (Dvořák et al., 2017), was the second most abundant phylum in the PSBR biomass and its effluent, accounting for 10 % and 14 % of the community, respectively. Conversely, Cyanobacteria showed a relative abundance between 0.1 % and 0.3 % in WW, AS biomass and effluent, confirming the absence of suitable conditions for their development in the deep AS tanks (usually with depth of 4 m). While AS systems have a long history and their composition can be considered well known, MBC have been rarely explored and are considered an emerging research topic with cyanobacterial interactions remaining largely unexplored in this context (Romanis et al., 2021).

The phyla of Nitrospirae and Planctomycetes were retrieved at immediately lower abundances mainly within the biomass and effluent of the PSBR (1 % and 2 %, respectively for Nitrospirae and at 1 % in both samples for Planctomycetes). The phylum Nitrospirae belongs to nitrite oxidizing bacteria (NOB) (additionally, a subset of the *Nitrospira* genus is able to carry out the complete ammonia oxidation (comammox)) and thus it is involved in nitrification and N removal (Xia et al., 2018). The role of comammox bacteria in full-scale WWTPs is still under study however, they seem to dominate in N removal systems under low nitrite and low dissolved oxygen conditions (Mehrani et al., 2022). On the other hand, microalgae have been shown to possibly enrich functional groups associated with comammox (Jin et al., 2023). At the same time, *Planctomycetes* are also involved in N removal due to the presence of anaerobic ammonium oxidation (anammox) bacteria (Fuerst and Sagulenko, 2011). These two phyla differ for the aerobic (Nitrospirae) or generally anaerobic (Planctomycetes) pathways for N (more specifically NH_4^+ and NO_2^-) removal and their requirements of dissolved oxygen (anoxic conditions for anammox (Cho et al., 2020), while high DO for comammox (Gonzalez-Martinez et al., 2016)). The alternation of light and dark within PBRs could possibly favour the availability of oxygen with high variations during the day, favouring the growth and metabolism of both groups possibly favouring the higher removal of TN seen

in the PSBR.

When looking at the totality of the genera, all samples were characterised by a high abundance of unclassified genera (82–84 %) (see supplementary materials). Changes in the bacterial community structure were analysed for the top OTUs accounting for more than 1 % of the abundance in at least one sample (Fig. 2). In all samples the most abundant OTU was an unclassified Bacteria (10–13 %) followed by an unclassified Proteobacteria (4–8 %) and by an unclassified Betaproteobacteria (3–7 %). The samples from the PSBR were characterised, at highest classified level, by OTUs belonging to Cyanobacteria, Rhodocyclales, Actinobacteria, *Thauera* (common in polluted soil and WW (Liu et al., 2006)) and Zoogloeaceae. The order Rhodocyclales is widely distributed in WW treatment systems with its sub-lineages showing denitrifying activity (e.g. *Thauera*) (Wang et al., 2020). AS samples were defined by species belonging to Burkholderiales, *Arcobacter,* Flavobacteriaceae, all commonly detected in AS-based WWTPs (Shchegolkova et al., 2016). Together with the influent WW, AS were further characterised by bacteria such as Campylobacteraceae and Pseudomonadales, which comprise commensal or pathogenic bacteria (Rinninella et al., 2019).

In general, biomasses showed lower observed and Chao1 richness but higher Shannon diversity and Pielou's evenness than influents and effluents ($p < 0.05$) while no differences were seen in terms of Simpson diversity (see supplementary materials).

Solid effluents originate from a small fraction of floating biomass flakes escaping from the reactors after the secondary sedimentation. Within this study, TSS concentration between influent and effluent has a ratio of 16:1 for the AS and of 3.3:1 in the PSBR (i.e. WW: 0.133 g/L; AS biomass: 4.767 g/L; AS effluent: 0.008 g/L; PSBR biomass: 2.056 g/L; PSBR effluent: 0.040 g/L). In the AS reactor, the contribution of the microorganisms of the WW inlet is prevalent in the effluent (Fig. 2b); microorganisms that are not stopped by primary sedimentation do not bioflocculate and are possibly not trapped or incorporated within the AS flocs and will therefore bypass secondary sedimentation and remain in suspension and come out in the effluent. On the contrary, when considering the PSBR, biomass and effluent had a higher similarity than the WW.

Permanova analysis showed a further influence of the system and of the sampling points and their interaction in shaping bacterial diversity ($p < 0.05$).

Summarizing, both reactors showed the presence of bacterial genera commonly present in WW with a higher abundance of genera related to pathogens in the AS while photosynthetic and denitrifying microorganisms in the PSBR. Bacteria connected to the anammox and nitrification pathway were further highlighted in connection to N-cycling within the PSBR. Whitin the AS, bacteria seemed to bypass

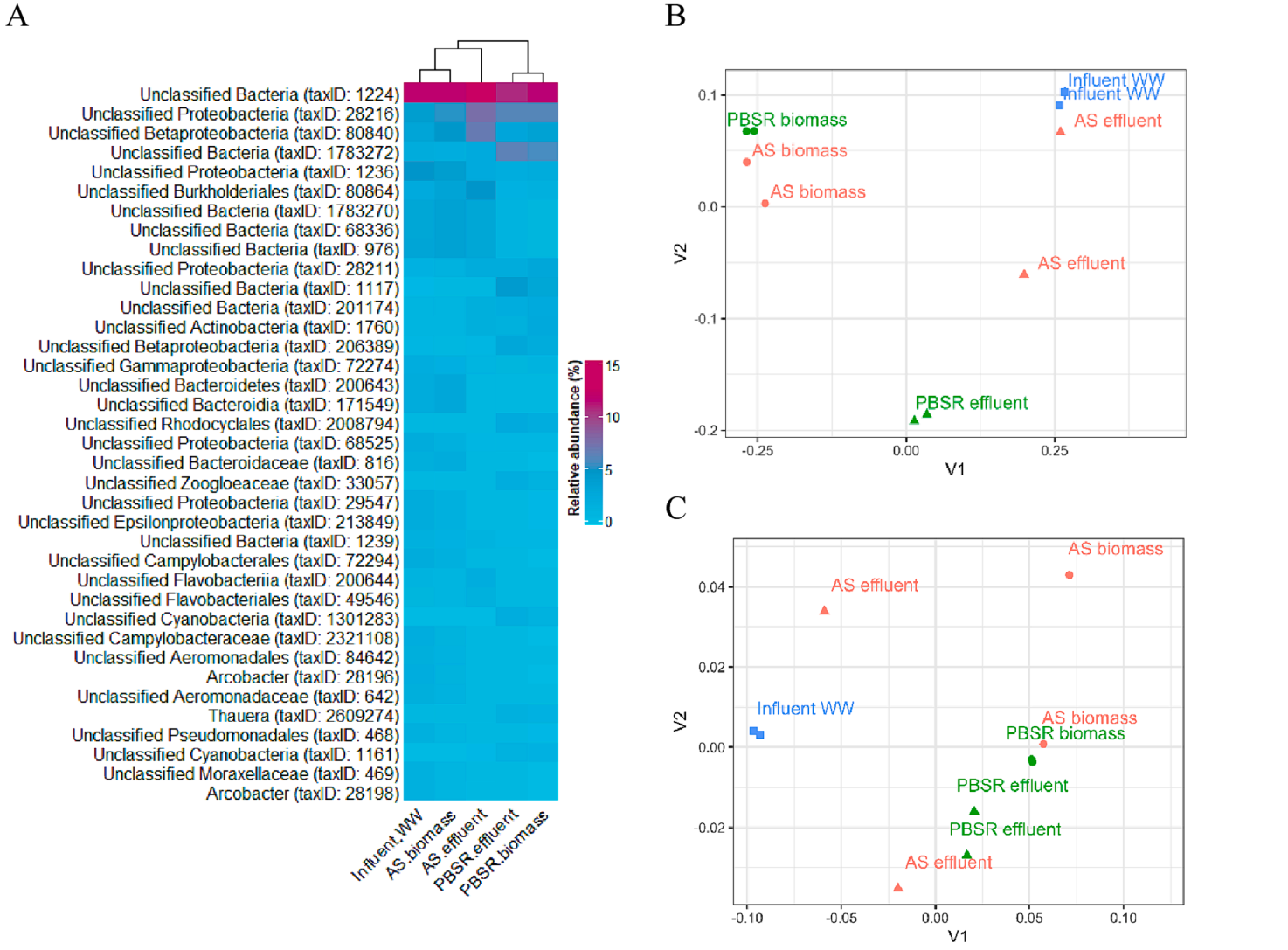

Fig. 2. Heatmap representation of the prokaryotic (A) community at highest classification level for the top OTUs (>1% in at least one sample) (Av., n = 2). Principal coordinates analysis of prokaryotic (B) and eukaryotic (C) diversity community patterns of influent WW, PSBR and AS biomasses and effluents.

sedimentation leading to a higher similarity between WW and effluent while WW and effluent differed more within the PSBR.

3.3. Bacterial pathogens and biohazard risks

Wastewater harbours many pathogenic microorganisms that may affect workers or people living in the surrounding areas through contact or aerosol inhalation (Sarker, 2022). The presence and relative abundance of human pathogens, in this case bacteria possessing one or more virulence factors against humans was examined, as by Krustok et al. (2015), in both PSBR and AS systems.

Pseudomonas aeruginosa was the most abundant pathogen at all the sampling points. When compared to influent WW, pathogenic bacteria showed a decrease in abundance (−30 %) within the PSBR effluent while an increase (+15 %) within the AS effluent indicating that PSBR was overall more capable to reduce the number of pathogens similarly to other studies (Ruas et al., 2021) (Table 2). In particular, the environment and the community of the PSBR were more efficient in reducing *Enterococcus faecalis, Vibrio cholerae, Yersinia enterocolitica, Haemophilus parainfluenzae*, while it mostly increased the number of *Clostridium perfringens, Mycobacterium tuberculosis, Helicobacter pylori, Campylobacter jejuni*. Conversely, the AS system reduced to a greater extent the number of *Streptococcus agalactiae, Staphylococcus aureus, Enterococcus faecalis, Haemophilus parainfluenzae* while increased the number of *Clostridium perfringens, Helicobacter pylori, Haemophilus influenzae, Vibrio cholerae*. Different increase or decrease of various species within the same genera, might be linked to different sensitivity across species for certain environmental parameters (e.g. oxygen concentration for anaerobic bacteria) or differential resistance to other compound such as toxins and bactericidal substances (Ruas et al., 2022).

It is worth noting that Firmicutes, which contains bacteria reported as opportunistic pathogens in human gut (Rinninella et al., 2019), showed higher abundance in influent WW and in the AS effluent (4.6 % and 3.7 %, respectively) while its abundance was below 3 % in the other samples.

According to the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Lundholm et al., 2009), several toxic cyanobacteria highlighted in this list were encountered across all samples but at highest abundance in the PBSR (e.g., *Microcystis aeruginosa, Microcystis paniformis, Microcystis viridis, Planktothrix agardhii, Anabaena cylindrica, Calothrix parietina, Cylindrospermum stagnale, Nodularia spumigena, Raphidiopsis curvata* and *Trichormus variabilis*).

Since only a limited percentage of bacteria are culturable, it is important to monitor the system not only with traditional culture methods but also with the metagenomic analyses to understand the pathogenic potential of the system, i.e. how many and what potentially dangerous bacteria are present and released from the system, possibly not culturable, that can have an health and/or environmental impact in case of the reuse of water and biomass.

In general, and in accordance to literature, PSBR are more efficient in pathogen removal than AS reactors however personal protection systems need to be put in place when working with these system.

Table 2
Pathogen reduction from the inlet WW to the effluent of both PSBR and AS reactors (n = 2).

	Pathogen abundance − WW influent (%)	Pathogen removal − PSBR effluent (%)	Pathogen removal − AS effluent (%)
Bordetella parapertussis	3.7×10^4	+52	−3
Campylobacter jejuni	6.9×10^4	+118	+25
Clostridium botulinum	1.2×10^3	-33	−4
Clostridium novyi	6.1×10^4	−83	−10
Clostridium perfringens	5.7×10^5	+593	+256
Clostridium tetani	2.3×10^4	−71	+49
Corynebacterium diphtheriae	3.1×10^5	+66	−40
Enterococcus faecalis	6.4×10^5	−100	−81
Escherichia coli	6.5×10^4	−80	-33
Haemophilus influenzae	2.0×10^5	−24	+146
Haemophilus parainfluenzae	9.0×10^4	−90	−62
Helicobacter pylori	7.9×10^5	+173	+249
Klebsiella pneumoniae	4.2×10^3	−88	-23
Legionella pneumophila	5.7×10^4	−41	−40
Mycobacterium tuberculosis	4.8×10^4	+344	+25
Neisseria meningitidis	1.8×10^4	−75	−30
Pseudomonas aeruginosa	7.6×10^2	−26	+26
Salmonella enterica	6.8×10^3	−57	−25
Staphylococcus aureus	1.3×10^4	−84	−85
Streptococcus agalactiae	2.0×10^5	+4	−100
Streptococcus pseudopneumoniae	4.6×10^5	−1	−7
Vibrio cholerae	2.9×10^5	−100	+50
Vibrio parahaemolyticus	1.0×10^4	−41	+25
Yersinia enterocolitica	1.6×10^3	−99	−52
Yersinia pseudotuberculosis	2.0×10^3	−68	−50
Total	9.7×10^2	−31	+15

3.4. Bacterial and cyanobacterial interactions

Co-occurrence networks were analysed to understand bacterial-cyanobacterial interactions within the PSBR and AS context. Bacterial networks were characterized by the dominance of negative co-occurrences within all sampling points (55 for the influent WW, 29 and 54 for AS biomass and effluent respectively and 14 and 48 for PBSR biomass and effluent) (see supplementary materials). In terms of similarity of the retrieved co-occurrences, the two effluents seemed to have more in common between them and to the WW influent than to the two biomasses.

Most cyanobacterial genera (42 out of 51) had a negative interaction with influent WW and the two effluents, meaning that they developed within the reactor and were retained in the biomass.

Focusing on the positive co-occurrences, Nostoc had the highest interactions (24) in terms of cyanobacteria. In general, most cyanobacteria presented highest number of positive interactions across the whole dataset. Among non-photosynthetic bacteria, bacteria found having a high amount of positive interaction were mainly characterised by microalgal growth stimulating properties. The highest number of positive interactions were retrieved for *Azospirillum* (21), generally known plant growth promoting bacteria, followed by *Streptomyces* (18), and other Actinobacteria. *Streptomyces,* known antibiotic producers, have already shown a symbiotic interaction with microalgae, probably due to phytohormones producing characteristics (Lakshmikandan et al., 2021). Similarly, the presence of Actinobacteria has shown to enhance microalgal growth (Perera et al., 2022). The highest number of negative interactions were achieved by *Blautia* (50), followed by *Sulfurospirillum* (16) and *Anaerobutyricum* (15), all bacteria widely occurring in mammals' guts (Liu et al., 2021; Seegers et al., 2022). This negative interaction with intestinal bacteria might be due to the WW depuration technique that can occur from the presence of microalgae (Mohsenpour et al., 2021). Considering the main pathogens found in Section 3.3, no particular co-occurrences were highlighted indicating no significant interaction with other bacteria or cyanobacteria and no particular relationship with the five sampling points.

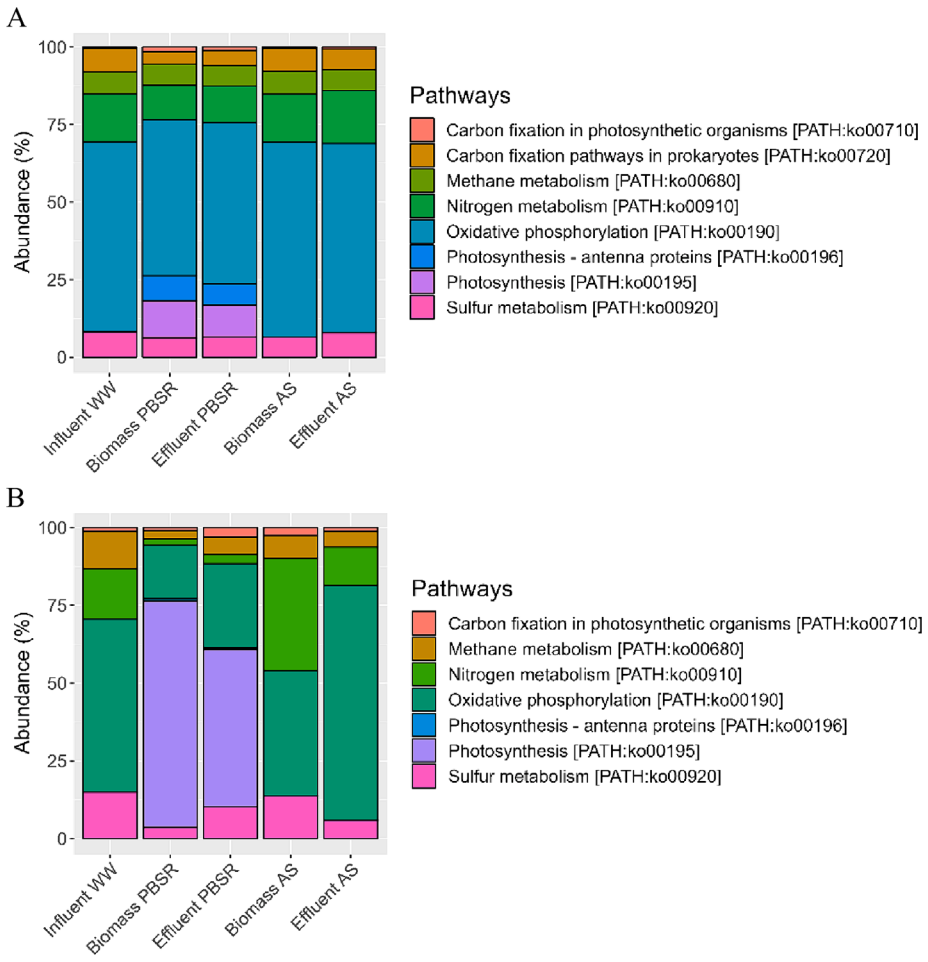

Fig. 3. Variation in the relative abundances of energy metabolism pathways for bacteria (A) and eukaryotes (Plants and Protozoa) (B) in influent WW, PBSR and AS biomasses and effluents (Av., n = 2).

3.5. Phototrophic eukaryotic community

In addition to Cyanobacteria, the phototrophic community, although more limited in number and abundance, was further investigated for diatoms and microalgae.

Regarding the kingdom of Plantae, the phylum of Rhodophyta (red algae) accounted for 0.0001–0.002 % of Eukaryota within WW, AS biomass and AS effluent and 0.002 % in the PSBR biomass and its effluent, while the rest belonged to Viridiplantae (19.4–7.5 %) (see supplementary materials). *Rhodophyta* were represented by a single species, *Cyanidioschyzon merolae*, a unicellular haploid red alga mainly found in sulphate-rich hot springs (Matsuzaki et al., 2004). Of the Viridiplantae group, the vast majority of the species belonged to Streptophyta (7.5–19.4 %) and in particular to the phylum of Tracheophyta with a small percentage of Bryophyta (*Physcomitrella*: 0.010–0.020 %). Almost all the Tracheophyta identified were species attributed to human consumption and use (i.e. *Malus* sp., *Prunus* sp., *Coffea* sp., *Quercus* sp., *Oryza sativa, Populus* sp.) and thus present originally in the raw WW coming from the sewerage. A small portion of *Viridiplantae* belonged to the phylum of *Chlorophyta* which was slightly more abundant within the PSBR biomass (0.089 %) and its effluent (0.080 %) rather than in WW (0.052 %) or in AS biomass (0.058 %) or its effluent (0.069 %). In more detail, within the Chlorophyta phylum two main families were present: (1) *Mamiellaceae* (small green algae mostly dominating in temperate coastal as well as arctic pelagic waters (Worden et al., 2009)) represented by the genus *Micromonas* (a marine picoeukaryote), and (2) the Bathycoccaceae family. The family of Bathycoccaceae (green algae in both fresh and marine waters) was mainly present with the genera *Bathycoccus* and *Ostreococcus*. Unsurprisingly, *Chlorella* spp., which have been frequently reported in the literature in PBRs and very frequently used as inocula in these systems (Wang et al., 2016), was not present in the PSBR or its effluent. The PSBR used in this study was inoculated at the beginning with *Chlorella vulgaris*, but after an acclimation of a couple of months and under steady-state conditions, it was demonstrated that the inoculation of microalgae was not essential and the selective pressure induced by real WW was able to change completely the composition of microalgae and *C. vulgaris* became negligible (Petrini et al., 2020). In particular, the present study investigated the PSBR biomass after two years of operation and continuous feeding with real WW from inoculation with *C. vulgaris*.

Protozoa and Chromista accounted for 0.19–0.22 % of Eukaryota. Most of the genera encountered in all samples were parasitic (i.e. *Plasmodium*, *Theileria*, *Babesia*, *Eimeriorina*, *Leishmania*, *Trypanozoon*), generally from animal origin (e.g. *Plasmodium chabaudi, Theileria equi, Leishmania infantum*). *Dictyostelium* and *Paramecium* (possibly photosynthetic by endosymbiosis of algae) were also found as free-living organisms (Table 2) (Kodama and Fujishima, 2022). A phototrophic community was present only in the PSBR biomass and its effluent and it was mainly composed by four phyla. Of these, three main phyla were identified: *Cryptophyta* (marine and freshwater nanoalgae), *Bacillariophyta* (diatoms) and *Cercozoa* (*Chlorarachniophyceae*, marine algae). Main genera for *Cryptophyta* were *Guillardia*, *Cryptomonas* (microalgae containing species of interest to WW remediation and biofuel, Tawfik et al., 2022) and *Hemiselmis*. *Bacillariophyta* was mainly characterised by *Thalassiosira pseudonana* (both marine and freshwater diatom) and *Phaeodactylum tricornutum* (used as biofuel precursor and recombinant

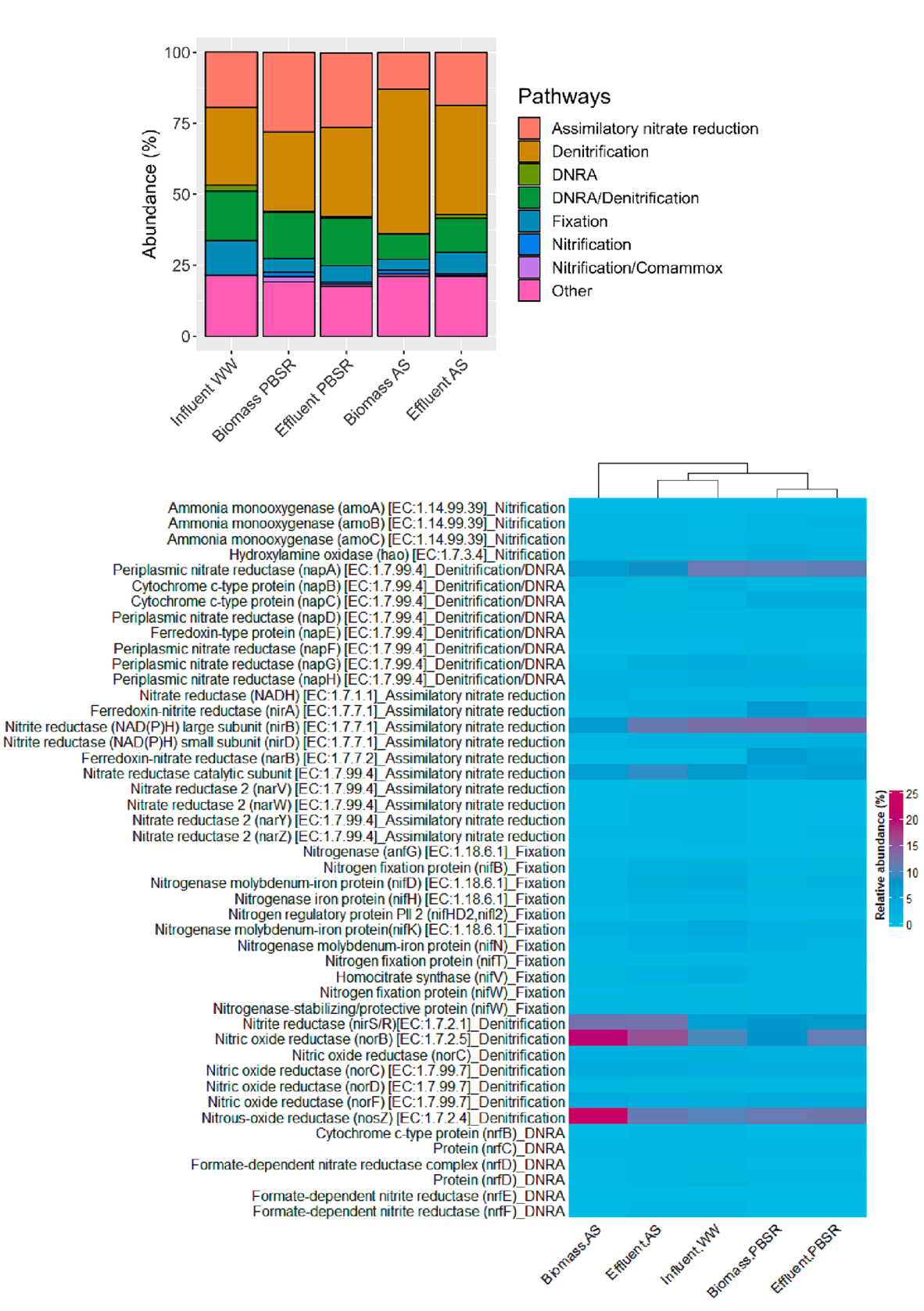

Fig. 4. Variation in the relative abundance of main bacterial N-cycle pathways in influent WW, PBSR and AS biomasses and effluents (A) (Av., n = 2). Heatmap representation of the gene abundances for the main bacterial N- cycle pathways (B) (Av., n = 2).

protein expression host) while *Bigelowiella* was the main genus of *Cercozoa* (Hempel et al., 2011).

As expected, samples that were mainly impacted by the phototrophic eukaryotic community of both Protozoa/Chromista and Plantae were the PSBR biomass and its effluent (see supplementary materials).

Similarly to the bacterial communities, an influence of system, sampling points and their interaction was identified in modifying the bacterial communities ($p < 0.05$).

In summary, PSBR was characterised by spontaneous bloom eukaryotic mainly composed by Rhodophyta and Chlorophyta. As expected, numerous parasitic microorganisms highlighting the need for the adoption of preventive and protective measures following the control hierarchy as per Clagnan et al. (2022).

3.6. Energy metabolisms and nitrogen bioremediation

When considering the energy metabolisms using MG-RAST, the most common pathway across all samples was the classic oxidative phosphorylation. Additionally, 44 genes were retrieved for the photosynthetic process in bacteria (PATH: ko00195). This photosynthetic pathway accounted for the 12.1 % and 10.2 % of the energy metabolism genes in PSBR biomass and its effluent, respectively, while between 0.00 % and 0.04 % within the other samples (Fig. 3). Additionally, the genes for the antenna proteins [PATH: ko00196] accounted for 8.06 % and 6.98 % of the energy metabolism genes in PSBR biomass and effluent and for 0.00 %-0.01 % in the other samples. Within PSBR biomass and its effluent, the photosynthetic pathway accounted for 72.82 % and 50.62 % of the energy metabolism genes for Eukarya, while in the other samples these genes were not present.

An important role especially in WW and AS samples was played by the N metabolism [PATH: ko00910] (Figs. 3 and 4). The N cycle is a group of reductive and oxidative transformational processes controlling the N distribution in global ecosystems and is highly influenced by the water continuum (Cabello et al., 2004).

Predominant pathways of N bioremediation are nitrification, followed by denitrification; through the former, NH_4^+ is oxidised to NO_3^-, while the latter reduces NO_3^- to N_2 (Rivett et al., 2008). These two processes are carried out through a set of sequential reactions and may produce nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) as undesirable intermediate compounds, which could be released in the environment. The outcome of these processes and attenuation of N-species is regulated by many environmental factors and by a large set of minor alternative pathways (e.g. dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation (Anammox) and complete ammonia oxidation (Commamox) (Saggar et al., 2013). DNRA is a process where NO_3^- is reduced to NO_2^- and then to NH_4^+ generally under anaerobic conditions, Anammox consist in the anaerobic reduction of NO_2– to NO, which in turn is oxidized to N_2 while Commamox is the complete nitrification where the whole pathway is carried out within a single bacterium.

Whitin this study, denitrification (in terms of gene abundance) was particularly enhanced within the AS reactor while fixation in the PSBR. In general, 50 genes were retrieved for the N metabolic pathways; among these, 4 were removed as not immediately correlated to the N cycle, these 4 genes accounted for the 17.7–21.3 % of the abundance. Considering the genes specific for the N cycle, the gene abundances and composition were similar between biomass and effluent of the PSBR and between influent WW and AS effluent (Fig. 4). Considering influent WW as a starting point, the abundance of the genes for assimilatory nitrate reduction increased in the PSBR and its effluent while decreased in the AS biomass (Fig. 4). Denitrification genes had a slightly increasing trend from influent WW to PBSR effluent and AS effluent with the highest number within the AS biomass. Dissimilatory nitrate reduction to ammonium (DNRA) genes showed a decreasing trend from WW in both PSBR and AS biomasses. Regarding shared genes between DNRA and denitrification, influent WW had a higher concentration than activated sludge. Fixation genes also showed a trend of reduction in abundance from influent WW to both effluents. Nitrification genes showed an increasing trend from influent WW to both effluents. A small portion of genes (Other) was further correlated to the N cycle (i.e. nitronate monooxygenase, carbonic anhydrase and hydroxylamine reductase) but not directly to a specific pathway.

The AS biomass, and to a lesser extent the effluent, showed a higher abundance of norB and nosZ (and a trend of higher nirS/R) genes (Fig. 4); these genes code for the two final steps of denitrification, respectively, the reduction of NO to N_2O and of N_2O to N_2 (nir codes for the reduction of NO_2^- to NO). This might lead to an enhanced production of greenhouse gasses (i.e. NO and N_2O) withing the AS biomass and higher environmental pollution. However, under optimal condition for denitrification the high amount of nosZ might suggest that the process can be carried out completely with the release of only N_2.

4. Conclusions

Stable nitrification occurred in PSBR ensuring TKN and ammonium removal (>95 %). TN reduction was higher in PSBR (73 %) than in AS (62 %) as larger microalgal-bacterial flocs might enhance denitrification. Efficient nitrification and N removal, without external aeration, occurs when photosynthetic oxidation sustains both heterotrophic bacteria and nitrifiers. Long HRTs are necessary (here, 48 h) for optimal photosynthetic biomass growth; short HRTs and high COD loads favor heterotrophic bacteria and hinder autotrophic microorganisms. AS showed higher pathogens' abundance, stressing the need of personal protection systems. The PSBR was characterised by anammox and nitrification bacteria highlighting the presence of alternative N pathways.

5. Statements and declarations

Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors.

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Elisa Clagnan: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Serena Petrini:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Pioli:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Federica Piergiacomo:** Investigation. **Atif Aziz Chowdhury:** Investigation. **Lorenzo Brusetti:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Paola Foladori:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paola Foladori reports financial support was provided by University of Trento. Serena Petrini reports financial support was provided by University of Trento. Silvia Pioli reports financial support was provided by Free University of Bozen-Bolzano. Federica Piergiacomo reports financial support was provided by Free University of Bozen-Bolzano. Atif Aziz Chowdhury reports financial support was provided by Free University of Bozen-Bolzano. Clagnan Elisa reports financial support was provided by Free University of Bozen-Bolzano. Brusetti Lorenzo reports financial

support was provided by Free University of Bozen-Bolzano. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

Data availability

Data will be made available on request.

Acknowledgement

Special thank goes to Dr Andrea Polo and Dr Luigimaria Borruso for handling the samples at the initial stages.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130735.

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