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Effect of polyethylene microplastics on activated sludge process - accumulation in the sludge and influence on the process and on biomass characteristics

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Abstract

According to previous research, it has been proved that wastewater treatment plants (WWTPs) can retain more than 90% of the MP contained in wastewater. However, a significant fraction of the MP removed in WWTPs is retained in the sludge floc and this may lead to an environmental issue, as biosolids are used as fertilizers. The purpose of this research was to evaluate how the presence of polyethylene (PE) could interfere with activated sludge performance. For this, a sequencing batch reactor (SBR) was

continuously fed during 93 days with synthetic sewage and PE microbeads. It was observed that 98±2% of the total amount of MPs that entered SBR was accumulated in the activated sludge. Despite the high accumulation of MP in the sludge the depuration performance of the reactor was not compromised, the presence of MPs decreased the richness (Chao1) and abundance-based coverage estimators (ACE) and diversity (Shannon) of the bacterial community on day 93. Based on the analysis of the diversity indices and the relative abundances of microbial taxa, it was concluded that MPs had selective effects on activated sludge microbial community. However, MPs did not affect the abundance of nitrifying and denitrifying bacteria in the sludge.

1. Introduction

Municipal wastewater treatment plants (WWTPs) treat daily thousands of liters of wastewater generated from human activity, andamong other pollutants, it has been proven that microplastics (MPs) reach the WWTPs and a large part (more than 90%) is retained during the depuration process (Carr et al., 2016; Edo et al., 2020; Gies et al., 2018; Li et al., 2018; Murphy et al., 2016; Simon et al., 2018; Talvitie et al., 2017). However, since a complete removal is not achieved the MPs are also present in the final effluent (Sun et al., 2019). The variation in the quantity reported by previous studies may be related to the treatment units involved in the WWTPs, the size of the population served, consumption patterns and also to the lack of standardization of MPs separation, quantification and identification protocols. Although preliminary and primary treatments are responsible for the greatest removal of MPs (Talvitie et al.,2017, Hou et al., 2021; Jiang et al., 2020; Liu et al., 2019; Murphy et al., 2016, Ziajahromi et al., 2021) the secondary treatment is drawing the attention of researchers due to the accumulation of these particles in the sludge (Hou et al., 2021; Kalčíková et al., 2017;

Lares et al., 2018; Talvitie et al., 2017). Activated sludge from WWTPs can be treated and applied as biosolids, which has been causing terrestrial contamination by MPs in agricultural soils. For instance, Corradini et al. (2019) measured concentrations of 1.1-3.5 particles/g of dry soil in agricultural soils in Chile that received different applications of wastewater sludge. Liu et al. (2018) also reported the occurrence of microplastics in agricultural soils in Shanghai (China) at concentrations of 78.00±12.91MP/kg and 62.50±12.97MP/kg. The authors correlated the presence of MPs to both application of sewage sludge and the use of plastic mulching.

The surface of the MPs can serve as a substrate for the growth of bacterial communities, for nutrient cycles and biofilm formation. These environments have been called 'plastisphere' (Delacuvellerie, et al., 2019, Eckert et al., 2018; Zettler et al., 2013). Despite this symbiotic relation, the presence of microplastics in organic matrices can disturb microbiological activities and communities and can have toxic effects on some cultures (Sun et al., 2019; Huang et al., 2019; Wei et al., 2019a; Zhang et al., 2020). Zhang et al. (2020) investigated the influence of the presence of PET microplastics (0.2mm of size) in anaerobic granular sludge and reported that when the UASB systems were fed with PET-MPs concentrations of 75, 150 and 300MP/L, a reduction in the efficiency of COD removal was observed. In addition, when subjected to a concentration of 300MP/L, a reduction of 28.4% in the methane production, compared to the control, was also recorded. Bacterial community analyzes showed that populations of key acidogens and methanogens decreased after the exposure to PET with concentration of 300MP/L. Although being considered emerging contaminants in aquatic and terrestrial environments, the consequences of the presence of MPs in the microbial communities of activated sludge systems still have many gaps and require a lot of investigation.

Information of biomass characteristics allows to assess how the presence of MPs impacts the biomass, whether by compromising cell viability, bacterial growth and/or metabolic activities. Based on the study of biological parameters, it is possible to identify whether the quality of the effluent or whether the nitrification process suffers some type of inhibition and allows strategic action to improve WWTPs performance. Therefore, this study was carried out in order to evaluate the effect of PE microplastics on the biological process performed in a sequencing batch reactor (SBR). PE was selected because it is a plastic widely used commercially (PlasticsEurope, 2019) and commonly found in WWTPs (Simon et al., 2018; Lares et al., 2018; Bayo et al., 2020; Ziajahromi et al., 2017). Thus, the effects of MPs on the biomass characteristics and its capacity for depuration were assessed. In addition, since few studies have been carried out to characterize the effects of MPs on activated sludge microorganisms (Tang et al., 2021), next-generation amplicon sequencing has been applied to better understand the ecological effect of MPs contamination on bacterial communities in activated sludge.

2. Materials and methods

2.1. Activated sludge

The activated sludge used in this study was collected from the aeration basin of a WWTP located in Comunitat Valenciana (Spain). The facility was designed to treat 40,000 m^3/day and consists of pre-treatment (coarse and fine screens, and basins for removal of grease and sand), primary settling followed by activated sludge system, including secondary settling (secondary treatment), and tertiary treatment.

2.2. Sequencing Batch Reactor (SBR) operation

To evaluate the influence of the presence of polyethylene MPs on activated sludge process, two SBRs were fed with simulated urban wastewater and they were operated for 93 days. One SBR was used as control (SBR-Control), i.e. without PE microspheres addition in the feed, and the other one was doped with PE microspheres (SBR-MP). Table 1 details the operational conditions established for the reactors. The reactors consisted of cylindrical tanks measuring 30x20 cm of height and diameter, respectively. The oxygen was supplied by air diffusers positioned at the bottom of the tanks, and the concentration of dissolved oxygen was 1.8-2.2mg/L. In addition, the equalization of the systems was provided by mechanical stirrers. The SBR feeding and the effluent drawing were performed by peristaltic pumps.

Table 1 - Operational SBR condition

Cycles/day	3
Hydraulic retention time (HRT)	24h
Filling and aerobic reaction	6h
Sedimentation	90min
Draw and idle	30min
Operating days	93

As mentioned above, the reactors were fed with synthetic wastewater to minimize external interference. The synthetic wastewater was prepared with peptone, meat extract and K_2HPO_4 (supplied by Panreac) diluted in tap water, in proportions that guaranteed a feed of 500mg/L of COD, as reported by Ferrer-Polonio et al. (2019) and a supply of nutrients following the COD:N:P of 100:5:1 ratio. To maintain a food/microorganisms (F/M) ratio of 0.2gCOD/(gSS·d) (Eq.1), the reaction volume (V_R) was 6L, the daily

feeding volume ($V_{feed|draw}$) was 6L and the concentration of mixed liquor suspended solids (MLSS) was maintained around 2.5g/L. Periodic sludge withdrawals were performed to maintain the MLSS concentration around that value.

$$F/M = \frac{COD \times V_{Feed|draw}}{V_R \times MLSS} \quad (1)$$

2.3. Microspheres monitorization in the SBR

Green PE microspheres, with a diameter between 150µm-180µm and density around 1.00g/cc, were purchased from the Cospheric provider. The microspheres were dosed directly into the SBR feed tank and the equalization of the systems was provided by mechanical stirrers, providing a feed concentration of 50MP/L (0.2mg/L). The SBR was operated for 70 days with this concentration. To assess the eventual effects of an abrupt increase in MPs concentration on the SBR performance, on day 70 the concentration of MPs in the feed was increased to 253MP/L (1mg/L). The SBR was operated under that concentration for 23 days.

To assist the separation of MPs, a filtration system consisting of a 150µm stainless steel mesh placed inside a 50mm diameter PVC tube was coupled to the peristaltic effluent drain pump (Fig.S1). Once a week the mesh was removed and the retained material was rinsed with distilled water and stored in a beaker, which was covered with aluminum foil to avoid possible contamination. When the presence of suspended solids made the identification of the MPs unfeasible, a chemical digestion procedure based on peroxidation (H_2O_2 35%wt) was applied. In this case, the retained material on the mesh was rinsed with distilled water and treated for 2 hours at 60±2ºC, using a volumetric ratio (mL) of 1:100 [H_2O_2: distilled water]. Afterwards, the material was filtered

through a glass fiber membrane (1µm) and dried in an oven at 50ºC for 2 hours for the visual identification of microparticles.

The mixed liquor was also collected and pretreated by a chemical digestion with H_2O_2 35%wt once a week. For the pretreatment, a volumetric ratio[mL] of 2:1 [H_2O_2: sludge] was used and the chemical digestion was performed at 60±2ºC for 4 hours. This chemical digestion protocol proved to be sufficient to remove more than 90% of suspend solids of the mixed liquor with an initial concentration of 2.5g/L. After digested, the sample was passed through a 150µm mesh and the retained material was removed with distilled water. Finally, the material was filtered through a glass fiber membrane (1µm) and dried in an oven at 50ºC for 2 hours for visual identification of microparticles. The methods of chemical digestion applied in this study, for both effluent and activated sludge, were thoroughly described in our previous research (Bretas Alvim et al., 2020). The material retained on the filter was carefully analyzed by stereomicroscope (LEICA MZ APO), with the magnification adjusted between 8X and 80X, and microparticle were counted. This previous sample characterization was also important to ascertain the presence of microspheres in the initial sludge, which could influence later the monitoring of the SBR-MP.

In order to control possible losses of microspheres in the feed doping, in the separation and in the identification process, the theoretical concentration of microspheres in the sludge was estimated based on the amount of MPs that entered in the SBR via feed, and amount that was removed from the SBR during the sludge withdrawals and the MPs output through the effluent. The number of MPs was expressed as a function of the volume for the effluent (MP/L) and as a function of dry matter (d.w) for the sludge (MP/d.w).

2.4. Analysis

2.4.1. SBR performance

Throughout the three months of monitoring, physical and chemical characterizations were carried out both in the effluent and in the sludge. The effluent was characterized in terms of pH, turbidity, conductivity and soluble COD three times a week, and TN, NH_4-N, NO_3-N, NO_2-N, TP and PO_4-P once a week. The sludge was monitored for MLSS (three times a week), mixed liquor volatile suspended solids (MLVSS) and soluble microbial products ($SMP_{proteins}$, $SMP_{carbohydrates}$) once a week. Samples for SMP analysis were obtained from the MLSS centrifuging at 12,000rpm for 15min and filtering the supernatant through 0.45µm cellulose acetate filters. $SMP_{proteins}$ were measured by BCA method (Zuriaga-Agustí et al., 2013) and carbohydrates by anthrone method (Frølund et al., 1996). Sludge samples were immediately observed after sampling with Carl Zeiss phase contrast microscope, Axiostar Plus model.

2.4.2. Adenosine triphosphate (ATP) and Cellular viability

During the SBRs operation, nine samples of the mixed liquor were collected periodically from the SBRs, at the end of the reaction time, to determine ATP and cell viability. ATP measurements were performed using PhotonMaster ™ Luminometer from Luminultra® (Ferrer-Polonio et al., 2019). The ATP detection method is based on the production of light when reacting a sample containing ATP with the enzyme luciferase. The light produced is detected in a luminometer and is proportional to the amount of ATP in the sample. The detected light response is given in units of relative light (RLU). The results obtained were converted to ng ATP/mL using the Standard ATP solution UltraCheck ™ and LumiCaptureTMLite software.

From ATP measurements, the bio stress index (BSI), Eq.2, has been calculated. This parameter indicates eventual toxic effects on the microbial community.

$$BSI\ (\%) = {}^{dATP}/_{tATP} \times 100\% \quad (2)$$

Cellular viability was performed by Film Tracer™LIVE/DEAD™Biofilm viability kit (Molecular Probes Eugene, OR, USA) using the methodology reported by Ferrer-Polonio et al. (2019). The BX50F microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury lamp was used to evaluate the samples.

2.4.3. Microbial hydrolytic enzymatic activities (MHEA)

Six microbial hydrolytic enzymatic activities were determined: Lipase, Acid phosphatase, Alkaline phosphatase, α-D-Glucosidase, Dehydrogenase and Protease in the SBR-MP and SBR-Control. They were analyzed using the same samples taken for ATP and cell viability analyses. For this purpose, the methodology reported by Ferrer-Polonio et al. (2019) was used.

2.4.4. Statistical analysis

Statistical significance was evaluated by *t*-test analysis (confidence level of 95%) with Statgraphics Centurion XVII. The following parameters were assessed in both reactors: %removal of COD, MHEA, ATP, BSI.

2.4.5. Microbial community analysis

DNA from SBR samples was extracted in duplicate, as previously described (Luján-Facundo et al., 2018) using a FastDNA® SPIN kit for soil (MP Biomedicals, OH, USA),

according to the manufacturer's protocol. DNA quality was measured using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, DE, USA). DNA concentration was measured using Qubit® dsDNA BR Assay Kit (Molecular probes, Eugene,OR, USA). DNA samples were sent to Fundación FISABIO sequencing service (Valencia, Spain) for V3-V4 16S rRNA gene amplification using the primers PRO341F and PRO805R. The subsequent amplicon sequencing on the Illumina Miseq platform was also performed by Fundación FISABIO sequencing service (Valencia, Spain) using a 2 × 300 nucleotide paired-end reads protocol.

2.4.6. Bioinformatics analysis of Illumina-generated amplicons

Sequence data processing and operational taxonomic units (OTU) picking were described in an earlier work (Luján-Facundo et al., 2018). Briefly, the Microbiome Helper standard operating procedure was used to process and analyse the sequencing data (Comeau et al., 2017). Raw Illumina sequences were analyzed using QIIME™ 1.8.0 (Caporaso et al. 2010). The most abundant sequence of each OTU was picked as its representative and it was used for taxonomic assignment against MiDAS v3.6 (Nierychlo et al., 2020) at 97% identity (3% cutoff level) using default parameters. Alpha-diversity (Chao1, ACE and Jacniffe indicators of species richness, Shannon and Phylogenetic diversity) were generated using CL_OPEN_REF_UCLUST_MC2 method (OTU-picking method) against EzBioCloud PKSSU4.0 database (Yoon et al., 2017).

3. Results

3.1. Behavior of polyethylene MPs in the SBR

The microspheres used in SBR-MP had a spherical shape and a very evident green color (Fig.1), which facilitated their separation and quantification.

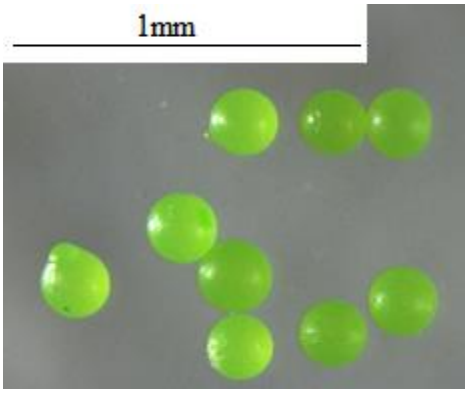

Fig.1 – Polyethylene Microspheres

During the operation of the SBR-MP reactor, a continuous accumulation of MPs was observed in the mixed sludge, reaching a final concentration of 6260MP/L (2607MP/g d.w) (Fig.2). This trend was observed throughout the monitoring period of the SBR-MP and a percentage of 98±2% (n=13) of the MPs were accumulated in the activated sludge. MPs could serve as a substrate for the formation of biofilms by bacterial communities, which would decrease their buoyancy (Zettler et al., 2013; Andrady, 2011, Lobelle and Cunliffe, 2011). Kalčíková et al. (2019) also suggested that the high affinity between PE microparticles and sludge could be attributed to the negative charge of the sludge flakes and the positive surface charge of PE. These factors could be the reason why MP were found in the activated sludge rather than in the secondary effluent.

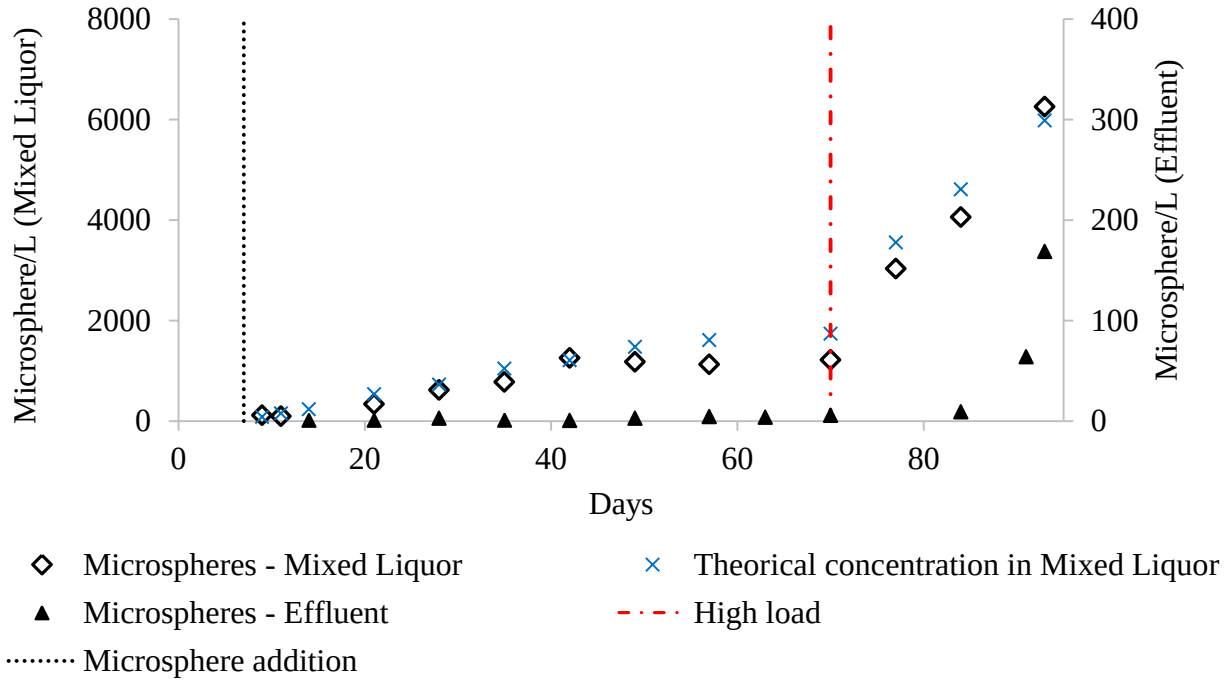

Fig.2 – Temporal evolution of the disposal of microspheres in mixed liquor and effluent

Some authors also describe similar results, where a huge portion of MPs was removed during secondary treatment due the affinity between MPs and sludge. Edo et al. (2020) reported 94% removal efficiency in the secondary settler in a WWTP in Spain preceded by an Anaerobic-Anoxic-Oxic (A2O) biological process. During the secondary treatment in a WWTP in Finland, around 87% of microparticles were removed in the activated sludge process (Talvitie et al., 2017). Kalčíková et al. (2017), although reported lower removal percentage (of 56±16% and 47±17%) of PE microbeads in two SBRs, also observed that the MPs were visibly retained in mixed liquor.

During the feeding of the SBR-MP with low MPs load (50MP/L), concentrations of 3±2MP/L in the SBR-MP effluent were counted. When the MPs concentration in the feed was raised (253MP/L), an increase in the MPs concentration in the effluent was also observed (64±81MP/L), but in terms of percentage the activated sludge still accumulated around 98% of the MPs. Since biosolids can be used as fertilizer, MP

retained on this biological material can lead to contamination of the terrestrial environment (Corradini et al., 2019; Liu et al., 2018). Talvitie et al. (2017) estimated that 80% of microparticles, including MP, removed by a WWTP (Finland) are contained in the dried sludge. That WWTP annually produced about 60,000 tons of dry sludge. Thus, considerable amounts of microparticles are allocated to the environment by the application of the sludge as biosolids. Van den Berg et al. (2020) reported that in Spain agricultural soils receiving biosolids from sewage sludge, presented on average 256% more MPs than soils without application.

3.2. Effluents characterization (SBR-MP and SBR-Control)

In general terms, the effluents from both reactors SBR-Control and SBR-MP were very similar in characteristics. A percentage of COD removal of 97.35±0.81% (n=33) and 96.35±1.56 (n=33) was obtained in SBR-MP and SBR-Control, respectively, representing a high purification capacity in the two reactors. The results of the microbial community, which will be better discussed in the section 3.3.1, demonstrated that the presence of nitrifying and denitrifying bacteria increased in the SBR-MP during the reactor performance, hence the high ammonium concentration reported in the Fig.3 (days 9-16 and 37-45) was attributed to a malfunction in the aeration system. It explains the high standard deviation of the values of the parameters NH_4-N and NO_3-N. When the aeration was corrected, the ammonium nitrogen concentration decreased due to its oxidation and consequently the nitrate concentration increased. Furthermore, the aeration problems associated with the MPs did not compromise the COD removal efficiency and even implied nitrogen removal by biological denitrification due to short anoxic periods in the reactor, which is proved by the sum of the concentrations of the nitrogen species in the effluent. Table 2 shows the characteristics of the effluents of

both SBRs. Results are expressed in average values and their respective standard deviations (±SD).

Table 2 – Average values of the physical-chemical parameters of the effluents

Parameter	**SBR-MP**	**SBR-Control**
pH	7.45±0.31	7.31±0.20
Conductivity (mS/cm)	1.16±0.12	1.10±0.07
Turbidity (NTU)	0.82±0.96	1.50±1.27
COD (mg O_2/L)	13.25±4.06	18.24±7.78
COD removal efficiency (%)	97.35±0.81	96.35±1.56
NH_4-N (mg/L)	11.61±12.00	4.00±0.00
NO_3-N (mg/L)	20.20±15.19	38.38±5.05
NO_2-N(mg/L)	0.15±0.12	0.11±0.11
TP (mg/L)	5.66±1.42	5.52±1.34

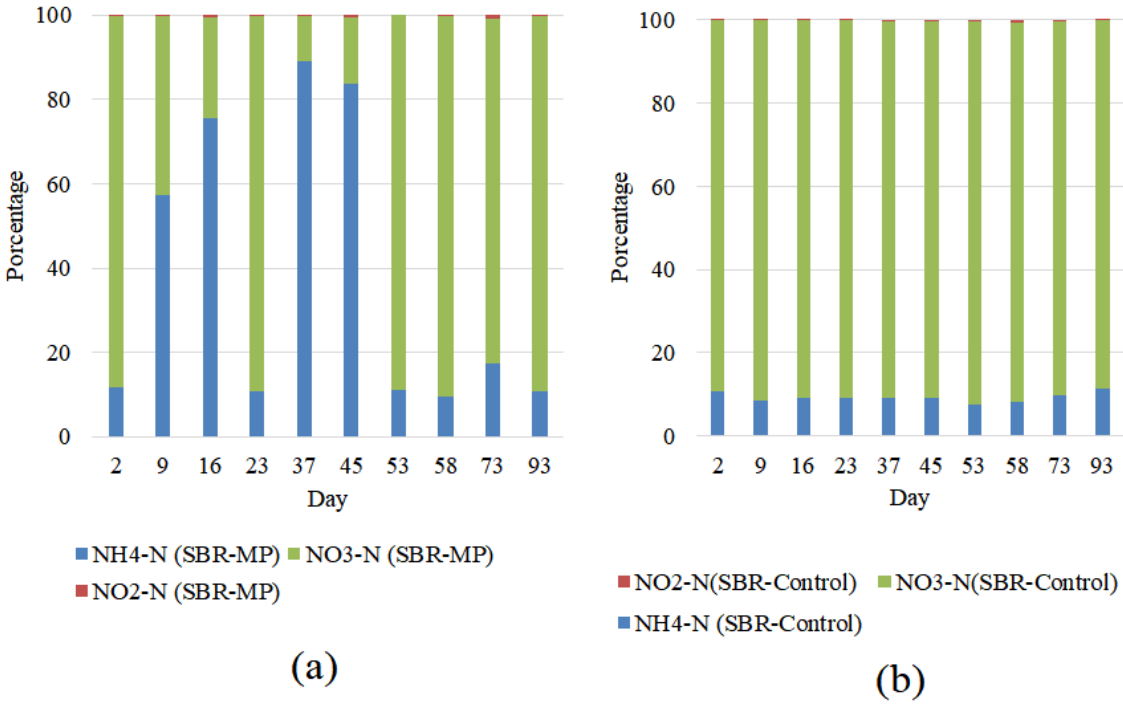

Fig.3- Nitrification in the SBR-MP (a) SBR-Control (b)

In the depuration process based on biological treatments, the microbial soluble products (SMP) correspond to the mayor fraction of the COD in the effluents and may be related to cell lysis, substrate metabolism and biomass growth (Laspidou and Rittmann, 2002; Potvin and Zhou, 2011; Xie et al., 2013). These substances mainly correspond to proteins (SMPp) and carbohydrates (SMPc). Despite the discrepancy in the average values of SMPp (6.76±0.81mg/L SBR-Control and 5.86±1.35mg/L SBR-MP) and SMPc (16.60±4.10mg/L SBR-Control and 4.59±2.12mg/L SBR-MP) between the reactors, the SBR-MP showed an average concentration of SMP lower than the SMP-Control. It suggests that the presence of microspheres did not promote biomass disruption, which could release more SMP in the system.

With all these results, the presence of PE does not seem to be a stress factor to the biological process, as the depuration capacity of SBR-MP was high and it was very similar to that measured for the SBR-Control. Kalčíková et al. (2017) also evaluated the possible impacts of the presence of PE microbeads (0.5g/L), extracted from cosmetics, on the performance of the SBR, reporting that the nitrification process was not inhibited with the presence of PE, moreover, removals of 96±8% (n=6), 95±7% (n=6), and 93±7% (n=6) of dissolved organic carbon were achieved by these authors in the SBRs with microbeads (MP1 and MP2) and in the control, respectively. Li et al. (2020) evaluated the effect of microplastics (PP, PVC, PE, PS and polyester - PES) with concentrations of 1000, 5000 and 10000MP/L in the nitrification process in bench tests with activated sludge, and reported that the presence of MPs did not stop nitrification, but impacted in different ways this process. For instance, 1000MP/L of PP and PVC slightly improved the ammonia oxidation rate compared with the control (without

adding MPs), but concentrations of 5000 and 10000MP/L negatively impacted the nitrification. The presence of PE, PS and PES also decreased the ammonia oxidation rate, but regarding the denitrification was concluded that 5000MP/L of PVC and PES increased expressively the denitrification rate compared with the control. Liu et al. (2019) also assessed the impacts of PE, PVC and PES MPs on the nitrification and denitrification process and their results demonstrated that at concentrations of 50 – 10,000particles/L, both ammonium-oxidizing bacteria activity and nitrite oxidizing bacteria activity and also the denitrifiers activity were not significantly affect by the presence of MPs. However, Tang et al. (2021) observed controversial results when studied the influence of PVC microplastics (PVC-MPs) on an ANAMMOX process. The nitrification process was negatively impacted by the presence of 0.1g/L and 0.5g/L PVC-MPs, the average ammonia nitrogen removal rates decreased by 3.8 % and 6.2 %, respectively, compared with the control. These results suggest that the MPs may have different impacts in the nitrification process and this may be due to the polymer type, the concentration of the MPs, the presence of additives in the formulation of the polymer and the structure of the microbial community. Therefore, the possibility of the inhibition of the nitrification and denitrification must not be neglected especially considering the continuous production of MPs and the high amount of MPs that reaches the WWTPs and accumulate in the activated sludge.

3.3. Biomass characterization

Due to the high retention of MPs in the activated sludge, parameters to assess MPs toxicity on the biomass were evaluated. Firstly, cell viability based on the images obtained by the epifluorescence microscope (Fig.4) -which shows green and red zones corresponding to the viable and damaged cells, respectively– was measured. It can be

observed that the presence of 50MP/L (days 31, 52, 65) and 253MP/L (day 93) did not increase the percentage of dead cells in the SBR-MPs. Hence, the cell viability in SBR-MP was not affected by the MPs presence (p-Value = 0.9494), representing 75±7% and 75±8% in the SBR-Control and SBR-MP, respectively during the monitoring period.

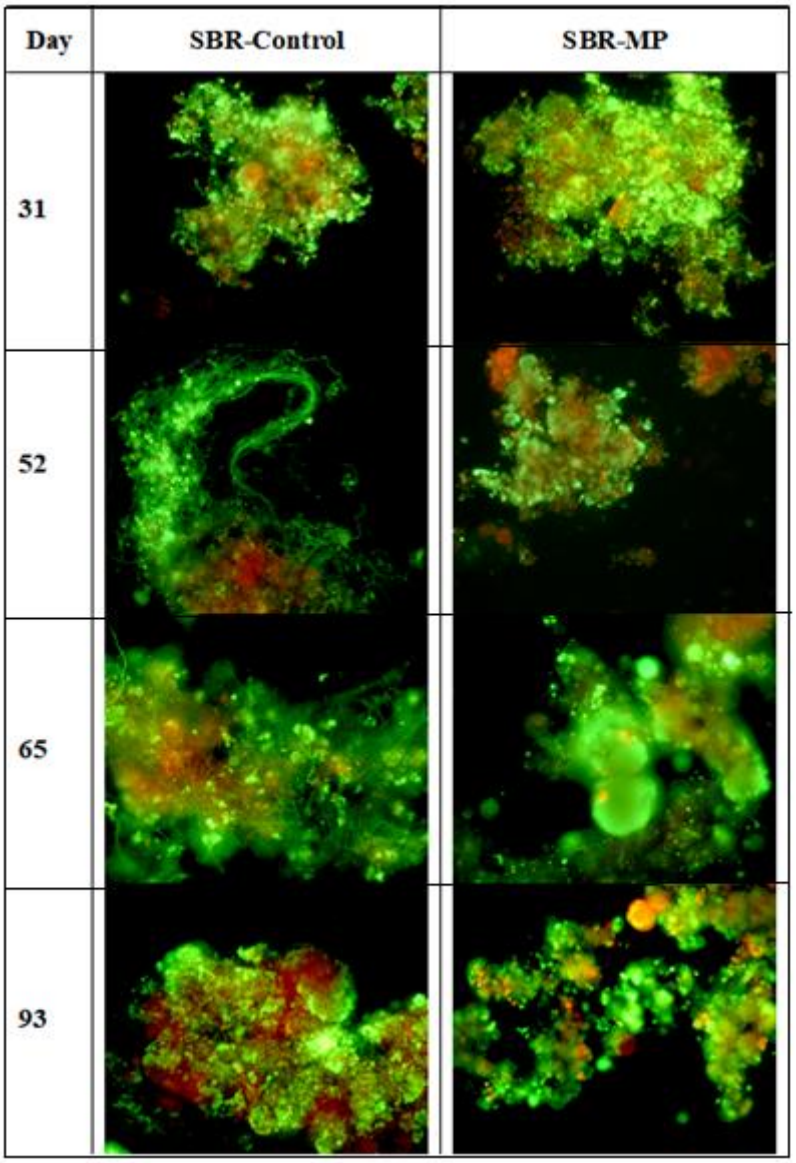

Fig.4 – Epifluorescence microscope images (200x magnification) of SBR - Control and SBR-MP

In addition to the cell viability, c-ATP and BSI were also measured to ascertain the impacts of the PE-MPs on the SBR biomass. The amount of ATP in biological systems is related to the metabolic activity of viable organisms. Therefore, the introduction of a new component in the SBR (in this case PE) may affect the bacterial metabolism, resulting in changes in cellular ATP (c-ATP) (Bäckman and Gytel, 2015; Dalzell and Christofi, 2002). However, according to the c-ATP values measured in this work, the addition of MPs in the SBR did not result in significant differences in the microbiological metabolic activity over the days of monitoring, comparing to the SBR-Control (p-Value=0.1083). Furthermore, the values of BSI also showed that the presence of MPs did not result in toxic impacts on the microbial community, with values of 13.88±3.98% and 14.00±5.18% (SBR-Control and SBR-MP, respectively) without statistically significant differences, at 95% confidence level, between the reactors (p-Value=0.9576).

Despite no toxic impacts on the microbial community was assessed, in the period from day 70 to day 93 (period in which the SBR-MP was operating with a high load of MPs, 253MP/L), it was observed that the biomass growth was impacted (Fig.5). In the Fig.5, the initial period (from day 0 to 7) is related to the acclimation of biomass in the reactors. From day 7 it is observed that both SBR-Control and SBR-MP maintained their growth rate achieving the steady state. When SBR-MP was subjected to an increase in the load of microspheres (from day 70), it suffered an abrupt growth rate reduction (from 0.30gSS/d to 0.14gSS/d).

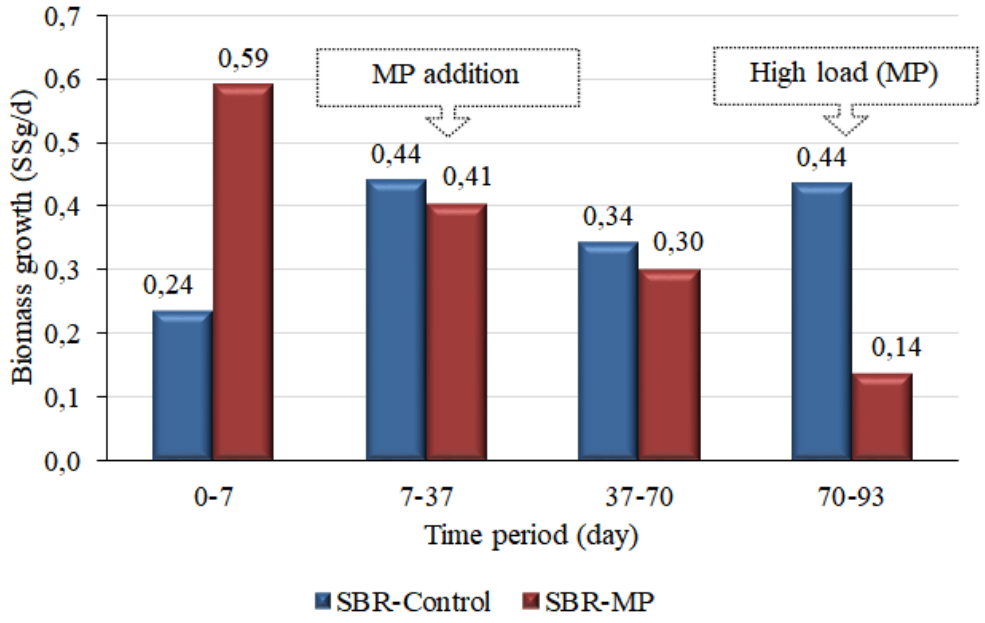

Fig.5 – Biomass growth in SBR-Control and SBR-MP

To explain this growth rate reduction, it is necessary to consider that the microorganisms used the surface of the MPs for the development of microbial communities (biofilms). Once the formation of biofilms around MPs had started, it was expected an increase of the production of bound EPS to keep these structures well established. Therefore, the external substrate, in addition to being used for the cell growth, was also required for the bound EPS formation (Laspidou and Rittmann, 2002). Once the external substrate was depleted, the active sludge reached the endogenous phase, and the oxidation of the biomass started in order to obtain nutrients for maintaining the microbial community. In brief, on day 93, due to the high accumulation of MPs in the sludge, it was assumed that the microorganisms were able to use the polymer surface extensively for the establishment of biofilms, and for this reason the endogenous phase was more quickly reached.

The lower growth rate of biomass in the SBR-MP, and consequently greater solid retention time (SRT), was corroborated by the microscopic analysis of the sludge structure at the beginning (a, b), middle (c,d) and end (e,f) of the experiments (Fig.6). In

the SBR-MP the presence of protozoa at a higher extent was observed, mainly at the end of the monitoring (Fig.6f). These organisms are commonly found in activated sludge systems with longer SRT (Ghyoot and Verstraete, 1999; Hao et al., 2010; Madoni, 2011).

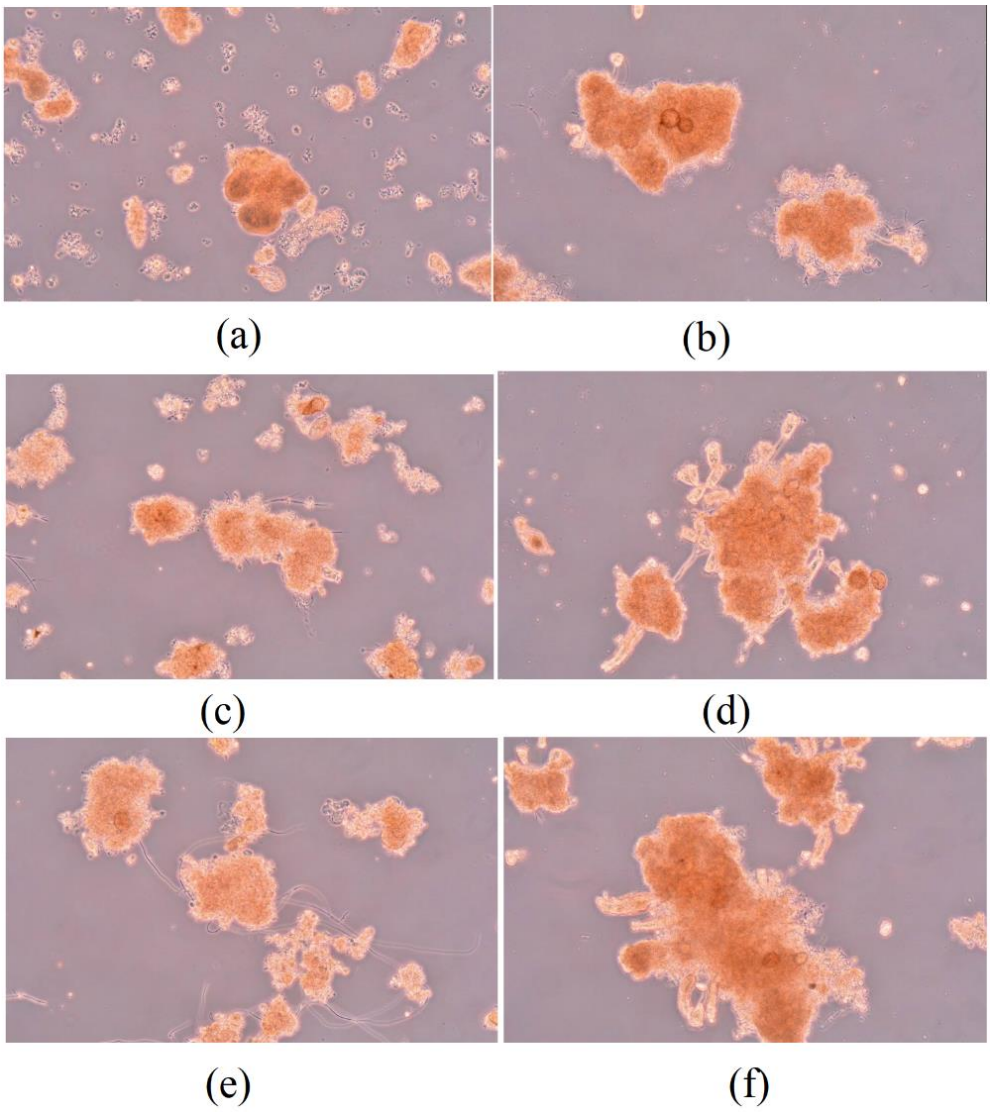

Fig.6 – Biomass images 100x from SBR-Control (Left) and SBR-MP (Right)

The SBR-Control started with a high protozoa diversity but with a low population density of individual microorganisms. At the end of the study, in the SBR-Control, the

protist diversity was almost completely declined with occasional observation of *Opercularia articulata* and *Arcella vulgaris*. *Opercularia articulata* is usually associated with discharges of high Food/Microorganisms ratio. On the contrary, at the end of the study, the SBR-MP presented a low protozoa diversity, but a high population density. In this last stage was also observed in the SBR-MP the coexistence of *Rotaria* sp. and *Zoothamnium* spp. species, and occasionally *Arcella vulgaris*. The *Zoothamnium* spp. is usually associated with high COD removal, high quality of the wastewater effluent and is an excellent indicator of low Food/Microorganisms ratio in biological reactors. Microscopically, the activated sludge of the SBR-MP presented a more compact floc structure with larger flocs and slightly more mineralized than SBR-Control. These results confirmed that the SRT in SBR-MP was higher than in SBR-Control, which showed a slightly open and less mineralized floccular structure. Another revealing aspect observed was the absence of filaments in SBR-MP, while in SBR-Control their presence was quite evident (Fig. 6e). This result might suggest that, in the SBR-MP, the structures of filamentous organisms were destroyed by mechanical shocks caused by microspheres.

3.3.1. Microbial community analysis

The performance of the activated sludge during the wastewater treatment is related to the sludge microbiome; therefore Illumina sequencing was performed in both reactors SBR-Control and SBR-MP to assess the impacts of the presence of PE-MPs on the microbial community. The MIDAS 3 database was used to analyse the microbiome in 8 SBR sludge samples. The 8 Illumina libraries of bacterial 16S rRNA gene yielded 703,359 reads after quality filtering and removal of chimeric sequences from 1,008,864 raw reads. The species richness, library coverage and diversity estimation were

calculated for each library and shown in Table 3. The Chao1 demonstrated that the richness of bacteria in SBR-MP samples on day 93 was lower than that in SBR-Control. Furthermore, the bacterial biodiversity in SBR-MP was also lower than that of SBR-Control on day 93, according to the ACE and Jackniffe indices. Based on these results, our findings suggest that MPs can have selective effects on activated sludge microbial community.

Table 3 – Index of microbial community diversity of 16S rRNA gene amplicon analysis for SBR sludges

Sample	Day	Clean reads	OTU	ACE	Chao1	Jackniffe	Shannon	Phylogenetic diversity	Coverage
SBR-Control	23	92,980	1958	2139	2090	2281	5.466	2755	0.930
SBR-Control	52	93,049	1415	2221	2166	2355	5.790	2782	0.930
SBR-Control	65	63,348	1425	1838	1791	1962	5.604	2425	0.921
SBR-Control	93	93,196	2423	1999	1945	2128	5.562	2629	0.932
SBR-MP	23	93,494	1682	2165	2068	2245	5.545	2735	0.935
SBR-MP	52	93,007	1270	1808	1755	1935	5.222	2413	0.930
SBR-MP	65	93,419	1604	1697	1639	1820	5.195	2289	0.934
SBR-MP	93	76,284	2084	1608	1567	1727	5.330	2180	0.924

The predominant phyla in both reactors SBR-Control and SBR-MP belonged to *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi* and *Patescibacteria* (Fig.7a). The dominant presence of these phyla is usually reported in the microbial community of activated sludge (Guo et al., 2017; Nguyen et al., 2019). During the performance of the SBR-MP was observed an expressive reduction of the relative abundance of *Chloroflexi* from 9.6% (Day 23) to 4.4% (Day 93). Once these microorganisms correspond to filamentous bacteria, this decrease could be linked to the bacteria disruption by the mechanical shocks caused by the MPs, as explained in the

section 3.2. Unlike *Chloroflexi* phylum, the relative abundance of *Proteobacteria* raised from 26.4% (Day 23) to 39.3% (Day 93) in the SBR-MP. Delacuvellerie, et al. (2019) studied the formation of 'plastispheres' on LDPE, PET and PS macroplastics (>5cm) and observed that the microbial community on the plastic surface was mainly formed by *Proteobacteria* (especially *Gammaproteobacteria* and *Alphaproteobacteria*), which is considered the primary colonizers in a plastic biofilm in the marine environment (De Tender et al., 2015). In our study we did not evaluate the biofilm on the PE-MPs surface, but results suggest that these bacteria can play an important role in the biofilm formation on PE-MPs since the activated sludge is the only source of bacterial colonization and it is mainly compound by *Proteobacteria*.

Another important aspect about the presence of *Proteobacteria* is that this phylum includes bacteria groups that are responsible for ammonia (*Nitrosomonas*, AOB) and nitrite oxidation (*Nitrospira*, NOB) and for the denitrification process (denitrifying bacteria, *Thauera*). Their presence in the activated sludge contributes to the good depuration performance of the SBR. In this context, *Nitrosomonas*, *Nitrospira* and *Thauera* were detected during all the studied period of SBR-Control and SBR-MP (Fig.7b) and their relative abundance were not affected by the presence of MP. In addition, an increase of these groups in the SBR-MP during the experimental time these bacteria can play an important role in the biofilm formation on PE-MPs. Therefore, these results indicate that the nitrification and denitrification process in the SBR-MP were not inhibited by the presence of MPs, even under higher PE-MPs concentration (Day 93). More detailed information is included in Fig. 2S.

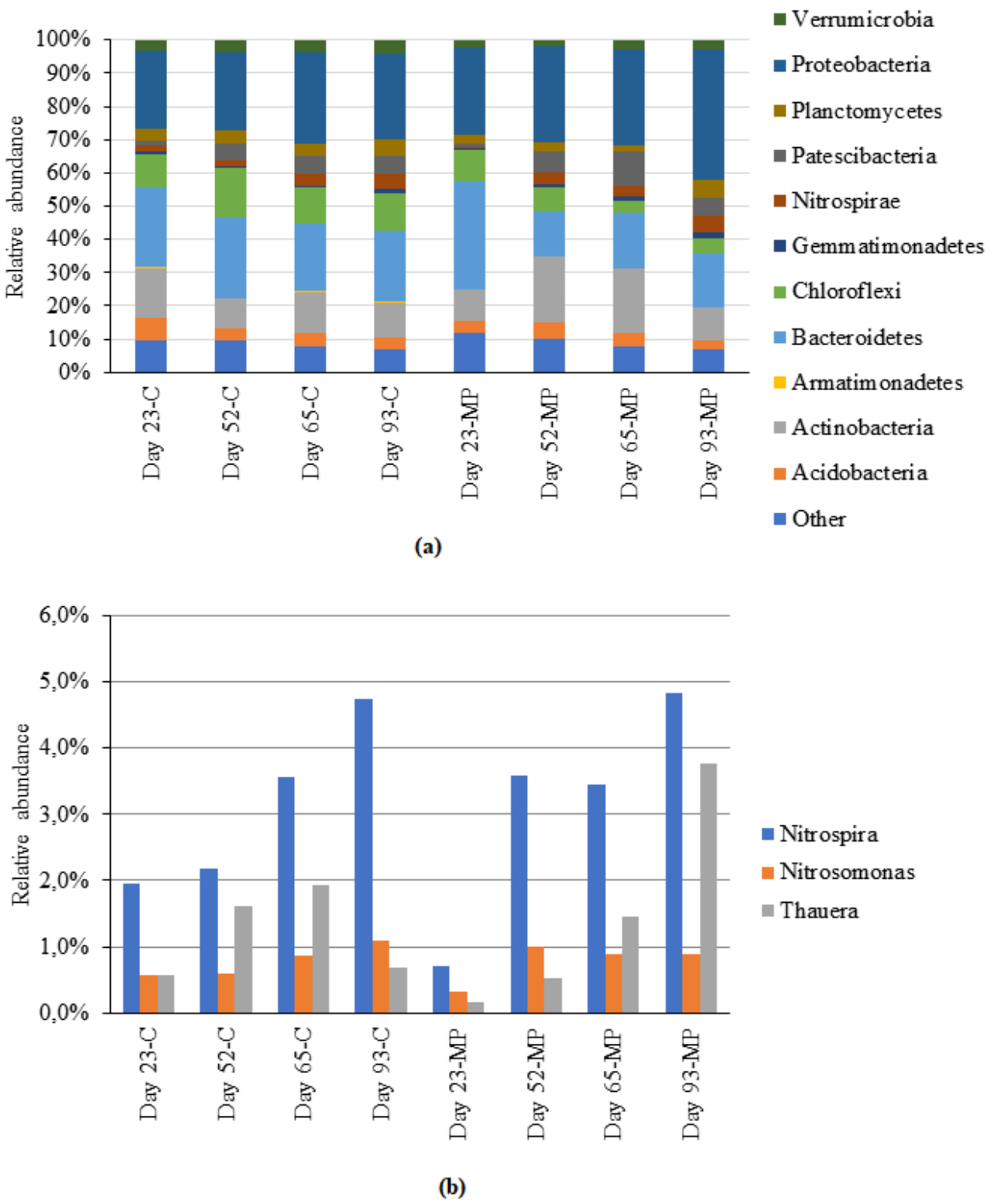

Fig.7 – (a) Bacterial community at phylum level by MiSeq sequencing (C: SBR-Control and MP: SBR-MP samples); (b) Relative abundances of nitrifying and denitrifying bacteria by MiSeq sequencing (C: SBR-Control and MP: SBR-MP samples)

Across all 8 libraries, 57 and 51 different bacterial classes were found in SBR-Control and SBR-MP, respectively. The dominant class was *Bacteroidia* with relative abundance of 23.0-19.7% in SBR-Control and 31.2-16.4% in SBR-MP. *Actinobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria* accounted for 14.2-10,0%, 11.7-12.1%, 8.9-9.5%, respectively, in the SBR-control. The other three dominant classes in SBR-MP were also *Actinobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria* accounting for 8.6-19.3%, 14.4-11.0%, 11.9-12.0%, respectively. Relative abundances at genus level can be observed in supplementary materials (Fig. 3S)

Until now there is a lack of knowledge about how and why the MPs can impact the microbial population in activated sludge. However, it has been reported that the presence of MPs in organic matrices can indeed alter their microbial community. For instance, Wei et al. (2019a) observed that the exposure of the waste activated sludge (which was submitted to anaerobic digestion) to PE-MPs (200MPs/g-TS) reduced the total microbial population, without affecting the microbial diversity. In another study Zhao et al. (2020) assessed the effects of polyamide 66 (PA66) on the aerobic granular sludge and observed that 0.1g/L PA66 leaded to a reduction of the diversity of the bacterial communities and an increase of microbial richness. The authors also observed that 0.2g/L of PA66 contributed to increase the abundance of *Proteobacteria*, however, 0.5g/L of PA66 negatively impacted the abundance of this phyla. Tang et al. (2021) reported that 0.5 g/L of polybutylene succinate (PBS) stimulated the enrichment of the *Proteobacteria* on 10.23 % on the anaerobic ammonium oxidation (ANAMMOX) sludge. However, the authors also observed that PVC microplastics inhibited the growth of anaerobic ammonia-oxidizing bacteria on the anammox process. Since the biosolids from WWTPs can be applied in the farmlands, the presence of MPs in this organic matrix can also impact on the microbiome of soils, an in this context Wang et al. (2020)

observed that the presence of LDPE-MP altered the soil microbial community and selectively enriched specific members of bacteria. After 90 days exposure, it was noticed that the phyla *Acidobacteria, Armatimonadetes, Bacteroidetes, Gemmatimonadetes,* and *Proteobacteria* were significantly abundant in the microplastic amended soils.

3.4. Enzymatic activities

The results of MHEA (Fig.7) show that SBR-MP had slightly lower phosphatase activity and equal dehydrogenase and α-D-glucosidase enzyme activity compare to SBR-Control, and almost all activities remained constant throughout the monitoring period. However, the increase in protease activity over time is noteworthy. Few studies have reported how the presence of MPs can affect microbiology communities and their enzymatic activities; thereby conclusions that justify the increase in certain enzyme activities are not clear. Wei et al. (2019b) demonstrated that the presence of PVC microplastics (60particles/g Total Solid) can reduce the enzyme activities Protease, AK and F420, which are considered key enzymes for the anaerobic digestion process. In addition, they reported that the additive bisphenol-A (BPA), when leached from PVC microplastics inhibits methane production. In another investigation Wei et al. (2019a) observed that during the waste activated sludge anaerobic digestion, fed with MPs of PE in concentrations of 200particles/g of Total Solids, methane production can be reduced by more than 20% also resulting in a reduction of microbial population. A study carried out by Huang et al. (2019) showed that the presence of MPs of PE in soils (200fragments per 100g of soil dw = 0.076 g/kg) stimulated an increase in urease and catalase activity (175% and 139%, respectively) after being exposed to the plastic for 90 days. Unfortunately, we cannot perform a direct comparison between the results

obtained in our work and those presented by the authors previously cited because different conditions and different types of enzymatic activities have been measured. In this way, further research about how the presence of MPs in activated sludge would affect microbial communities and their enzymatic activities should be developed. Thus, due to the lack of knowledge of the impacts of MPs on enzymatic activities of activated sludge, two hypotheses are stipulated to justify the increase in protease in the SBR-MP. The first hypothesis is based on the EPS hydrolysis and the second one on the PE biodegradation.

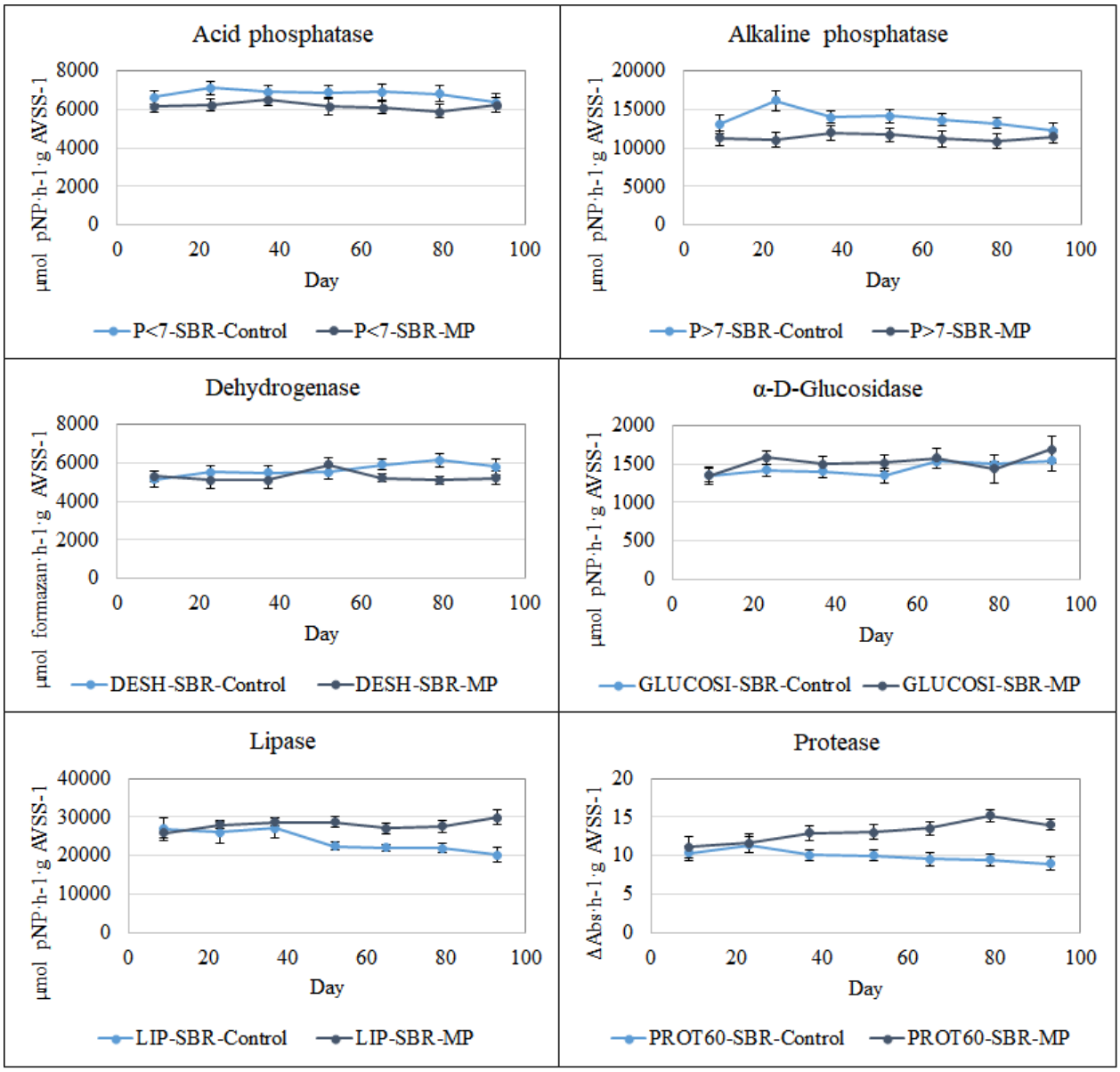

Fig. 7- Microbial hydrolytic enzymatic activities in SBRs

The biomass growth decline in the SBR-MP was attributed to the biofilm formation on the MPs surface, as explained in the section 3.3. The faster substrate consumption makes that the SBR-MP reaches the endogenous phase earlier. In this phase, to guarantee the maintenance of active biomass, hydrolysis of bound EPS is enhanced (Laspidou and Rittmann, 2002). Therefore, in the SBR-MP the hydrolysis of EPS would

lead to an increase in protease enzymatic activities. However, EPS are mainly formed by carbohydrates and proteins (Sheng et al., 2010); thereby an increase of the enzymatic activity for carbohydrates depletion would be also expected. It cannot be discarded because other enzymes in addition to glucosidase may play a role in carbohydrates degradation.

Therefore, a second hypothesis was elaborated considering the possible biodegradation of polyethylene after reach the endogenous phase as an attempted to use the PE as carbon source. The biodegradation process could be started with the formation of colonies of microorganisms on the polymer surface, followed by the secretion of extracellular enzymes in order to produce low molecular weight products which could be used as a carbon source (Arutchelvi et al., 2008; Das and Kumar, 2015). The enzymes proteases, ureases and esterases could be involved in the biodegradation process (Kumar et al., 2020).

Tribedi and Sil (2013) reported the biodegradation of polyethylene film by *Pseudomonas* sp. In this process, besides the presence of biofilm on LDPE surface, the authors also suggested that *Pseudomonas* sp. have enzymatic activities which lead to polyethylene biodegradation. Park and Ki (2019) evaluated the biodegradation of PE microspheres (40µm-600µm) as sole carbon source, by mixed bacterial strains mainly composed of *Bacillus sp.* and *Paenibacillus sp.* isolated from a municipal solid waste disposal site in Korea. In this study it was observed that the microorganisms, besides colonizing the surface of the PE, promoted a weight loss of 14.7% of the microplastic after 60 days of incubation. In another work, where *Bacillus amyloliquefaciens* isolated from municipal solid soil (India) were used to assess the biodegradation of LDPE film microplastic, showed that these bacteria were capable of colonizing the microplastic surface and degrading it, achieving 16% of its weight loss (Das and Kumar, 2015). The

degree of biodegradation may vary according to the type of polymer, experimental protocols and microorganisms present in the process. The two hypotheses proposed were based on the results reported in previous studies, nevertheless, more specific investigations regarding the exposure of activated sludge to MPs must be performed to validate them. The measure of EPS in biological systems fed with microplastics can provide relevant information about the microbial structure, and analyses such as scanning electron microscopy can validate the formation of microbial colonies on the microplastic surface. Moreover, spectroscopy analyses, FTIR for example, on the MPs surface can assess their biodegradation and their use as carbon source.

4. Conclusion

During the operation of the SBR-MP was observed that 98±2% of the PE-MPs were accounted in the mixed liquor, and the remaining fraction in the effluent. Despite the high and continuous accumulation of MPs in the activated sludge, reaching a final concentration of 6260MP/L (or 2607MP/g d.w) on day 93, the SBR-MP maintained a high depuration capacity, with a COD removal efficiency of the same order of magnitude as the control reactor. In addition, since the relative abundance of the bacteria responsible for the nitrification and denitrification did not decrease during the operation of the SBR-MP, was conclude that the nitrification was not inhibited by the presence of PE-MPs. Concerning the biomass, neither cell viability nor c-ATP production were affected by the PE microspheres. However, the results of microbial community suggest that the PE-MPs may had selective effects on activated sludge microbiome. An important observation regarding it was the increase of the relative abundance of *Proteobacteria* recorded on day 93. This phyla is considered the primary colonizers in a plastic biofilm in the marine environment, its high presence in the

activated sludge suggests that the MPs surface can be colonized by these bacteria that try to use the polymer as carbon source in the endogenous phase and, therefore, the possible biodegradation of the PE-MPs may justify the increase of the protease activity in the SBR-MP.

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Supplementary Material

Effect of polyethylene microplastics on activated sludge process - accumulation in the sludge and influence on the process and on biomass characteristics

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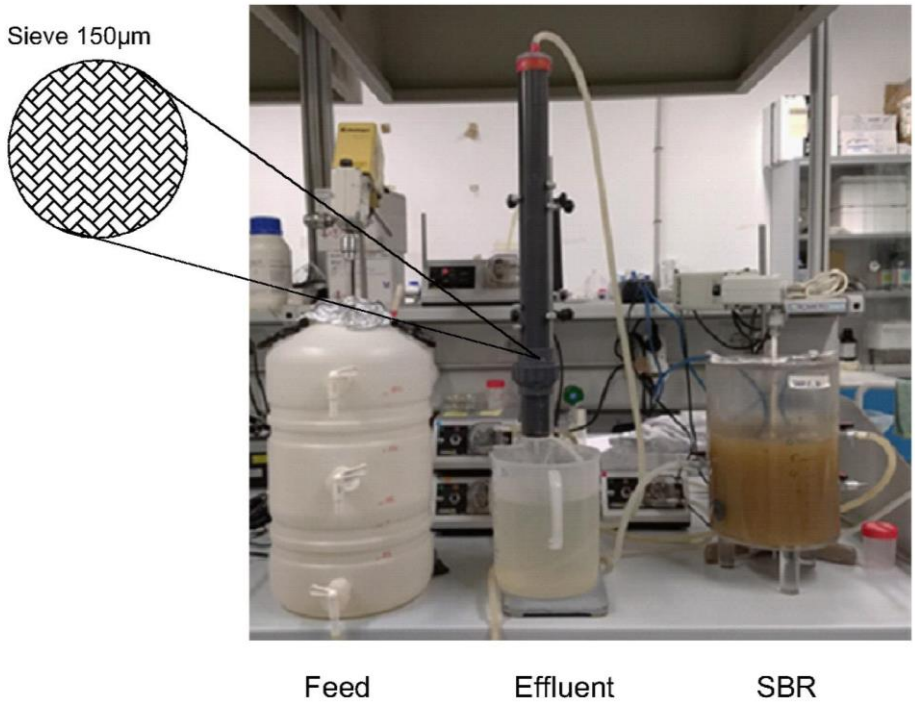

Fig.S1 – Filtration system coupled to the SBR

Nitrosomonas, *Nitrospira*, *Thauera* and *Tetrasphaera* sequences were assigned unique MIDAS names to species level. The most abundant AOB, NOB, denitrifiers, and PAOs species identified in SBR-MP were s_midas_s_723 (*Nitrosomonas*) (0.15-0.39%), *Nitrospira defluvii* (0.70-4.79%), *Thauera phenylacetica* (0.15-3.10%) and *Tetrasphaera jenkinsii* (4.14%-0.60%), respectively. Thauera and Nitrosomonas exhibited higher diversity in SBR-MP, each having 11 and 10 species, respectively (Fig. 2S). The MIDAS 3 database propose unique provisional names for all unclassified microorganisms down to species level providing *de-novo* taxonomy names (midas_x_y names).

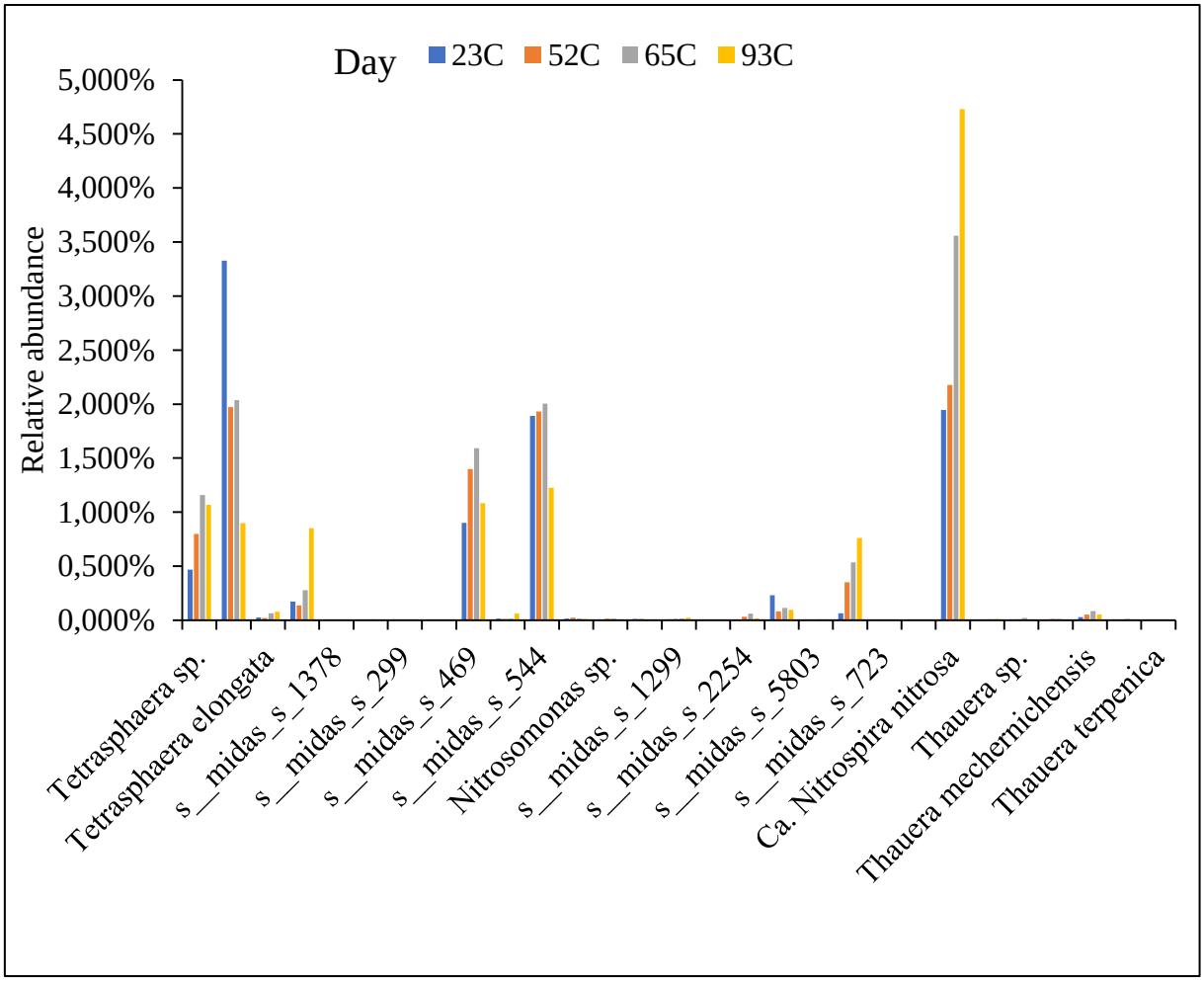

Fig. 2Sa- Relative abundance of *Tetrasphaera*, *Nitrosomonas*, *Nitrospira* and *Thauera* species in SBR-C during the studied period

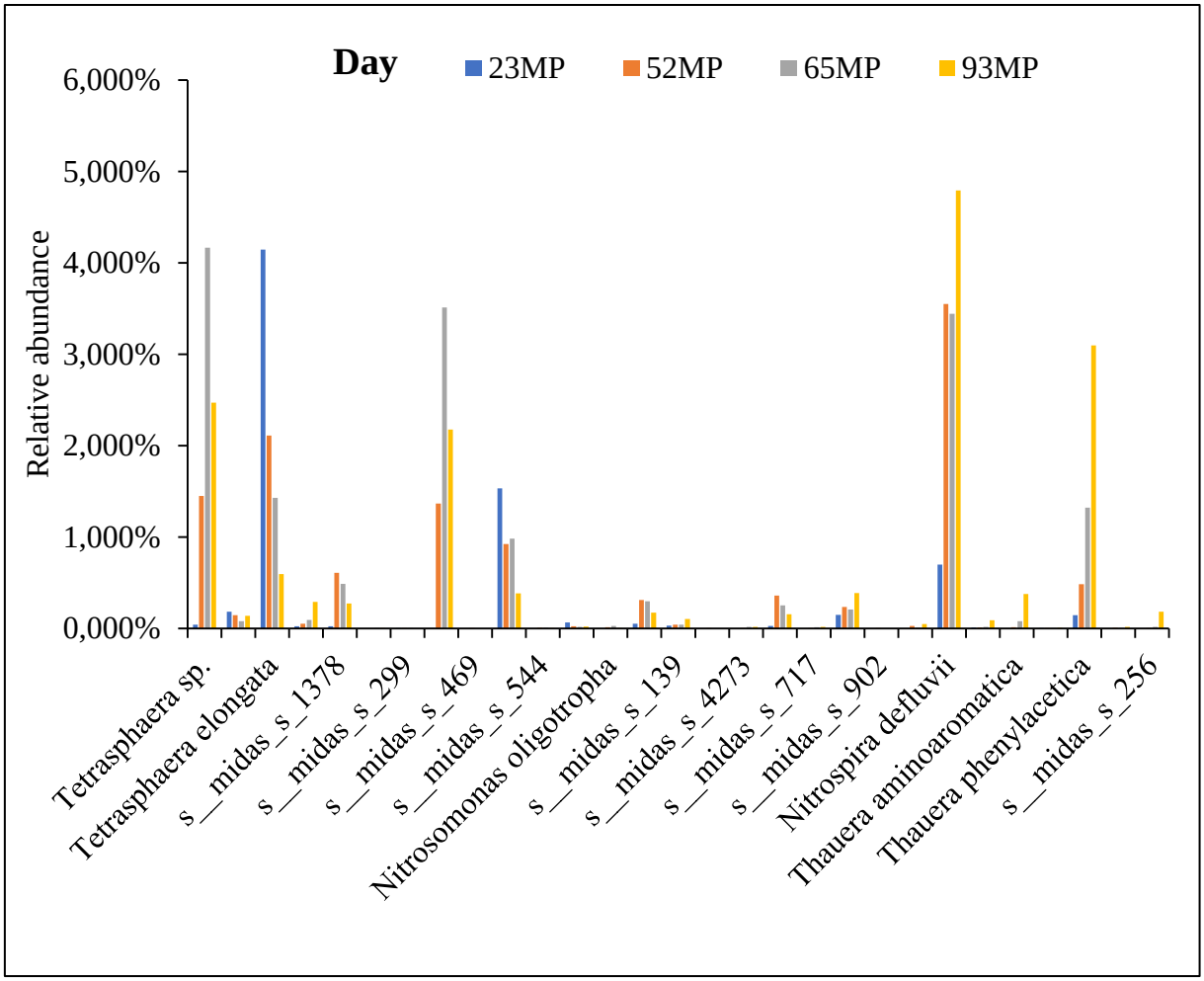

Fig.2Sb - Relative abundance of *Tetrasphaera*, *Nitrosomonas*, *Nitrospira* and *Thauera* species in SBR-MP during the studied period

At the genus level a proportion of 5.4-8.6% and 6.3-10.2% in sludges of SBR-Control and SBR-MP remained unclassified, respectively. *Tetrasphaera* (7.1%), *Nitrospira* (4.7%) and *Ferruginibacter* (2.7%) were the main genera in SBR-Control sludge samples at genus level on day 93 (Fig.3S.a). Other identified genera (>1%) at day 57 were *Rhodobacter* (1.8%), *Shinella* (1.7%), *Terrimonas* (1.4%), *Haliangium* (1.3%), *Dokdonella* (2.0%) and *Nitrosomonas* (1.1%). The proportions of genera (>1%) in SBR-MP sludge on day 93 were *Zooglea* (6.4%), *Tetrasphaera* (6.3%), *Nitrospira* (4.8%), *Ferruginibacter* (3.9%), *Thauera* (3.8%), *Thiothrix* (3.0%), *Candidatus*

Jidaibacter (2.5%), *Novosphingobium* (2.3%), *Haliangium* (2.1%), *Nocardioides* (1.7%), *Paracoccus* (1.3%), *Terrimonas* (1.3%) and *Gemmata* (1.2%) (Fig.3Sb).

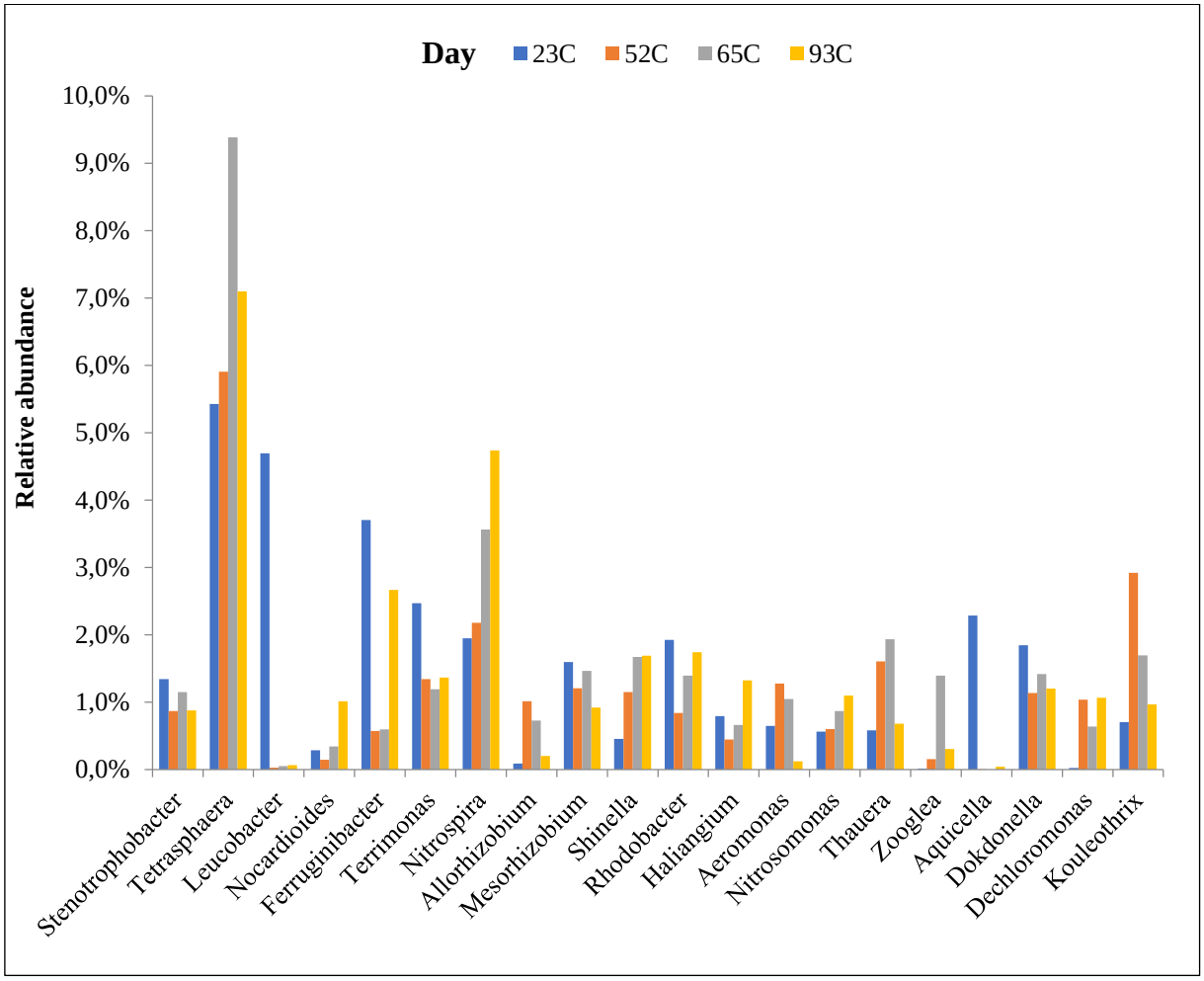

Fig.3Sa - Relative abundance of the most abundant genera (>1%) in at least one of sludge samples) in SBR-Control during the studied period

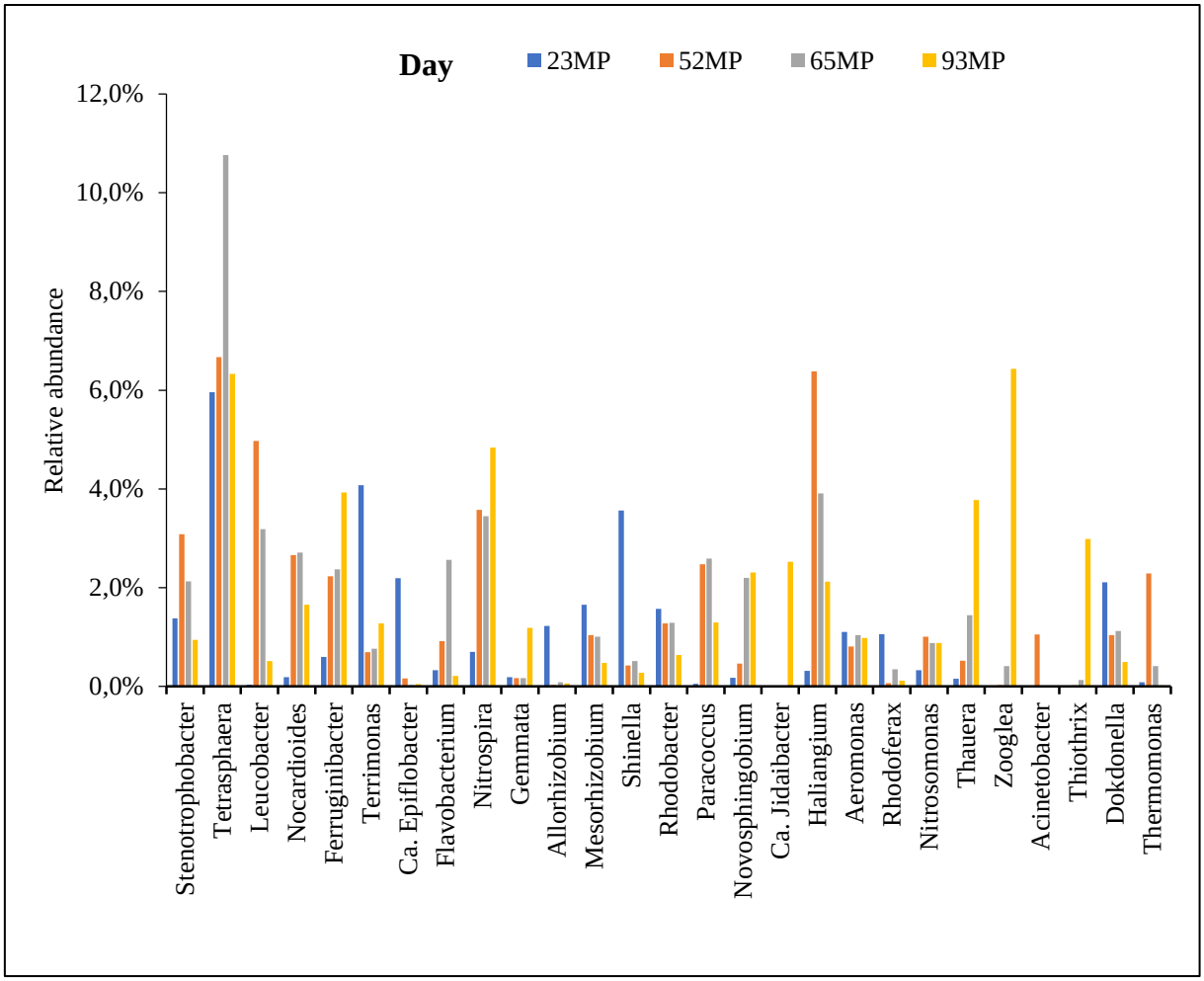

Fig.3Sb - Relative abundance of the most abundant genera (>1%) in at least one of sludge samples) in SBR-MP during the studied period