Journal of Environmental Management 371 (2024) 123186

Contents lists available at ScienceDirect

Journal of Environmental Management

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

Research article

Microalgae-bacteria consortia dynamics in a long term operated membrane-coupled high-rate algal pond (MHRAP)

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ARTICLE INFO

Keywords:
16S/18S rDNA
Microalgae-bacteria consortia
Hydraulic retention time (HRT)
Wastewater stream
Microbial ecology

ABSTRACT

Traditional activated sludge-based technologies have significant drawbacks, including high energy requirements and greenhouse gas emissions. Microalgae-based processes offer a promising, low-cost, and environmentally friendly alternative. However, the knowledge of treatment systems based on microalgae-bacteria consortia is limited, and even more so is their microbial composition and its relationship with operational parameters. Thus, this study explores the dynamics of microalgae-bacteria consortia in a long-term operated membrane-coupled high-rate algal pond (MHRAP) for wastewater treatment. For this, a pilot-scale MHRAP plant, located in a wastewater treatment plant in Valencia (Spain), was monitored under various hydraulic retention times (HRT) and wastewater influents: i) effluent from a primary settler and ii) effluent form pre-treatment. The biomass retention time was kept constant at 6 days. The composition of the bacterial community was studied through 16S rDNA sequencing, while 18S rDNA sequencing was used to study the microalgae. The results indicate that shorter HRTs significantly increased bacterial diversity, but not eukarya. Principal Co-ordinates Analysis (PCoA) revealed that the HRT and the incoming wastewater quality control the type of the bacterial populations. However, this effect was not observed in eukaryotic organisms. The dominant microalgae genera identified were *Desmodesmus* and *Coelastrella*, with *Coelastrella* becoming more prevalent at shorter HRTs. For bacteria, *Verrumicrobiota* dominated (18–56%) at high HRT while Proteobacteria was dominant (28–44%) at HRTs below 6 days. The changes observed in the bacterial composition, including the ammonia oxidizing bacteria (AOB) community (mainly Nitrosomonas), suggest that photo-inhibition could be taking place. The nitrite oxidizing bacteria (NOB) community was dominated by *Nitrospira* and *Candidatus Nitrotoga*. Operational parameters such as light intensity, pH, and nitrite concentration were found to significantly influence the microbial community structure. Higher light intensity and alkaline pH favored the growth of *Desmodesmus*, while *Coelastrella* thrived under lower HRTs. Bacterial diversity plays a crucial role in the treatment process, while microalgae primarily support aerobic bacterial processes by providing oxygen. These findings contribute to a deeper understanding of the complex biological processes in microalgae-bacteria consortia and offer insights into improving wastewater treatment technologies.

1. Introduction

Using activated sludge-based wastewater technologies involves certain disadvantages, including high energy requirements, loss of nutrients and the environmental impact of greenhouse gas emissions (Chai et al., 2015; Ozgun et al., 2013; Rahman et al., 2016; Sabeen et al., 2018). However, interesting low-cost and environmentally friendly alternatives to conventional activated sludge are now being considered, especially those using microalgae processes (Acién et al., 2016; González-Camejo et al., 2020; Seco et al., 2018). The microalgae genera used for treating wastewater reproduce rapidly and can tolerate intense light and high temperatures (Bernard and Rémond, 2012). Types such as *Scenedesmus* and *Chlorella* the most commonly for wastewater treatment (González-Camejo et al., 2020; Mantovani et al., 2020; Marazzi et al., 2019) in combination with bacteria.

The microalgae-bacteria interactions include a wide range of ecological relationships (mutualism, amensalism, competition, etc.), which can be either beneficial or detrimental to microalgal growth

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https://doi.org/10.1016/j.jenvman.2024.123186
Received 30 July 2024; Received in revised form 3 October 2024; Accepted 31 October 2024

(Ramanan et al., 2016; Zhang et al., 2020). Together, they can enhance nutrient removal and recovery through mutualistic relationships (Zhang et al., 2020), including the widely used self-sustaining gas exchange (Foladori et al., 2018; Zhang et al., 2020). Microalgae provide oxygen to aerobic bacteria and in return receive inorganic carbon for photosynthesis (Zhang et al., 2020). As mentioned above, their interactions are not only restricted to mutualism, but also include amensalism and competition, which can negatively affect microalgae growth. Some bacteria can influence microalgae activity by synthesizing strong algaecidal metabolites (Lian et al., 2018), while nitrifying bacteria compete with microalgae for ammonium.

The microorganisms in the microalgae-bacteria consortium could clearly have unpredictable and sometimes detrimental effects on microalgae growth and on effectively removing pollutants from wastewater. This Interrelationship is strongly affected by the operating conditions in the reactor and ever-changing environmental conditions. Identifying the most important groups of microalgae and bacteria for wastewater treatment and analyzing the impact of operational and environmental parameters on the ecological structure, could thus contribute to improving treatment process. Few studies have been made to date on associating the biological diversity obtained from the operating parameters and efficient pollutant removal by means of microalgae-bacteria treatment systems (Sánchez-Zurano et al., 2020a; Ye et al., 2016). Nevertheless, none of the previous studies have examined the impact of hydraulic retention time (HRT), when operating at constant BRT, on the ecology of the microalgae-bacteria community and the efficiency of wastewater treatment in an outdoor pilot-scale reactor, without external aeration, artificial lighting, or control of culture pH and temperature in an outdoor pilot-scale membrane high rate algal pond (MHRAP).

The effectiveness of integrating microalgae-bacteria consortia in wastewater treatments depends on establishing a favourable biotic community. If this balance is not maintained, the efficiency of the microalgal and bacterial communities may decline. Microalgae blooms can cause the culture pH to exceed the optimal range for bacteria (Foladori et al., 2018), and photosynthesis alone may not sufficiently support bacterial activity in a bacteria-dominated structure. The balance of microalgae-bacteria consortia fluctuates over time due to constantly changing environmental conditions (e.g., temperature, light intensity, influent composition, etc.) and operational parameters (HRT and biomass retention time (BRT)) (González-Camejo et al., 2018)). By optimizing HRT and/or BRT, the nitrogen and phosphorus availability can be adjusted to support the appropriate microalgae-bacteria communities, thereby enhancing removal efficiency. Similarly, different influent water qualities allow studying the suitability of the microalgae-bacteria consortium for treating different streams in a wastewater treatment plant (WWTP).

The aim of this work is therefore to assess the diversity and composition of the microalgae-bacteria community diversity created in a MHRAP to evaluate the changes in the diversity and structure of the microalgae and bacteria groups at different hydraulic retention times (HRT) and with a variety of wastewater influents. This approach is expected to improve our understanding of the complex biological processes occurring in microalgae-bacteria consortia for wastewater treatment.

2. Materials and methods

2.1. MHRAP pilot plant

The continuous pilot-scale MHRAP plant at the "Cuenca del Carraixet" wastewater treatment plant, WWTP (39°30′04.0''N 0°20′00.1''W, Valencia, Spain) was operated outdoors, so that the culture temperature and sunlight irradiance depended on the current weather. The plant consisted of a high-rate algal pond (HRAP) connected to a membrane tank (MT) module. The HRAP had a working volume of 318.75 L, a solar irradiance area of 1.275 m^2 (2.55 × 0.5 m) and a liquid depth of 0.25 m. The reactor was continuously stirred by a single paddle wheel. The volume of the MT module was 14 L and the filtration surface covered 3.4 m^2 in the hollow-fiber membrane system (PURON® Koch Membrane Systems (PUR-PSH31), with 0.03 μm pore size). An air-sparging was used to reduce membrane fouling.

Various on-line sensors were installed in the MHRAP pilot plant to obtain real-time information on the process performance, including: a pH sensor (pHD sc DPD1R1, Hach Lange); a dissolved oxygen-temperature sensor (LDO Hach Lange); an AN-ISE sensor (AN-ISE LXV440.99.00001, Hach Lange) to record NH_4-N and NO_X-N (sum of NO_2-N and NO_3-N); and a light irradiance sensor (Apogee Quantum SQ-200) set up on the surface of the HRAP for measuring the photosynthetic active radiation (PAR).

2.2. MHRAP pilot-plant operation

A natural process of the initial ecological succession of the indigenous microorganisms was allowed to take place, first operated in batch mode until the pseudo-stationary phase (i.e. lack of significant variations in the biomass concentration measured as volatile suspended solids (VSS)), followed by continuous feeding at the desired HRT. After the calculation of the theoretical biomass retention time (BRT) which promotes maximum biomass productivity (Ruiz et al., 2013), the BRT was set to 6 days. It must be highlighted that microalgae growth is directly related with its nutrient removal capability. The HRAP was fed with two wastewater streams from the "Cuenca del Carraixet" WWTP: effluent from primary settling (EPS) and from pre-treatment (screening, de-gritter, and grease removal) (EPT) (see Table 1). Five different HRT were considered: 6, 4 and 2-day HRTs for effluent from the primary settling unit; and 2 and 1-day HRT with the pre-treatment effluent (Table 2). The following labels were used to refer each period: HRT_(operating HRT)_EP(S for effluent from primary settling or T for pre-treatment effluent), so that the 6-day HRT period with primary settling effluent is referred as HRT_6_EPS. The order in which experiment were conducted can be seen in Table 2, column "Date". First three experiments were done with EPS and HRTs of 6, 4 and 2, consecutively. Next two experiments were performed with EPT and HRTs of 2 and 1, in that order.

2.3. Sampling and analytical methods

Samples from the MHRAP culture, influent and permeate were collected in duplicate for each pseudo-stationary state, i.e., biomass concentration measured as volatile suspended solids (VSS) remained constant. Ammonium (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N) and

Table 1
Average composition of effluent from primary settling and pre-treatment supplied to MHRAP pilot plant. Abbreviations: total suspended solids (TSS), volatile suspended solids (VSS), total and soluble chemical oxygen demand (T-COD and S-COD, respectively), total and soluble biological oxygen demand (T-BOD and S-BOD, respectively), total nitrogen (T-N), ammonium nitrogen (NH_4-N), total phosphorus (T-P) and phosphate phosphorus (PO_4-P).

Parameter		Effluent from primary settling (EPS)			Effluent from pre-treatment (EPT)		
pH		7.7	±	0.5	7.9	±	0.3
TSS	(g TSS·m^{-3})	107	±	61	308	±	86
VSS	(g VSS·m^{-3})	92	±	46	258	±	73
T-COD	(g COD·m^{-3})	252	±	71	692	±	139
S-COD	(g COD·m^{-3})	100	±	27	123	±	29
T-BOD	(g BOD·m^{-3})	105	±	18	290	±	30
S-BOD	(g BOD·m^{-3})	36	±	5	106	±	21
T-N	(g N·m^{-3})	40	±	10	47	±	4
NH_4-N	(g N·m^{-3})	37	±	8	39	±	3
T-P	(g P·m^{-3})	4.2	±	1.2	7.7	±	1.4
PO_4-P	(g P·m^{-3})	3.7	±	0.9	4.4	±	1.3

Table 2
Samples analyzed and characterization of the samples' main abiotic parameters. Abbreviations: photosynthetically active radiation (PAR), temperature (T), average light intensity (I_{av}), dissolved oxygen (DO), organic loading rate (OLR), ammonium, nitrite and nitrate (NH_4-N, NO_2-N and NO_3-N, respectively) in the effluent, total nitrogen and ammonium removal efficiencies (T-N-RE and T-NH_4-RE).

Steady state group	Date	Bacteria sample code	Microalgae sample code	Wastewater stream	HRT (d)	T (ºC)	PAR ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Average I_{av} ($\mu mol \cdot m^2 \cdot s^{-1}$)	Maximum I_{av} ($\mu mol \cdot m^2 \cdot s^{-1}$)	pH	OD	OLR ($g \cdot m^{-3} \cdot d^{-1}$)	NH_4-N (g $N \cdot m^{-3}$)	NO_2-N (g $N \cdot m^{-3}$)	NO_3-N (g $N \cdot m^{-3}$)	T-N-RE (%)	T-NH_4-RE (%)
HRT_6_EPS	19/08/2019	BRW101		EPS	6	30.3	281.5	16 ± 5	186 ± 33	8.56	6.15	41.0	25.0	0.0	0.0	26.3 ± 0.6	75 ± 2
	16/10/2019	BRW102	ARW102		6	17.5	276.1			8.27	10.24	44.0	0.2	10.8	1.5		
	18/10/2019	BRW103			6	21.1	227.2			8.85	8.83	39.0	0.3	9.2	2.0		
	23/10/2019	BRW104	ARW104		6	15.4	136.7			8.53	9.03	46.0	0.0	6.9	0.0		
	25/10/2019	BRW105	ARW105		6	22.0	245.7			8.57	8.05	42.0	0.8	9.4	0.0		
	28/10/2019	BRW106	ARW106		6	22.8	99.6			8.55	7.42	48.0	0.4	5.5	0.0		
	06/11/2020	BRW128	ARW127		6	16.5	132.1			8.33	4.02	40.0	9.6	15.1	2.8		
	18/11/2019	BRW107	ARW107		6	16.4	132.0			8.22	3.91	38.0	10.5	16.2	2.8		
	20/11/2019	BRW108			6	16.4	152.0			8.25	2.82	52.0	9.0	16.5	8.5		
	25/11/2019	BRW109	ARW109		6	19.0	145.7			8.03	2.51	32.0	10.6	16.0	0.4		
HRT_4_EPS	20/12/2019	BRW110	ARW110	EPS	4	18.9	152.5	91 ± 73	14 ± 5	7.30	8.25	62.0	0.0	1.2	39.3	5.5 ± 0.9	98 ± 3
	23/12/2019	BRW111			4	19.1	165.2			7.56	8.63	59.0	0.0	1.4	39.1		
	26/12/2019	BRW112	ARW112		4	16.9	123.7			7.26	5.74	61.0	0.0	1.6	38.7		
	27/12/2019	BRW113	ARW113		4	16.1	140.2			7.57	8.79	60.0	0.5	1.1	42.8		
	02/01/2020	BRW114	ARW128		4	14.7	24.5			7.32	4.13	61.0	0.0	1.0	47.7		
HRT_2_EPS	13/01/2020	BRW115	ARW114	EPS	2	12.9	144.2	12 ± 2	105 ± 40	7.69	6.15	137.0	15.7	0.8	24.0	5.5 ± 0.8	99 ± 4

(*continued on next page*)

Table 2 (*continued*)

Steady state group	Date	Bacteria sample code	Microalgae sample code	Wastewater stream	HRT (d)	T (^{o}C)	PAR ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Average I_{av} ($\mu mol \cdot m^{2} \cdot s^{-1}$)	Maximum I_{av} ($\mu mol \cdot m^{2} \cdot s^{-1}$)	pH	OD	OLR ($g \cdot m^{-3} \cdot d^{-1}$)	NH_4-N (g $N \cdot m^{-3}$)	NO_2-N (g $N \cdot m^{-3}$)	NO_3-N (g $N \cdot m^{-3}$)	T-N-RE (%)	T-NH_4-RE (%)
	15/01/2020	BRW116			2	12.8	81.0			7.70	5.86	128.0	16.7	0.8	22.5		
	20/01/2020	BRW117	ARW116		2	9.6	43.5			7.31	8.92	120.0	1.6	0.8	28.1		
	31/01/2020	BRW118	ARW117		2	20.3	145.5			7.19	4.49	132.0	0.5	1.6	42.7		
	11/02/2020	BRW119	ARW118		2	18.6	152.0			7.23	4.35	127.0	0.0	0.4	42.0		
	30/02/2020	BRW129	ARW129		2	17.7	151.1			7.14	4.26	118.0	0.0	0.4	30.2		
HRT_2_EPT	03/07/2020	BRW120	ARW119	EPT	2	28.4	117.8	4.3 ± 0.8	19.7 ± 0.6	7.35	2.54	339.0	1.5	0.0	3.8	91 ± 10	95 ± 6
	08/07/2020	BRW121	ARW120		2	30.8	269.5			7.43	1.00	337.0	4.6	0.0	1.9		
HRT_1_EPT	10/07/2020	BRW122	ARW122	EPT	1	29.8	259.6	3.1 ± 1.2	7.2 ± 0.8	7.64	0.13	678.0	1.4	0.4	1.0	1.12 ± 0.7	1.1 ± 0.5
	13/07/2020	BRW123			1	28.6	193.1			7.41	0.16	641.0	3.9	0.5	0.8		
	15/07/2020	BRW124	ARW123		1	27.6	265.6			7.36	0.17	666.0	0.1	2.1	2.0		
	17/07/2020	BRW125	ARW124		1	28.6	269.1			7.39	0.16	672.0	2.7	0.4	0.9		
	12/08/2020	BRW126	ARW125		1	29.6	253.4			7.42	0.15	681.0	0.1	0.4	2.2		
	17/08/2020	BRW127	ARW126		1	28.9	246.8			7.38	0.29	672.0	0.2	1.0	4.5		

phosphate (PO_4-P) were analyzed according to the Standard Methods (APHA-AWWA-WPCF, 2012): 4500- NH3-G, 4500-NO2-B, 4500-NO3-H and 4500-P-F respectively, with an automatic analyzer (Smartchem 200, WestcoScientifi Instruments, Westco). Total suspended solids (TSS), VSS, total and soluble chemical oxygen demand (T-COD and S-COD) and total and soluble biological oxygen demand (T-BOD and S-BOD) were measured according 2540-TSS-D, VSS 2540-VSS-E, 5220-COD-C, 522-COD-D and 5210-BOD-C, respectively (APHA-AWWA-WPCF, 2012).

2.4. Extracting nucleic material and sequencing 16S and 18S rDNA genes

A total of 29 samples (Table 2) were extracted from the MHRAP plus one each of the experiments working with primary settling and pre-treatment effluents. Pseudo-stationary state samples were taken from the HRAP culture in duplicate/triplicate (biological replicates). Following the procedures form Zamorano-López et al. (2019), aliquots of 1 mL sample were stored in 2 mL cryotubes at −20 °C. For bacteria, the E.Z.N.A® DNA Extraction Kit for Soil (Omega-Biotek, USA) was used to extract nucleic acid material from 1 g of biomass, in accordance with the manufacturer's protocol, while the E.Z.N.A® Plant DNA Kit (Omega-Biotek, USA) was used for microalgae. Those analyzed for bacteria were labeled BRW, while those for microalgae were labeled ARW. DNA concentration and purity were measured at wavelengths of 260, 230 and 280 nm with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Samples with a A260/280 ratio below 1.8 and a A260/230 ratio out of the range 2.0–2.2 were discarded, to avoid humic acids or other compounds contamination.

16S rDNA gene bacteria and archaea microorganisms were analyzed by amplicon sequencing using specific primers for the v3-4 hyper variable region of the target gene (341F 5′-CCTACGGGNGGCWGCAG-3′ and 805R 5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). The V4 hyper-variable region of the 18S rDNA gene was amplified using the universal primers set TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3_modified (5′-ACTTTCGTTCTTGATYRATGA-3′) to identify the microalgal groups in the eukarya domain (Tragin and Vaulot, 2018). The sequencing run was carried out in a 2 × 300 base pairs paired-end run using v3 chemistry in an Illumina MiSeq Sequencer (FISABIO, Valencia, Spain).

2.5. Illumina data processing and statistical analysis

The resulting raw sequences were downstream processed following the MiSeq_SOP pipeline (website accession data September 5, 2021) using open source mothur software (v.1.46.1) (Schloss et al., 2009). The taxonomy was assigned according to the v138.1 release of SILVA database. The contingency table generated was used as the input for microbial ecology analysis using R-studio v.2021.09.0 software and vegan packages. For bacteria quantification, chloroplasts were not considered for microbial ecology analysis as they were supposed to belong to eukaryotic microalgae and not to the bacteria domain.

Alpha diversity was calculated through the evenness and richness index indicators (Shannon-Wiener and Simpson) using R-studio v.2021.09.0 software and vegan packages. A principal co-ordinate analysis (PCoA) based on the Bray-Curtis distances matrix was performed on mothur to explore the beta diversity of the different samples processed for bacteria and microalgae quantification.

The microbial and operational results obtained were statistically analyzed by IBM SPSS Statistics 26 software (Armonk, NY: IBM Corp). An analysis of variance (ANOVA) was performed to evaluate the significance of the differences in the mean values. Differences were considered statistically significant at p-values below 0.01.

2.6. Photo-respirometric tests

The nitrite-oxidizing bacteria (NOB) oxygen uptake rate (OUR_{NOB}) was measured using a modified version of the standardized photo-respirometric protocol developed by (Sánchez-Zurano et al., 2020b) , which allows differentiation between the activities of microalgae, heterotrophic, and nitrifying bacteria based on oxygen production and uptake rates.

For the measurement of OURNOB (mg O2·g VSS^{-1} h^{-1}), a sample of microalgae-bacteria culture was placed inside the photo-respirometer described in Chapter 3. A solution of NaNO2 was dosed to reach an NO2-N concentration of 10 g $N{\cdot}m^{-3}$, facilitating the assessment of NOB activity. Ammonia-oxidizing bacteria (AOB) activity was inhibited by dosing an inhibitor solution of allylthiourea (ATU) at a concentration of 5 g m^{-3}.

3. Results and discussion

Table 2 gives details of the samples analyzed and the operational and environmental values. The analysis of the sequences obtained revealed shallow depths in samples BRW122 and 123, in which only 466 and 3858 sequences were detected, respectively, so that both were excluded from further analysis. Subsampling was then applied to the minimum level of sequences obtained (94,204 sequences for bacteria and 59,919 for eukarya).

3.1. Diversity and operational parameters

To analyze the samples' bacterial and microalgal structure, diversity was measured by the inverse Simpson and Shannon-Wiener indices. Diversity gives the number of different microorganism types present in the sample and their evenness, i.e their relative abundance. The Shannon-Wiener index focuses on the species' richness and can have any value, the higher the value, the more taxonomic levels present and the more uniform their distribution. The inverse Simpson index gives the species' uniformity and is stated in values between 0 and 1, the greater the value the higher the diversity. Fig. S1, supplementary material) gives the Simpson and Shannon-Wiener diversity indexes for the bacteria and eukarya domains of all the samples, together with the number of genera found. For bacteria (Fig. S1A), it seems that both the diversity and number of genera appear to grow as the HRT is reduced, although this trend was not found in the eukarya domain (Fig. S1B).

Genera diversity can be related to both interspecific and intraspecific competition. The conventional theory of competition and niche differentiation assumes that competitive interactions can modify community structures by limiting the number of genera that can exploit the available resources/substrates. The role of competition is based on the competitive exclusion principle, i.e. if two or more genera compete for the same limited resource, the genus best adapted to the boundary conditions outcompetes the others.

Important to note is that the use of membranes for decoupling the BRT from the HRT avoids biomass washout at low HRTs, thus allowing the development of slow-growing microorganisms. The ANOVA test for diversity and operational parameters revealed that diversity increases as the HRT is reduced($p < 0.01$) at all bacterial taxonomic levels. The lower the HRT, the higher the availability of substrate (organic matter, nutrients, micronutrients) for the bacteria, which can increase both the genera richness and relative abundance. This effect has been previously observed in other treatment systems using membranes in the biological process (Greses et al., 2018). The test also revealed that as this diversity increases, the effluent COD decreases ($p < 0.01$), indicating that the more diverse the bacteria community, the better able it is to consume COD, whatever its form. It was also found that bacterial diversity is reduced as nitrite concentration rises ($p < 0.01$), since NO_2-N not only affects microalgae growth but also affects certain bacterial taxonomic groups (Blackburne et al., 2007; Claros et al., 2013; Pijuan et al., 2010).

When considering the phylum or class levels in the eukarya domain, the ANOVA test revealed that diversity increases ($p < 0.01$) when the PAR is increased. This is because, as the PAR increases, more microalgae

can grow not only in number but in diversity. This rise is also related to temperature ($p < 0.01$).

It must be highlighted that when working with EPS, all experiments accomplished with COD discharge limits (<125 with $gO_2 \cdot m^{-3}$), but none of the three HRTs studied allowed to remove enough nutrients. However, working with EPT and an HRT of 2 days led to an effluent which accomplishes not only COD discharge limits but also nutrient discharge limits (15 $gN \cdot m^{-3}$ and 2 $gP \cdot m^{-3}$), according to EU Directive 98/15/CEE (10,000 - 100,000 p.e.).

To explore the beta diversity of the different samples processed for bacteria and microalgae quantification, a PCoA was carried out based on the Bray-Curtis distance matrix (Figs. 1 and 2). This procedure can group the samples by the dissimilarity between the samples and calculate a distance matrix. From Fig. 1, it seems clear that the HRT and the incoming wastewater stream control the type of the bacterial populations. The bacterial structure of the samples analyzed can be divided into 5 clusters. Although the biological community of the EPT-fed samples was similar, regardless of the operating HRT, there were significant differences in the biological community in the EPS-fed samples. The HRT_6_EPS period shows a biological community different from those of the other HRTs. The main differences between these three periods is the concentration of NO_2-N, alkaline pH (and the consequent increase in free ammonia nitrogen (FAN)), which could have specialized the bacterial groups towards a nitrite- and high pH-tolerant community. The biological specialization against these abiotic factors explains the lower diversity found in the Shannon-Wiener and Simpson indexes. As fewer genera become adapted to growing in these conditions, the biological diversity is reduced in a 6-day HRT (Fig. S1).

The eukaryotic organisms did not seem be affected by the type of the influent or the operating HRT (Fig. 2). Eukaryotic composition can be grouped into two main clusters: those of the 6-day HRT and the remaining samples. The oxygen production rate measured was considerably lower at the 6-day HRT, indicating limited microalgal activity. Photosynthesis inhibition by NO_2-N was verified and assessed in a previous work (Aparicio et al., 2022), working at 6 days HRT. This abrupt division between both clusters (Fig. 2) could therefore be due to NO_2-N accumulation.

However, although the optimal pH for the microalgae range is between 8 and 9 (Barceló-Villalobos et al., 2019; Rossi et al., 2020), the slightly higher pH found when working at the 6-day HRT could have negatively affected the microalgae and also been responsible for the division between both groups, as can be seen in Fig. 3.

3.2. Bacteria and microalgae communities

3.2.1. Bacteria community structure

The bacteria and microalgae communities were characterized and quantified on the Illumina MiSeq platform. Fig. 3 contains a barplot showing the relative abundance of the bacteria at the phylum level. Microbial characterization of influent wastewaters with the same microbial composition is also included. Dominant phylum differences can be seen between the samples obtained from HRT_6_EPS and those from the other periods. In HRT_6_EPS, the dominant phylum was *Verrucomicrobiota* (relative abundance between 18 and 56% without taking into account sample BRW101), although in the other periods it was *Proteobacteria* (relative abundance between 28 and 44%), regardless of the operating conditions.

Phylum *Verrucomicrobiota* forms cytoplasmic appendages known as *protists*, which are attached to surfaces and involved in respiration. This phylum's morphological feature could have induced the floc formation present during most of the experimental period in the HRAP. The *Verrucomicrobiota* phylum has been widely reported in the upper layer of aquatic ecosystems due to its high resistance to UV irradiation (Ferrero et al., 2012). The maximum average light intensity (I_{av}) was reported at HRT_6_EPS (186 ± 33 µmol $m^{-2} \cdot s^{-1}$). The light intensity could therefore have acted as a microbial selection factor through UV tolerance. When HRT was reduced to 4 days and the maximum average irradiance was 91 ± 73 µmol $m^{-2} \cdot s^{-1}$ (see Table 2), the *Verrucomicrobiota* population decreased dramatically (7–13%) and *Proteobacteria* became the main bacteria phylum (32–35%). This shift between *Verrucomicrobiota* and *Proteobacteria* could be have been due to suppression of the "selective pressure". However, although the maximum I_{av} was significantly higher in HRT_6_EPS, the average I_{av} was similar in all three operating periods treating EPS (16 ± 5, 13 ± 5, 12 ± 2 µmol $m^{-2} \cdot s^{-1}$, at 6, 4 and 2 days of HRT, see Table 2). The phylum distribution cannot therefore be assumed to be the result of occasional high light intensities but may also have been influenced by NO_2-N accumulation and pH values close to 9. The taxonomic classification at the phylum level covers a wide range of organisms, which makes it impossible to lay down for pH and NO_2-N tolerance ranges.

The rest of the phyla also increased, but *Actinobacteriota* (12–16%) increased most in HRT_4_EPS and HRT_2_EPS. It is also worth noting the

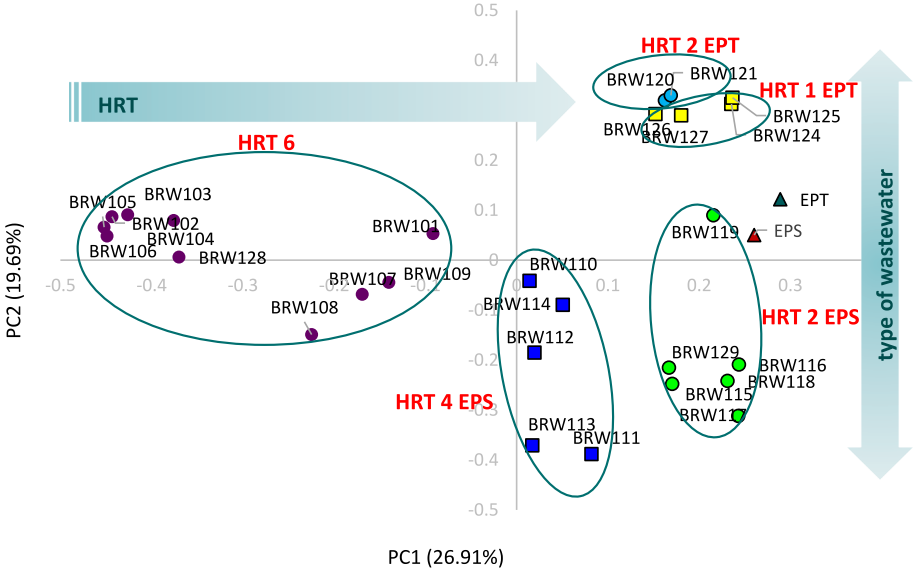

Fig. 1. Principal Co-ordinates Analysis (PCoA) based on the Bray-Curtis distances matrix for 16S rDNA (bacteria) sequenced samples. Explained variance by each component is indicated in each axis in percentages.

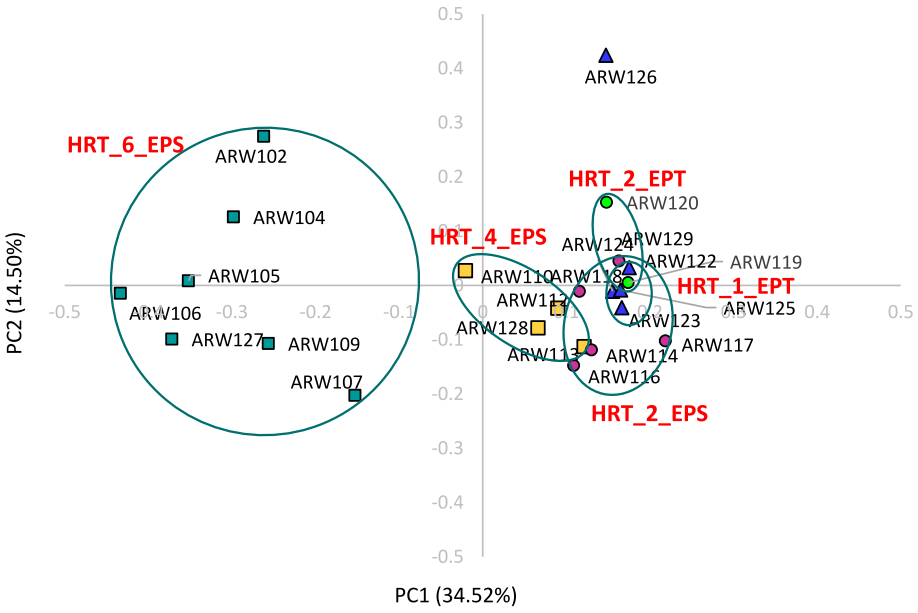

Fig. 2. Principal Co-ordinates Analysis (PCoA) based on the Bray-Curtis distances matrix for 18S rDNA (eukarya) sequenced samples. The variance explained by each component is indicated as a percentage in the axes.

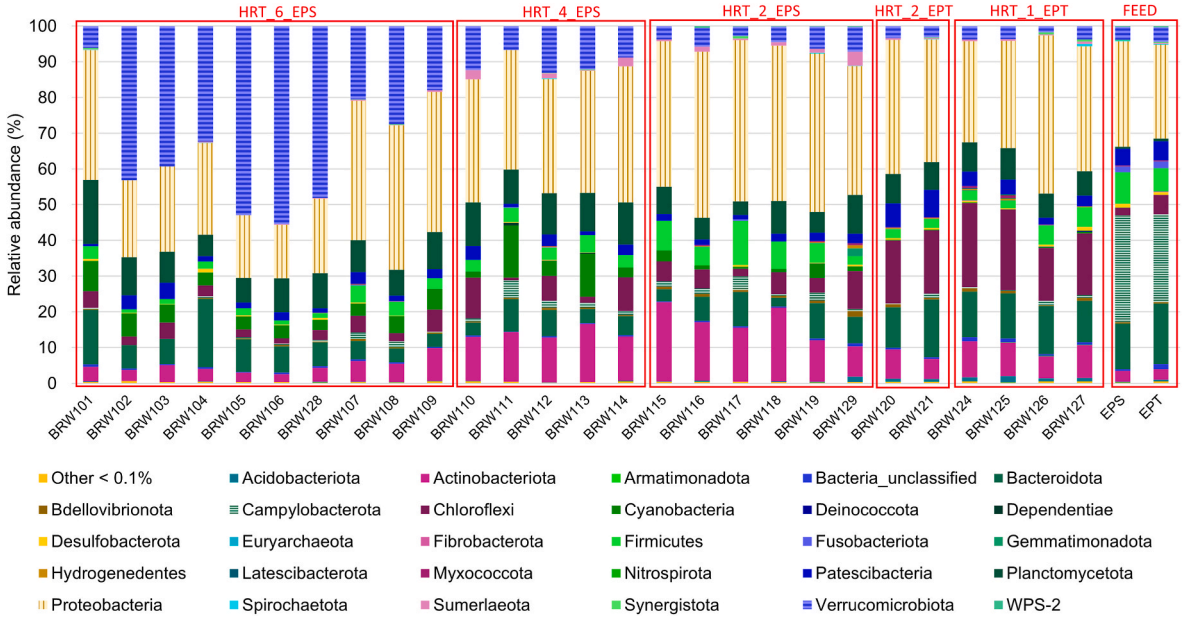

Fig. 3. Barplot of relative abundance of bacteria at phylum level. The "Other" series contains phyla with abundances below 0.1%. Samples grouped by HRT and influent wastewater (EPS or EPT). End columns represent the main phyla found in the EPS and in the EPT.

appearance of *Cyanobacteria*, reaching 15% in HRT_4_EPS. Similarly to previous works conducted in membrane photobioreactors operated at similar environmental conditions (see, e.g., González-Camejo et al., 2017, 2019), *Cyanobacteria* was not in a high abundance in the system. This could be attributed to the non-limiting nutrient concentration in the culture media (González-Camejo et al., 2017) and the BRT applied (González-Camejo et al., 2019). No major changes occurred in the populations when the HRT was reduced to 2 days. *Proteobacteria* remained dominant (36–47%) and *Firmicutes* slightly increased (3–13%).

The next change was to feed the reactor with EPT instead of EPS, thus increasing the organic load rate (OLR) to the MHRAP. The incoming wastewater stream markedly increased the relative abundance of *Chloroflexy* (15–24%). Phylum *Chloroflexy* usually coexist with annamox bacteria in wastewater systems and grow due to taking in organic substrates such as soluble microbial products and extracellular polymeric substances derived from anammox bacteria (Kindaichi et al., 2012; Nierychlo et al., 2019). The continual aerobic conditions in the EPS-periods, due to low OLR (Table 2), limited the anammox bacteria growth and, in turn, the phylum *Chloroflexy* bacteria, so that this phylum was barely detected when treating EPS.

The metagenomics data was further analyzed and compared at the genus level to examine the evolution of the influent's microbial community structure in greater depth throughout the operating HRT (see Fig. S2 in supplementary material). Genus LD29, belonging to the *Verrucomicrobiota* phylum, was the most abundant genera detected (up to 52%) in HRT_6_EPS. Although the LD29 physiology is unclear, it has been fond widely distributed in sea water and lakes, associated with

high oxygen concentrations and phytoplankton, suggesting that they use phytoplankton-derived organic matter (Bergen et al., 2014; Zhao et al., 2021). When the HRT was reduced, LD29 almost disappeared, diversity increased and most of the genera (between 26 and 60%) had an abundance below 1%. Abiotic stress controlled the relative abundance and diversity of the bacterial community. *Mycobacterium* (3–14%), from phylum *Actinobacteriota*, was the only larger genus in HRT_2_EPS. The *Mycobacterium* genus could be potentially pathogenic and harmful to humans (Ye and Zhang, 2013), although it is difficult to determine whether the *Mycobacterium* bacteria in these samples were pathogens since not all the species in these genera are pathogenic. Some strains of *Mycobacterium* were closely related to degrading organic carbon, such as the polycyclic aromatic ring (Jiang et al., 2021). In fact, Warshawsky et al. (2007) reported that the consortium of *Mycobacterium* and microalgal *Selanastrum capricornutum* was capable of degrading large recalcitrant polycyclic aromatic hydrocarbons.

When using pre-treated wastewater influent, an uncultured genus of the *Caldilineaceae* family became the most abundant (10–18%). The members of this family are positively correlated with the nitrite and nitrate removal rate (Cao et al., 2020; Liu and Li, 2019), indicating that this genus is an important active de-nitrifier. Zero oxygen concentrations were recorded during nighttime hours, which favored denitrification. Torres-Franco et al. (2021) also detected *Caldilineaceae* in an anoxic-aerobic microalgal-bacterial system treating digestate. The *Ahniella* genus, from phylum *Proteobacteria*, also increased considerably (3–13%). However, the members of this genus are not often found in activated sludge systems or microalgae-bacteria consortia and their role is almost unknown. They are considered to be heterotrophic bacteria, which are not involved in reducing nitrate (Hwang et al., 2018).

It should be noted that although bacteria related to the enhanced biological phosphorus removal (EBPR) process, i.e. Candidatus Accumulibacter Phosphatis (Yang et al., 2018), have been found in other microalgae-bacteria systems, they were not found in the present study, indicating the lack of suitable growth conditions, probably the lack of sufficient soluble phosphorus and/or volatile fatty acids (VFA).

3.2.1.1. Ammonia-oxidizing bacteria (AOB). The role of nitrifying bacteria in microalgae-bacteria systems has been previously discussed (González-Camejo et al., 2022). AOB not only compete with microalgae for ammonium, but also their metabolic product, NO_2-N, can inhibit microalgal photosynthesis. *Nitrosomonas* was the only AOB genus detected in in the samples obtained when treating primary effluent (Fig. 4), while both the *Nitrosospira* and *Nitrosococcus* genera were absent.

Nitrosococcus are often associated with marine ecosystems and salt lakes and this genus has also been found to be the dominant AOB with anammox bacteria in hypoxic layers (Campbell et al., 2011). The absence of the *Nitrosococcus* genus in the samples from the EPS-period was therefore consistent with the previously described continuous aerobic conditions.

Nitrosomonas and *Nitrosospira* are the main AOB genera in activated sludge (Park and Noguera, 2004), of which *Nitrosomonas* is often the dominant AOB in bioreactors (Limpiyakorn et al., 2006; Wells et al., 2009), since it can grow more than twice as fast as *Nitrospira* in the optimum abiotic boundary conditions (Siripong and Rittmann, 2007).

The most notable difference between the EPS- and EPT-fed MHRAP was the presence of the Ellin6067 spp genus, which is not usually found in biological nitrogen-removal systems. However, Wang et al. (2021) found that it steadily became more abundant in lighted systems and was tolerant and/or acclimatized to light irradiation, facilitating NH_4-N oxidation to NO_2-N in biological systems. Although light intensity on the reactor surface increased during the EPT and EPS periods, I_{av} was higher with EPS (Table 2), so that In spite of being acclimatized to light, it could be photo-inhibited at an I_{av} of 12 μmol $m^{-2}{\cdot}s^{-1}$. Also, *Nitrosomonas* can be more precisely controlled by instantaneous incident light than the average light intensity, *Ellin6067* spp. Being more competitive in the EPT periods and outcompeting *Nitrosomonas*. However, little information can be obtained on for *Ellin6067* spp in the scientific literature, while the growth of the *Ellin6067* genus could have been due to high temperatures.

The observed trend of a decreasing HRT with increasing AOB seems to be more important, while feeding with pre-treated wastewater instead of primary settled wastewater increases the abundance of both AOB and AOB genera (*Nitrosomonas* + *Ellin6067*).

3.2.1.2. Nitrite-oxidizing bacteria (NOB). As can be seen in Fig. 5, the NOB genera followed different evolutionary trends in the different experimental periods. *Candidatus Nitrotoga*, *Nitrobacter* and *Nitrospira* were the most common NOB genera in the MHRAP as *Nitrospira*-like microorganisms generally dominate in activated sludge systems thanks to their affinity for NO_2-N. The HRT_6_EPS sequencing showed that *Nistrospira* was the only NOB present in the system, although in very low proportions, which happens when NO_2-N accumulates (Fig. 6).

The dominant NOB in the system changed from *Nitrospira* to *Candidatus Nitrotoga* in HRT_4_EPS and HRT_2_EPS (Fig. 5). Zheng et al. (2020) found that *Nitrospira,* the dominant NOB under regular exposure to FAN, was the key factor that determined the composition of the NOB community. The culture pH was reduced (from approximately 9 to 7.5

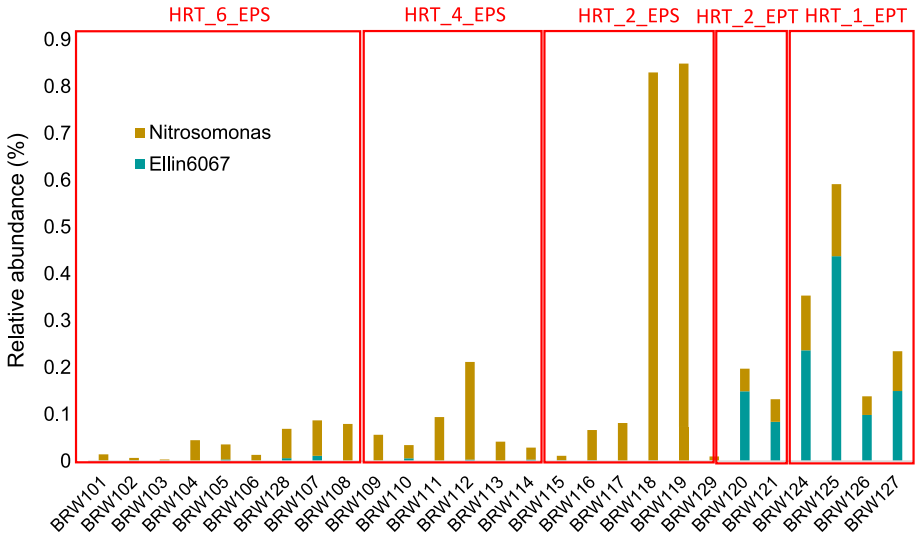

Fig. 4. Relative abundance of ammonia-oxidizing bacteria in each sample.

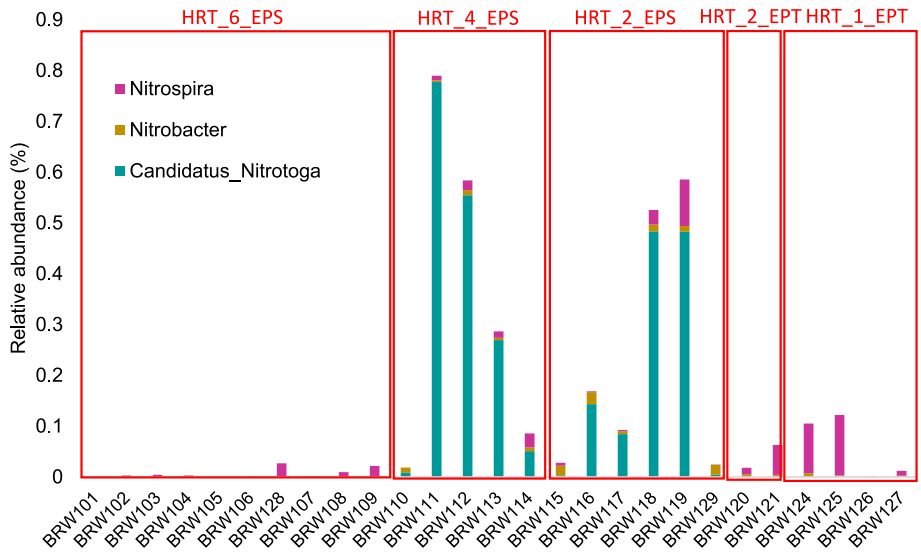

Fig. 5. Relative abundance of nitrite-oxidizing bacteria in samples.

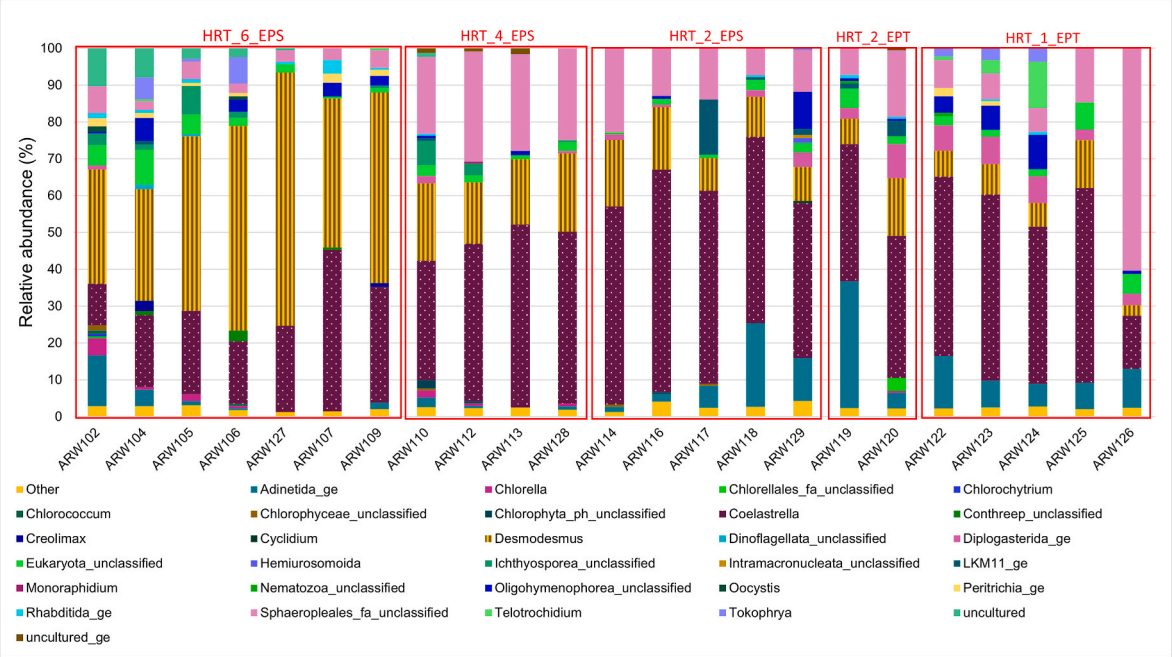

Fig. 6. Barplot of relative abundance of eukarya at the genus level. The "Other" series contains genera with less than 0.5% abundance. Samples are grouped by HRT and EPS or EPT influent wastewater.

(Table 2)) by reducing the HRT, when the FAN concentration also decreased. The different structure of the NOB community in these three periods suggested that *Candidatus Nitrotoga* may indeed be more sensitive to FAN and pH than *Nitrospira*. Comparing HRT_6_EPS with HRT_4_EPS and HRT_2_EPS, in the NOB and AOB were much more abundant. NOB was 0.02% relatively more abundant in HRT_6_EPS, while NOB was 0.77 and 0.50% was reached in HRT_4_EPS and HRT_2_EPS, respectively. The higher NOB relative abundance in HRT_4_EPS and HRT_2_EPS thus appears to be is responsible for NO_2-N oxidation to NO_3-N, avoiding the accumulation of the former.

Candidatus Nitrotoga was not found in the EPT-fed MHRAP samples and the NOB community structures were dominated by *Nitrospira*. The *Candidatus Nitrotoga* are less competitive than *Nitrospira* when oxygen is missing. Although oxygen concentrations close to 8 were reached in daylight, the night-time oxygen concentration was zero, which could have restricted the growth of *Candidatus Nitrotoga* in HRT_2_EPT. Also, oxygen concentration in HRT_1_EPT was nearly zero, which reduced the NOB relative abundance (0.01%) at the end of the period.

3.2.2. *Microalgae community structure*

Fig. 6 gives the relative abundance of the eukarya genera in the analyzed samples. The 6-day HRT produced a higher abundance of green microalage *Desmodesmus* (30–69%) over *Coelastrella* (11–44%), both from the *Sphaeropleales* order.

As in the bacterial community, abiotic stress in the HRT_6_EPS period samples conditioned microalgae growth. Although *Desmodesmus* could be less sensitive than *Coelastrella* to alkaline pH, high nitrite and FAN concentrations, no data can be found in the literature that describes suitable growing conditions for these genera.

The algal community structure was dominated by *Coelastrella* when

HRT was reduced, regardless of the incoming wastewater stream, more than 50% being from the *Coelastrella* genus. As previous studies found that *Chlorophyta* is widely used in treating wastewater (Abinandan and Shanthakumar, 2015), which suggests that *Coelastrella*, from the *Chlorophyta* phylum, contributed to removing the nitrogen. Luo et al. (2016) found a relatively high NH_4-N removal efficiency for *Coelastrella* (over 90%), while Abe et al. (2007) reported high nitrate removal, with a nitrate uptake reaching 0.4 mg h^{-1}. However, according to the nitrogen mass balance nitrification was the main NH_4-N removal mechanism, while no NO_3-N uptake rate by microalgae was found. This indicates that the microalgae played a supporting role and provided oxygen to the aerobic bacteria at all operating HRTs and influents. In the present study, the microalgae composition was not affected by environmental parameters, *Coelastrella* remained dominant even when the PAR or the temperature was raised.

Traditionally, microalgae have been identified according to their morphology. Although the populations have traditionally been found to vary between *Scenedesmus*- and *Chlorella*-like, the sequencing data in the present study indicates that the *Chlorellales* order barely makes up only 7% of the eukarya domain (sample ARW102). Almost all the microalgae found belonged to the *Sphaeropleales* order, including the *Scenedesmus*-like microalgae.

3.3. In-depth analysis of the evolution of microbiota community structure and the relationship with MHRAP performance

The effect of the bacterial and microalgal structure on MHRAP performance was analyzed according to the operating periods described in Table 2. Partial-nitrification accounted for 48.6% and gave a final NO_2-N concentration of 21 ± 3 g m^{-3} in HRT_6_EPS. The relative abundance of the NOB community in this period was only 0.03%, indicating an NOB oxygen uptake rate of 0.6 ± 0.2 mg O_2·g VSS^{-1}·h^{-1}, suggesting possible NOB inhibition. However, the abiotic stress in this period, e.g. alkaline pH, FAN concentration and even NO_2-N accumulation, restricted not only the NOB growth but also that of the other bacteria and microalgae. The biodiversity indexes (Fig. S1) and the relative abundance of bacteria phylum (Fig. 3) showed less diversity of the organisms than in other periods. The selective *Desmodesmus* microalgae genus was dominant in period HRT_6_EPS, but this type of genus only provide oxygen to aerobic bacteria. Reducing operating HRT reduces abiotic stress, as indicated above. Partial nitrification was avoided in both HRT_4_EPS and HRT_2_EPS, which is consistent with the greater NOB relative abundance, reaching values up to 0.7% in some samples, and activity (approximately 1.1 mg O_2·g VSS^{-1}·h^{-1} in both periods). MHRAP performance was similar in both periods (HRT_4_EPS and HRT_2_EPS) and removed over 95% of the ammonium (T-NH_4-RE)). This is consistent with the microbial dynamics, as Figs. 3 and 6 show similar compositions of the bacterial and microalgal community. Both periods were dominated by the same bacterial and microalgal groups.

The main difference between the EPS- and EPT-fed MHRAP was oxygen fluctuation. The oxygen concentration in HRT_2_EPT was near to saturation under lighting, while anoxic conditions were found at night, promoting the growth of denitrifying bacteria belonging to the *Chloroflexy* phylum (91 ± 10% of T-N was removed by coupled nitrification-denitrification). Nitrifying activity was greater in the EPT-operated periods (2.5 ± 0.4 mg O_2·g VSS^{-1}·h^{-1}) than in those with EPS, despite the lower relative NOB abundances in HRT_2_EPT. It should be noted that the relative abundance registered the number of copies in each group but did not provide any information on biological activity. Although similar microbial composition was found in HRT_2_EPT and HRT_1_EPT, Nitrogen removal eficiency accounted for 91 ± 1% and 1.1 ± 0.7%, respectively. The oxygen demand in theseconditions (HRT_1_EPT) exceeded the oxygen from microalgae and restricted aerobic processes such as nitrification. Relative abundance of the AOB and NOB genera were greater in some samples in the EPS- fed periods than in HRT_2_EPT, although there was significantly less nitrifiying activity, 0.3 mg O_2·g VSS^{-1}·h^{-1} compared to 1.3 mg O_2·g VSS^{-1}·h^{-1}, respectively. It should be noted that the metagenomic analysis is based on the number of copies of a target gene, which is not necessarily related to the organisms' activity.

The microalgal composition were not significantly different between the periods, indicating that microalgae selection was largely unaffected by HRT, (in the 4-1 day range) or type of wastewater stream under the abiotic conditions described in Table 2. The microalgae's main role was to provide oxygen to the aerobic bacteria. Bacteria are seen as the cornerstone of the microalgae-bacteria consortium. Although microalgae are necessary in an MHRAP system to provide oxygen and remove nutrients, it is the quantity and composition of bacteria that have a greater effect on the final water quality.

4. Conclusions

This study represents a significant advance in the in-depth understanding of using microalgal-bacterial consortia for wastewater treatment. The results indicate that the influent wastewater composition and HRT are primary factors shaping bacterial populations, while abiotic stressors such as light intensity, free ammonia nitrogen, pH, and nitrite accumulation influence bacterial diversity.

Changes in bacterial composition, including the AOB community, suggest the potential occurrence of photo-inhibition, which can regulate ecological structure. The NOB community was dominated by *Nitrospira* and *Candidatus Nitrotoga*, with *Nitrospira* being the dominant genus under regular exposure to free ammonia nitrogen and low average oxygen concentrations.

Regarding the microalgal community, the genera *Desmodesmus* and *Coelastrella* were predominant across the five periods studied. *Desmodesmus* thrived under alkaline pH conditions and exposure to nitrites and free ammonia nitrogen. The findings suggest that microalgal biodiversity was not affected by HRT or influent composition. Moreover, the main abiotic stress conditions appear to be light intensity, free ammonia nitrogen, pH, and nitrite accumulation.

Nitrogen removal efficiency was influenced by the bacterial community composition but not by that of the microalgae. Low relative NOB abundance resulted in partial nitrification, while daily fluctuations in oxygen concentration favored the growth of *Chloroflexy* and *Caldilineaceae*, leading to nitrogen removal through coupled nitrification-denitrification. Under the operational conditions studied, the key role of microalgae was to support aerobic bacterial processes.

CRediT authorship contribution statement

Stéphanie Aparicio: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alejandro Ríos-Mejía:** Writing – original draft, Investigation. **Juan Pablo Gallardo-Mejías:** Writing – original draft, Investigation. **Ángel Robles:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luis Borrás:** Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stephanie Aparicio reports financial support was provided by Spanish Ministry of Education, Culture and Sport. Luis Borras, Angel Robles reports equipment, drugs, or supplies was provided by Spanish Ministry of Economy and Competitiveness CTM2014-54980-C2-1-R and 2-R. Luis Borras, Angel Robles reports equipment, drugs, or supplies was provided by European Regional Development Fund. Luis Borras, Angel Robles reports equipment, drugs, or supplies was provided by Polytechnic

University of Valencia CE20180209. 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.

Acknowledgements

This research work was supported by: the Spanish Ministry of Economy and Competitiveness (MINECO, Projects CTM2014-54980-C2-1-R and CTM2014-54980-C2-2-R) jointly with the European Regional Development Fund (ERDF), both of which are gratefully acknowledged; and Universitat Politècnica de València (UPV, Project SISTEMA H-RAP, CE20180209). We also received support from the Spanish Ministry of Education, Culture and Sport via a pre-doctoral FPU fellowship to author Stéphanie Aparicio (FPU/15/02595).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.123186.

Data availability

Data will be made available on request.

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