Effect of oxic/anoxic conditions on the removal of organic micropollutants in the activated sludge process

Camilla Di Marcantonio[a,*] camilla.dimarcantonio@uniroma1.it, Agostina Chiavola[a], Amrita Bains[b], Naresh Singhal[b]

[a]Sapienza University of Rome, Department of Civil, Constructional and Environmental Engineering (DICEA), Via Eudossiana 18, Zip code 00184 Rome, Italy

[b]The University of Auckland, Department of Civil and Environmental Engineering, Private Bag 92019, Auckland 1142, New Zealand

*Corresponding author.

Abstract

Among the emerging issues in the field of wastewater treatments, reducing energy consumption and removal of new organic pollutants have become of primary concern. With respect to the first goal, alternating oxic/anoxic conditions in the bioreactor has demonstrated to be a feasible way to ensure the required efficiency of carbon and nitrogen removal along with energy saving. The aim of the present study was to investigate if these alternating oxic/anoxic conditions are also capable of boosting organic micropollutants degradation, by stimulating the appropriate enzymes. Three different aeration frequencies were tested in a laboratory scale activated sludge reactor and the effects evaluated in terms of removal of carbon, nitrogen and a mixture of OMPs (Sulfamethoxazole, Sulfadiazine, Lincomycin, Carbamazepine, Pyrazole, Naproxen, Atrazine and Sucralose). It was also evaluated if these aeration strategies could change the microbial community composition with respect to the control test conducted under continuous air supply. Among the tested strategies, the longest and shortest durations of anoxic conditions promoted the best removal for the majority of OMPs. This enhancement was statistically well correlated to the activity increase of Lignin Peroxidase and Cellulase enzymes whereas the microbial speciation did not change statistically. The same durations were also capable of maintaining high carbon and nitrogen removal rates within the same biological reactor.

Keywords: Biodegradation; Contaminants of emerging concern; Dissolved oxygen concentration; Enzymes; Wastewater

1 Introduction

Organic Micropollutants (OMPs) include a wide number of chemicals belonging to different classes, e.g. pesticides, pharmaceuticals, personal care products, drugs of abuse and many others (Barbosa et al., 2016; Chiavola et al., 2019). In the last two decades, OMPs have been considered as a new source of risk for public health and environment and therefore received increasing attention by researchers and legislators. Indeed, some of them have been demonstrated to be toxic, slowly biodegradable and resistant to degradation and to conventional removal treatments (Tiwari et al., 2017). For others, the real effects on humans and environment are still not fully defined. A common characteristic to all the classes of OMPs is the low concentration at which they are found in the environment (ng/L or µg/L in water). However, due to the low biodegradability and persistence, some OMPs can bioaccumulate in the aquatic environment giving rise to higher concentrations. Through agriculture products and drinking water, they enter the food chain (Clarke and Smith, 2011). Furthermore, they are being released into the sewage network as a consequence of a wide number of human activities, e.g. health care, home cleaning, recreation (Gogoi et al., 2018; Petrie et al., 2014) and then transferred to the Wastewater Treatment Plants (WWTPs). Most of the treatments commonly adopted in the WWTPs for domestic sewage for carbon and nitrogen removal are unable to remove OMPs or can only slightly reduce their concentrations, because not specifically designed and operated to address this type of contamination.

Furthermore, due to the low hydrophobicity, some of them are transferred from the liquid phase onto the sludge flocs by adsorption (Besha et al., 2017; Chiavola et al., 2016). Consequently, untreated OMPs return to the environment

through the effluent and/or waste sludge (Sousa et al., 2017). Therefore, a compelling need is the enhancement of the WWTPs performance, with respect to OMPs removal, in order to reduce the environmental risk associated to effluent discharge into the environment (Trapido et al., 2014). Since the biological processes represent the core of most of the WWTPs for domestic sewage, several studies focused on these treatments. These processes are potentially suitable for the degradation of relatively biodegradable OMPs (Ahmed et al., 2017). However, their removal rates are often too low to achieve a significant reduction of OMPs concentrations. To enhance the efficacy of the biological processes, several approaches have been tested (Alvarino et al., 2018).

The dissolved oxygen concentration in the aerobic biological tank of the WWTPs has been usually controlled and managed on a routine-base, providing a constant air flow rate. However, control of energy consumption in WWTPs has become a primary obligation for plant operators, also in view of the need of reducing carbon emissions. To this purpose, aeration, which represents the main energy consuming item in the plant, has been recently subjected to different management strategies. The aim was to provide the biological reactors only with the air flow which is strictly required for the main purpose of the processes, i.e. carbon and nitrogen removal, based on the limits for the final effluent to be complied with. Among the strategies implemented with the aim to ensure the optimal aeration control, alternating oxic/anoxic conditions within the activated sludge process has resulted in important energy savings without negatively affecting the overall removal achieved by the plant (Chiavola et al., 2017; Han et al., 2018; He et al., 2018; Metcalf and Eddy, 2015).

The aim of the present study was to evaluate if this type of aeration management can also provide an improvement of the OMPs removal in the biological reactor of a WWTP for domestic sewage, along with the advantages already demonstrated such as high efficiency of carbon and nitrogen removal and energy savings.

Several studies showed that an increase of OMPs degradation rate can be achieved by using specific enzymes under aerobic and anaerobic conditions (Alneyadi et al., 2018; Gonzalez-Gil et al., 2019). Particularly, some enzymes, especially oxidoreductase and hydrolase, have the ability to ~~catalyse~~ catalyze the oxidation and hydrolysis, respectively, of recalcitrant compounds, such as pharmaceuticals (i.e. Naproxen, Carbamazepine and Sulfamethoxazole) (Naghdi et al., 2018; Tran et al., 2013). However, in most of the studies biocatalytic processes were based on the use of specific living cultures or extracted enzymes (Naghdi et al., 2018). Bains et al. (2019) investigated the impact of dynamic DO variation on enzymatic response and bacterial community composition in biological reactors, along with OMPs degradation. They tested three oxygenation patterns, i.e. constant aeration, continuous cyclic aeration, and intermittent cyclic aeration, for both high and low aerobic conditions; they observed that DO variation activate the enzymes involved in the regulation of carbon metabolism and they obtained a better OMPs removal by using intermittent cyclic DO condition.

In the present experimental activity, three different aeration strategies were applied in a laboratory scale activated sludge reactor and the effects evaluated in terms of removal of carbon, nitrogen and a mixture of OMPs. Furthermore, it was investigated if these strategies could also affect the activity of the enzymes referred by the literature to be involved in the OMPs degradation. Finally, it was evaluated if there was a change in the microbial speciation of the activated sludge with respect to the control conducted under continuous aeration. The OMPs mixture included compounds belonging to the following classes: pharmaceuticals (Sulfamethoxazole (SMX), Sulfadiazine (SLD), Lincomycin (LNC), Carbamazepine (CBZ), and Naproxen (NPX)), pesticides (Atrazine (ATZ)), industrial compound (Pyrazole (PYZ)) and artificial sweeteners (Sucralose (SCL)). These compounds were selected based on their wide presence in the influent of WWTPs and because they are not easy biodegradable (Stevens-Garmon et al., 2011; Tiwari et al., 2017; Trapido et al., 2014).

With respect to the previous papers on OMPs removal which investigated the performance of selected biological technologies (e.g. activated sludge, membrane biological reactor, fungal processes), (Ahmed et al., 2017; Falås et al., 2016; Karigar and Rao, 2011; Naghdi et al., 2018; Tiwari et al., 2017), the present study focused on the control of the main operating parameter, i.e. the dissolved oxygen (DO) concentration, in the attempt of boosting biodegradation of all the contaminants of concern. The main novelty of this paper is represented by this all-inclusive approach, which considered all the main aspects of the biological process which might be affected by the operating parameter (i.e. DO). For instance, along with the effects on carbon and nitrogen removal, also the change of OMPs degradation was investigated in view of the future limits which will be likely posed also on this type of contaminants for the effluent, as suggested by the new European regulation on minimum requirement for water reuse Regulation (EU) 2020/741 (The European Parlament and the Council of the European Union, 2020); furthermore, to better understand this change, also enzyme activities and microbial speciation were studied. This study provides important data for operators of the WWTPs which have to be aware of the effects of the existing management strategies also on the fate of OMPs.

2 Materials and methods

2.1 Chemicals

The certified standards of the eight selected OMPs were purchased from Sigma Aldrich and Merck at purities >99% and dissolved in LC-MS methanol to obtain the OMPs solution at 1000 mg/L of each one. The isotopically labelled (> 99% purity) Carbamazepine-d_{10} (CBZ-d_{10}), Naproxen-d_3 (NPX-d_3) and Atrazine–d_5 (ATZ-d_5) supplied by Sigma-Aldrich were used as internal standards (IS). High purity analytical LC-MS grade solvents (Methanol, Acetonitrile and tert-Butyl methyl ether) were purchased from Merck. Sigma Aldrich also provided oxidoreductases, used as standards, such as lignin peroxidase (LiP), horseradish peroxidase (HRP), laccase (Lacc) derived from cultures of *Trametes versicolor*, cytochrome P450 (Cyt P450) from human 3A4 isozyme microsomes and beta-glucosidase (β-glu) from *Aspergillus niger* as well as their respective enzyme substrates (Methylene Blue (MB), Azure B (AB), 3,4-Dihydroxy-L-phenylalanine (L-DOPA), 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Sudan Orange G (SO), 4-nitrophenyl-dodecanoate (PNP-D), Indole (INDOLE) and 4-Aminoantipyrine (4-AAP), 4-nitrophenyl N-acetyl-β-D-glucosaminide (PNP-A) and 4-nitrophenyl-β-D-glucopyranoside (PNP-G)). Sodium acetate trihydrate, glacial acetic acid ($\geq$ 99% purity), di-potassium hydrogen phosphate, potassium di-hydrogen phosphate, dextrose and magnesium chloride hexahydrate of $\geq$ 99% purity (by Sigma Aldrich) were used to prepare enzyme buffers at pH = 5 and pH = 7, respectively.

A synthetic wastewater (SyWW) was used as a feed to the batch tests. For its preparation, the procedure proposed by Bassin et al. (2011) was followed, properly modified in order to have a C:N:P ratio equal to 100:5:1. Sodium acetate trihydrate was the main source of soluble organic carbon (81% of the total amount); besides, also the OMPs solution, being made in methanol, provided easy biodegradable organic carbon (19% of the total concentration of COD provided to the reactors). The SyWW was obtained by dissolving in MilliQ water the following ingredients: 1 mg/L of each OMP; 15.84 g/L $NaCH_3COO \cdot 3H_2O$; 0.89 g/L $MgSO_4 \cdot 7H_2O$; 0.4 g/L KCl; 1.53 g/L NH_4Cl; 0.30 g/L K_2HPO_4; 0.12 g/L KH_2PO_4; 10 mL/L trace elements. The trace elements solution contained the following components: 50 g/L EDTA; 22 g/L $ZnSO_4 \cdot 7H_2O$; 5.54 g/L $CaCl_2$; 5.06 g/L $MnCl_2 \cdot 4H_2O$; 4.99 g/L $FeSO_4 \cdot 7H_2O$; 1.1 g/L $(NH_4)6Mo_7O_{24} \cdot 4H_2O$; 1.57 g/L $CuSO_4 \cdot 5H_2O$; 1.61 g/L $CoCl_2 \cdot 6H_2O$ (Bassin et al., 2011). All the solutions were stored at 4 °C until their use.

2.2 Experimental tests

The activated sludge samples used as seed for the experimental activity were collected from the sludge recycle loop of the secondary settlement tank of the Mangere municipal WWTP in Auckland, New Zealand. Before the use, the sludge was always rinsed several times using tap water until the soluble compounds remaining from the treatment reached concentrations below detectable limits. Then, the samples were stored at the temperature T = −20 °C until the beginning of the tests.

The tests were performed in 1 L working volume bioreactors. The activated sludge concentration inside was maintained at 3000 mg/L MLVSS in all the tests, by adding 50 mL of 0.2 M PBS solution (Phosphate buffered saline, made according to Stoll and Blanchard (1990)) and tap water to the initial samples. Prior to the experimental study, biomass was acclimatized by maintaining it under aerobic conditions for at least 24 h at room temperature, fed with the same synthetic wastewater (SyWW) as that used in the experimental tests without OMPs. The acclimatization period was considered completed when steady state conditions of nitrogen and carbon removal was achieved. The average characterization of the mixer liquor at the end of this period was as follows: VSS 3 mg/L, nitrites 3 mg/L NO_2^-–N, nitrates 19 mg/L NO_3^-–N, acetate and ammonium below the limits of detection of the analytical methods (LOD = 0.1 mg/L). After the acclimatization phase, the experimental study could be started. Firstly, a proper volume of OMPs solution was added with the aim to obtain an initial concentration of 0.1 mg/L of each compound inside the bioreactors. Then, the synthetic wastewater (containing 1 mg/L of each OMPs and nutrients as above described) started to be supplied in a continuous feeding mode (at a flow rate equal to 0.0347 mL/min); this feeding continued for all the duration of the tests (i.e. 48 h). A summary of the experimental conditions and procedures applied to the tests is reported in Table S1 of Supplementary materials.

The pH value inside the reactors was maintained within the optimal range reported for nitrification, i.e. 7.2–8, through the addition of the PBS buffer solution at the beginning of the tests (Metcalf and Eddy, 2015).

Samples were collected during the tests at the following times: 0, 5, 24 and 48 h. This sampling schedule was designed in order to obtain information, during all the conditions of reactor operation, about: OMPs, nitrogen and carbon removal and enzymes activity. The target enzymes were selected according to previous studies (Alneyadi et al., 2018; Karigar and Rao, 2011). Nitrification and carbon removal were monitored by measuring the following parameters: acetate (the main source of carbon to the system), ammonia, nitrites and nitrates concentration. The samples for the microbial speciation were collected at the end of each test (t = 48 h). During the experiments, the aeration conditions inside the bioreactors were modified through the use of a logic controller (Millenium 3 CD 20) connected to solenoids valves, setting the times when aeration was active (ON phase) or absent (OFF phase); furthermore, the dissolved oxygen (DO) concentration was maintained in the range 0–7 mg/L by controlling the air flow rate (supplied by a pump). The monitoring and recording of DO concentration inside the reactors were performed, for the entire duration

of the tests, through a system composed by a Hamilton Device Manager 1.0.0 software equipped with a wireless sensor which was connected to VisiFerm DO sensors.

Cyclic changes of the ON and OFF phase were applied during the 48 h of the tests. In each cycle, the ON phase was always maintained equal to 11 min, whereas the OFF phase was changed in order to obtain three different aeration strategies. The duration of the ON phase was experimentally determined as the minimum time required to increase the DO concentration from about 0 mg/L to about 7 mg/L. The duration of the ON-phase was kept as shortest as possible to avoid the establishment of constant aerobic conditions. The time required to reach the desired DO level is a function of the substrate availability to bacteria; therefore, since the SyWW composition was maintained unchanged in all the reactors, the same duration of the ON phase could be applied.

Correspondingly, three values of the aeration frequencies, defined as the inverse of the total duration of an ~~ON-OFF~~ ON–OFF cycle per hour according to Bains et al. (2019), were obtained:

$$f = \frac{1}{ON + OFF} \tag{1}$$

A control test (C) was also run where oxygen concentration was constantly maintained at DO ≈ 7 mg/L O_2. Values of DO concentration recorded inside the reactors during the tests are reported in Supplementary materials.

The temporal characteristics of the aeration strategies are reported in Table 1.

Removal efficiency at different times was calculated based on the following equation:

$$R\% (t) = \frac{C_T (t) - C (t)}{C_T (t)} \cdot 100 \tag{2}$$

where C_T(t) and C(t) stand for the OMPs theoretical concentration and that measured at time t inside the reactor, respectively. Particularly, the theoretical concentration was the value expected at each contact time due to the continuous feeding mode in the reactor if only dilution of the feed within the liquid volume of the reactor was present and any other reaction was absent.

Table 1

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Temporal characteristics of the different aeration strategies.

Test	Per cycle		Total on 48 h		Frequency
	ON	OFF	ON	OFF	
	min	min	min	min	1/h ≡ 1/min
C	60	0	2880	0	/
E1	11	83	337	2543	0.6
E2	11	53	495	2385	0.9
E3	11	23	932	1948	1.8

All the tests were performed in duplicate and the results obtained averaged.

Statistical analysis of the removals measured under the different aeration strategies, for each contaminant, was carried out by using the one-way ANOVA test with the R package "stats", followed by the Tukey's honest significance test with the R package "agricolae" (R Core Team, 2019). The same statistical tools were applied to the enzymes' activity data. The microbial community composition measured at the end of each test (i.e. E1, E2, E3) was compared to the control (C) through paired Student's t-test to assess the statistical differences, the tool used is once again the aforementioned R package "stats".

2.3 Analytical methods

Analytical determination of nitrates, nitrites and acetate was performed by using the Thermo Scientific Dionex ICS-2100 Ion Chromatography System, following APHA methods 4110 B. NH_3–N was measured by the Thermo Scientific Orion 4 ammonia ion selective electrode, following the APHA methods 4500-NH3 D (Eaton et al., 2005).

Detection of OMPs concentrations

OMPs concentrations in the liquid phase were measured by a first solid phase extraction (SPE) on OASIS HLB cartridges (Waters, Milford, MA, USA) following the method described by Vanderford et al. (2003). Particularly, 20 mL of sludge sample was collected from the reactor and centrifuged to separate suspended particles (20 400 × g for 20 min at T = −4 °C) and stored for less than one week at T = −20 °C. The supernatant was spiked with 100 µg/L of internal standard, IS_1 (ATZ-d_5), and filtered through the cartridge (pre-conditioned with 5 mL of tert-methyl butyl ether, 5 mL of methanol and 5 mL of ~~deionised~~ deionized water). Then, the cartridge was rinsed with water and air-dried for 30 min. The OMPs were eluted in 5 mL 90/10 MTBE/MeOH (v/v) and 5 mL MeOH under a vacuum system. Total evaporation of the extracts was performed at T = 40 °C and 1200 rpm rotation speed using a rotary evaporator RVC 2–25 CO plus Christ. The dried phase was dissolved in 1 mL of MeOH and then filtered with a 0.2 µm membrane filter of regenerated cellulose. Just before the injection, other two internal standards, IS_2 (CBZ-d_{10} and NPX-d_3 at 100 µg/L), were added and used for the concentration quantification.

Quantitative analysis of OMPs was carried out by liquid chromatography coupled with mass spectrometry (LC-MS) using a Shimadzu 8040 Series LC-MS (Shimadzu, Japan) with an Agilent ZORBAX Eclipse Plus C18 column (2.1 mm × 100 mm, particle size 1.8 µm, Agilent Technologies, Germany). Two specific analytical methods were developed, one in negative mode for Naproxen and one in positive mode for all the other OMPs, based on EPA Method 1694 (Imma Ferrer, 2008). A binary gradient system of mobile phase A, 0.1% formic acid in ~~deionised~~ deionized water, and mobile phase B, 0.1% formic acid in acetonitrile, were used to separate analytes in positive ESI mode, while 5 mM ammonium acetate, pH-5.5 (mobile phase A) and methanol (mobile phase B), were used for analysis in negative ESI mode. The solvent gradient programme for positive ESI mode was as follows: 5% B held for 4 min, increased linearly to 50% by 5 min and then to 90% in 6 min and then dropped to 5% for 2 min. A 3 min equilibration step at 5% B was used at the end of each run to bring the total run time per sample to 17 min. For negative ESI mode, the gradient started with 30% B, and was increased linearly to 100% B over 8 min and held for 3 min, then maintained at 40% B for 3 min. The flow rate was 0.3 mL/min in the former and 0.2 mL/min in the latter mode and the injection volume was set to 3 µL and 10 µL for ± polarity modes, respectively. Limits of detection and limit of quantification (LOD and LOQ, respectively) were determined using signal/noise ratios of 3 and 10, respectively. The quality assurance and quality control were checked within each measurement series by recovery experiments both in deionized water (Recovery in Water) and SyWW (Recovery SyWW) spiking at 100 µg/L OMPs solution (n ≥ 3) and with 5 repeated injections of matrix recovery samples (Repeatability). The analytical method was also validated in terms of linearity (R_2 of the calibration curves). The validation results are summarized in Supplementary materials.

Enzyme activity assays

The activity of oxidoreductases in culture biomass samples was determined spectrophotometrically by measuring the degradation (oxidation and hydrolysis) of various chromogenic substrates used as surrogate xenobiotics (details in Supplementary materials). Specifically, 2 mL aliquots of microbial culture samples were centrifuged at 16 000 × g for 3 min in Eppendorf tubes. Ultrasonication (physical disruption) was applied as a standard method for the disruption of microbial cells from activated sludge. The pellets from each tube were individually homogenized by sonication in 600 µL of EDTA buffer (1 mM EDTA, 0.1% Surfact-Amps, 50 mM ammonium bicarbonate, pH 8) at 12 Hz for 30 s thrice. Sonication was performed with a sonication microtip (Qsonica Q-125, Alphatech Systems, New Zealand) to the ice-cold ~~homogenised~~ homogenized samples. Cell debris was removed by centrifugation at 16 000 × g for 10 min and the supernatant was used for the analysis of enzyme activity. Each well of a 96 well microplate was filled with 50 µL aliquots of buffers (50 mM acetate buffer (50 mM sodium acetate trihydrate adjusted to pH-5 with glacial acetic acid), 100 mM phosphate buffer (80 mM di-potassium hydrogen phosphate, 20 mM potassium dihydrogen phosphate, 10 mM dextrose, 6 mM magnesium acetate adjusted to pH-7.4), 50 µL chromogenic dye and 50 µL culture supernatant. Dyes and samples resuspended in double the amount of buffer served as controls. To start reactions of the sample enzymes with assay dyes, 10 µL of 0.3% H_2O_2 at 30% was added to the Methylene Blue, Azure B, L-DOPA and ABTS dye wells and 10 µL of 1M NaOH added to the para-nitrophenol dye wells (to stop the reaction) and vortex mixed. Changes in absorbance caused by chromogenic reactions were read on a Victor X3 Multimode Plate Reader (PerkinElmer, USA) at different wavelengths for 1 h with incubation at 30 °C.

Microbial DNA isolation and bacterial species identification

A PowerSoil DNA isolation kit (MoBio, Carlsbad, USA) was used for the isolation of bacterial total genomic DNA extracted from sludge samples (1 mL) following the manufacturer's protocol. All the extractions were performed in duplicate. Bacterial community composition was ~~characterised~~ characterized by amplifying and sequencing a fragment of the bacterial 16S ribosomal RNA (rRNA) gene following ~~a~~Illumina standard protocol ~~(Illumina 2013)~~. The V3 and

V4 regions of 16S rRNA genes were amplified from individual DNA extracts with the universal 16S Amplicon PCR Forward Primer (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 16S Amplicon PCR Reverse Primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). These primers have been validated to provide good bacterial phylum coverage as they are also modified to include Illumina adapter overhang sequences (in bold) required for downstream DNA sequencing. DNA amplification was conducted as follows: (i) 94 °C for 3 min; (ii) 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; (iii) 72 °C for 5 min (Klindworth et al., 2013). Following amplification, PCR products were purified using the AMPure XP beads kit (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions. The concentrations of purified amplicons were finally measured and recorded using a Qubit® dsDNA HS Assay Kit (Life technologies, Carlsbad, CA, USA) and submitted to New Zealand Genomics Ltd for sequencing by Illumina MiSeq machine. The resulting paired-end read DNA sequence data were merged and quality filtered using the USEARCH sequence analysis tool (Edgar, 2013). Data were dereplicated so that only one copy of each sequence was reported, and 'singleton' sequences represented by only one DNA sequence in the database were removed. Sequence data were then checked for chimeric sequences and clustered into groups of operational taxonomic units based on a sequence identity threshold equal to or greater than 97% (thereafter referred to as 97% OTUs) using the clustering pipeline UPARSE in QIIME v.1.6.0 as described in Ramirez et al. (2014). After that, prokaryote phylotypes were classified to their corresponding taxonomy by implementing the RDP classifier routine in QIIME v. 1.6.0 to interrogate the Greengenes 13 · 8 database. All sequences of chloroplast and mitochondrial DNA were removed. Finally, DNA sequence data were rarefied to a depth of 5600 randomly selected reads per sample and two samples per treatment to achieve a standard sequencing reads across all samples.

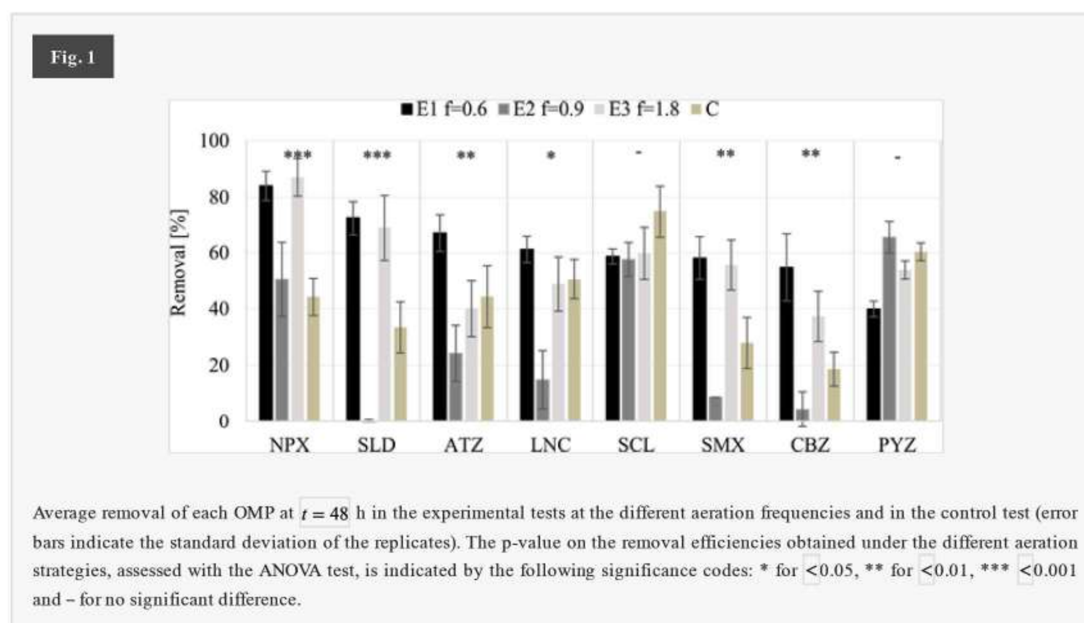
3 Results and discussion

3.1 OMPs, carbon and nitrogen removal

Fig. 1 depicts average removal efficiency, (R%(t), of each OMPs at $t = 48$ h for the different aeration frequencies. Concentrations of OMPs measured at each sampling time during the tests are reported in Supplementary materials.

It can be noted that changing duration of the OFF phase had a different effect on the removal of OMPs, depending on the specific value of the aeration frequency and the type of compound. The results obtained through the statistical analysis (ANOVA test) proved that the removals achieved at the end of the four tests are significantly different (p-value $<$ 0.05) except for SCL and PYZ. Thus, changing the aeration condition did not give effect only on the removal of these two compounds (SCL and PYZ).

Fig. 1



Average removal of each OMP at $t = 48$ h in the experimental tests at the different aeration frequencies and in the control test (error bars indicate the standard deviation of the replicates). The p-value on the removal efficiencies obtained under the different aeration strategies, assessed with the ANOVA test, is indicated by the following significance codes: * for $<$0.05, ** for $<$0.01, *** $<$0.001 and – for no significant difference.

For instance, the E1 frequency (i.e. f $=$ 0.6 1/h) determined an improvement of the removal for most of the contaminants (i.e. SLD, LNC, SMX, CBZ and ATZ). Particularly, this increase accounted of 39%, 11%, 30%, 36%, 23%, for SLD, LNC, SMX, CBZ and ATZ, respectively, as compared to the control test (C) where aeration was maintained always active.

In the case of SLD, SMX and CBZ, an increase was also measured by applying the E3 frequency (i.e. f $=$ 1.8 1/h), which accounted for 36%, 28%, 19% respectively. The same frequency improved by 43% removal of NPX. For CBZ and NPX, this results can partially be explained by the presence of electron donating groups such as an amine ($-NH_2$) and hydroxyl (-OH) groups, which are present in their molecular structure, respectively, which facilitate the electrophilic attack by oxygenase produced in the aerobic treatment (Tadkaew et al., 2011).

Considering the average removal of the OMPs measured in all the tests, it can be deemed that the E1 aeration strategy provided the best effect; furthermore, a reduced variability of the data was observed as compared to the other tests.

To improve the statistical analysis a post-hoc test was performed to better highlight the difference between the individual treatments. The Tukey's honest significance test was applied to this purpose (see Table 2); the difference was considered significant when the p-value was below 0.05, as indicated in the table by the codes "***", "**", "*". The statistical analysis proved that the differences between the control test and E1 are statistically relevant only for NPX, SLD and CBZ. Similar conclusion can be drawn by comparing the control and E3 tests, except for CBZ. The Tukey test also confirmed that E2 did not cause any appreciable variation on the OMPs removal with respect to the control frequency; indeed, it caused negative effects on the treatment performance for most of the compounds as compared to all the other aeration frequencies. Therefore, it is confirmed the capability of E1 and E3 aeration frequencies to improve the removal of some of the investigated OMPs.

Table 2

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Results of the multiple comparisons of the OMPs removals at the end of each treatment obtained by the ANOVA test and then the Tukey's honest significance test the.

Tests	ATZ	CBZ	LNC	NPX	PYZ	SCL	SLD	SMX
C – E1	·	*		**			*	·
C – E2	·		·				**	
C – E3				**			*	·
E1 – E2	**	**	*	**	·		***	*
E1 – E3	*							
E2 – E3		*	·	**			**	*

Table Footnotes

· The significance codes, related to the p-value, have the following meanings: ~0.05.

* The significance codes, related to the p-value, have the following meanings: <0.05.

** The significance codes, related to the p-value, have the following meanings: <0.01.

*** The significance codes, related to the p-value, have the following meanings: <0.001.

Since duration of the ON aeration phase was maintained unchanged in all the tests, the observed changes in the removal efficiency were likely due to the longer OFF phase. This might have favoured a different metabolic pathway of biodegradation under micro-aerobic or anoxic bacteria (being nitrate present in the mixed liquor). Indeed, other scientific studies observed biotransformation of some OMPs, such as SMX and Trimethoprim, under anaerobic conditions (Gonzalez-Gil et al., 2019). Bains et al. (2019) also found that perturbed DO conditions increased OMPs removal relative to constant non-perturbed conditions. OMPs biotransformation can potentially be linked to the presence of active oxidoreductases synthesized under these perturbation conditions (Rao et al., 2014).

Acetate concentrations measured during the same tests in the bioreactors were always found below the limits of detection of the analytical method (LOD = 0.1 mg/L). This indicates that the rate of carbon supply (acetate was the only external carbon source provided to the biological processes) through the continuous feeding mode likely corresponded to the microbial oxidation rate; consequently, no accumulation of acetate occurred within the bioreactors. This finding was the same as observed in the control test (not shown here).

The ammonia concentration in the synthetic wastewater solution was periodically measured confirming the expected concentration (400 mg/L NH_4^+–N). This solution was then fed in the continuous mode to the bioreactors to provide ammonia, along with the other components needed for the biological process. Since ammonia in the bioreactors during the tests was measured to be always below the limits of detection of the analytical method (LOD = 0.1 mg/L, data not shown here), it was concluded that it was completely oxidized ~~trough~~ through nitrification and used for bacterial cell synthesis. The rate of supply was equal to the rate of consumption through these two pathways. Since the pH value was maintained below 8, ammonia stripping was excluded as a source of loss from the system.

Nitrate formation is an intermediate step in the nitrification process; nitrites are then oxidized into nitrates if proper oxygen is available. Denitrification consists in the reduction of nitrates into nitrites and other nitrogen compounds until formation of nitrogen gas (Metcalf and Eddy, 2015).

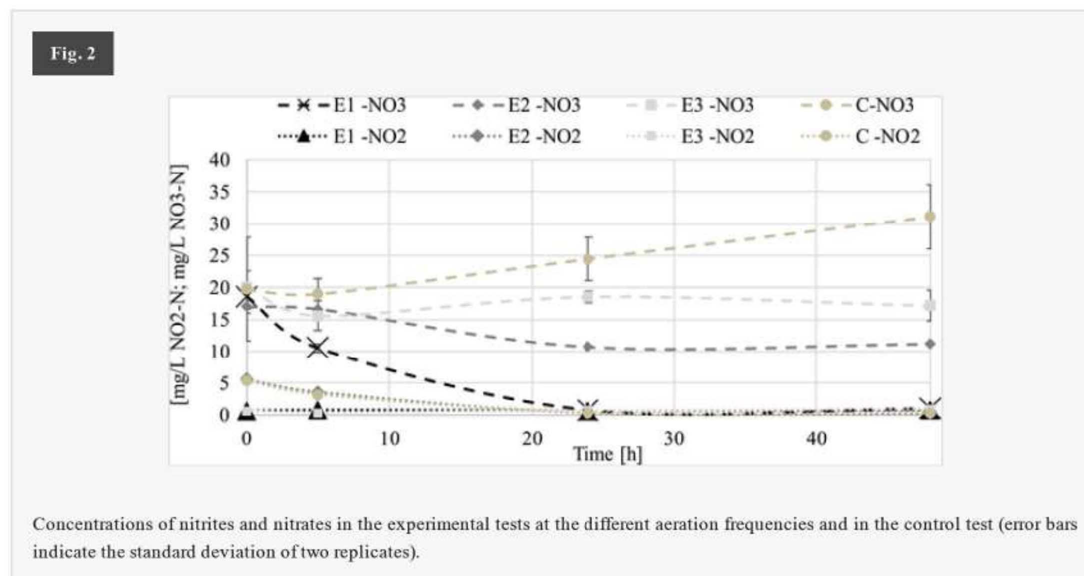
Concentration of nitrites and nitrates in the bioreactors showed different patterns depending on oxygen conditions, as reported in Fig. 2. The high concentration of nitrates measured at the beginning of all the tests was a consequence of the steady state nitrification process established during the acclimatization period conducted prior to the start of the experimental phase. Specifically, in the control test nitrates increased constantly, whereas nitrites decreased: therefore, it was assumed that complete nitrification was occurring based on the above considerations. In E1 experimental test, nitrates decreased continuously, whereas nitrites were always below LOD: these patterns were considered indicative of nitrification and denitrification occurring simultaneously. In E2 experimental test, nitrates decreased in the first 24 h and then remained pretty constant, whereas nitrites decreased during the same time interval until values below LOD: these patterns was considered indication of the incomplete denitrification due to the insufficient duration of the anoxic conditions which determined nitrification to prevail. Finally, in E3 experimental test, nitrates increased although at a slower rate than in the control whereas nitrites always remained below LOD: this aeration frequency implied the shortest duration of the anoxic phase and therefore denitrification could not occur at high extent and nitrates accumulated in the bioreactors.

Table 23 shows the values of the Specific Nitrogen Removal Rate (SNRR, g N/g MLVSS·d) which represents an adaptation of the Specific Denitrification Rate to the overall nitrogen removal processes with the aim to consider the combined effect of nitrification and denitrification under the different aeration conditions (Metcalf and Eddy, 2015; Raboni et al., 2014). The SNRR was calculated at $t = 24$ h, as the time at which the maximum reaction rate occurred, and at $t = 48$ h to obtain the average value along the test duration. The following equation was applied to determine the SNRR values:

$$\mathrm{SNRR}\,(t) = \frac{\Delta\left([\mathrm{NH}_4^+ - \mathrm{N}] + [\mathrm{NO}_2^- - \mathrm{N}] + [\mathrm{NO}_3^- - \mathrm{N}]\right)}{\Delta t \cdot X} \tag{3}$$

where $\Delta([NH_4^+-N] + [NO_2^- -N] + [NO_3^- -N])$ is the difference of the total nitrogen concentrations (mg/L N) measured inside the reactor at time $t = 0$ h and t (either 24 h or 48 h as above detailed), respectively; Δt is the time interval (d); X is the average MLVSS concentration inside the reactor (3000 mg/L MLVSS). The results obtained are reported in Table 23.

Fig. 2



Concentrations of nitrites and nitrates in the experimental tests at the different aeration frequencies and in the control test (error bars indicate the standard deviation of two replicates).

A decreasing trend of the SNRR values can be observed at increasing frequencies. The lowest value (below zero) of SNRR was calculated in the control test (C), indicating an accumulation of nitrate-nitrogen in the system due to the continuous oxidation of ammonium (provided through the feeding). In the test conducted at the highest frequency (f = 1.8 1/h), the SNRR was still very low and remained pretty constant between $t = 24$ h and $t = 48$ h, showing that the nitrification and denitrification rates were similar. This confirms the constant profile of nitrates concentration observed during the test and above highlighted. The SNRR value increased as the frequency decreased, thus validating the hypothesis that simultaneous ~~nitrification-denitrification~~ nitrification–denitrification was established within the bioreactors.

Based on these results, it can be concluded that, among the different aeration conditions tested in the study, the E1 allowed to achieve the better improvement of the OMPs degradation and also high removal of acetate and nitrogen removal~~ (see Table 3)~~.

Table 3

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Specific nitrogen removal rate (SNRR) at t = 24 h and t = 48 h in the experimental tests at the different aeration frequencies and in the control test.

Test	Nitrite [g/L NO_2^-–N]		Nitrate [g/L NO_3^-–N]		SNRR [g N/g MLVSS d]	
	t = 24 h	t = 48 h	t = 24 h	t = 48 h	t = 24 h	t =48 h
E1 (f = 0.6)	0.6283	0.7381	0.7667	0.9988	0.0058	0.0029
E2 (f = 0.9)	3.6006	0.2995	16.6633	10.7596	0.0038	0.0019
E3 (f = 1.8)	0.6459	0.8779	18.5611	17.1790	0.0004	0.0004
C	0.2903	0.3349	24.4714	31.0904	−0.0001	−0.0011

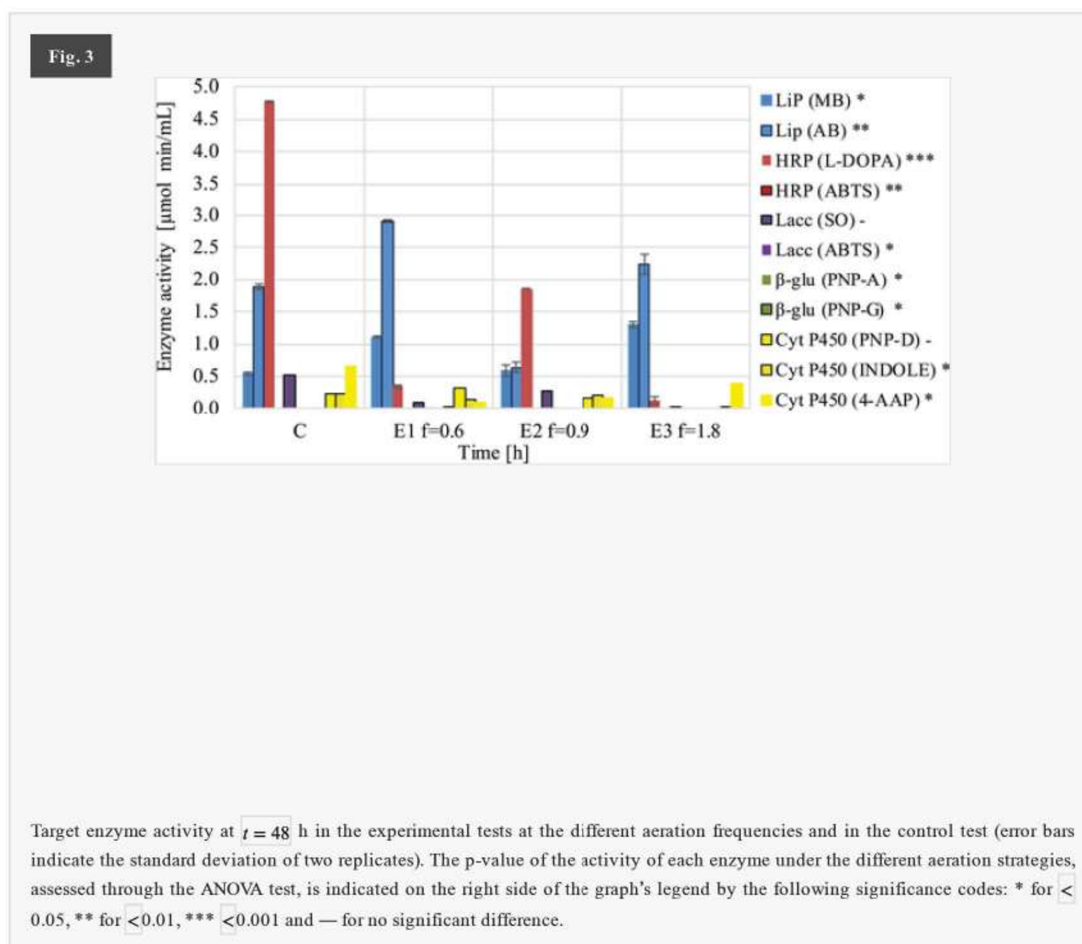
3.2 Activity of target enzymes

All the biochemical reactions are catalysed catalyzed by enzymes, as well as those occurring in the biological processes of WWTPs. Despite the high relevance of the role played by enzymes, however there is still a great lack of knowledge about the specific enzymes involved in the degradation of OMPs (Gonzalez-Gil et al., 2019). Therefore, in the present study, the activity of five target enzymes was also investigated during the tests. Particularly, LiP showed the capability to degrade several recalcitrant aromatic pollutants. Lac is of great interest because of its low specificity, ability to use atmospheric oxygen as the electron acceptor and good yields. Cytochrome P450 (Cy tP450) was found to play a significant role in the degradation of some pollutants (Naghdi et al., 2018).

Fig. 3 shows the values obtained at the end of the experiments, i.e. at $t = 48$ h, whereas the data determined at each sampling time are reported in Supplementary Materials.

Data of Fig. 3 show that aeration frequency affected in a different way the activity of the investigated enzymes. Specifically, LiP was favourably stimulated by both E1 and E3 frequencies, with an increase for both dyes as compared to the control tests. By contrast, HRP and Lacc were negatively affected, whereas negligible differences were observed in the case of Cyt P450. Similarly to the finding by Bains et al. (2019), LiP (AB, MB) was active in all the aeration conditions.

Fig. 3



Target enzyme activity at $t = 48$ h in the experimental tests at the different aeration frequencies and in the control test (error bars indicate the standard deviation of two replicates). The p-value of the activity of each enzyme under the different aeration strategies, assessed through the ANOVA test, is indicated on the right side of the graph's legend by the following significance codes: * for < 0.05, ** for <0.01, *** <0.001 and — for no significant difference.

The statistical analysis of the enzymes activity data was performed by applying the same method as used for the OMPs removal efficiency (i.e. by the ANOVA and then the Tukey's honest significance tests). The results obtained are shown in Fig. 3 and Table 4. The ANOVA proved that the activity of each enzyme was modified at the end of the four tests (p-value < 0.05): HRP and β-glu were the mostly affected whereas Lacc (SO) and Cyt P450 (PNP-D) were very little influenced (as showed in Fig. 3).

Table 4

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Results of the multiple comparisons of the enzymes activity at the end of each treatment obtained by the ANOVA test and then the Tukey's honest significance tests.

Tests	LiP		HRP		Lacc		β-glu		Cyt P450		
	(MB)	(AB)	(L-DOPA)	(ABTS)	(SO)	(ABTS)	(PNP-A)	(PNP-G)	(PNP-D)	(INDOLE)	(4-AAP)
C – E1	·	·	***	*		·	**	*			*
C – E2		*	***								*
C – E3	*		***		·					*	
E1 – E2		**	**	*		·	**	*			
E1 – E3				**		*	**	*	·		
E2 – E3	*	*	**							*	

Table Footnotes

· The significance codes, related to the p-value, have the following meanings: for ~ 0.05.

* The significance codes, related to the p-value, have the following meanings: < 0.05.

** The significance codes, related to the p-value, have the following meanings: < 0.01.

*** The significance codes, related to the p-value, have the following meanings: < 0.001.

Particularly, the Tukey test (see Table 4) confirmed that the HRP's activity was significantly affected by all the E1, E2 and E3 aeration strategies with respect to the control; the main effect was observed in the E1 test. The β-glu's activity was also mainly modified in the E1 test. By contrast, the statistical analysis did not confirm a significant difference for LiP and Lacc activities as compared to the control frequency.

The enzyme activity was then correlated to the OMPs removal measured at the end of all the tests. To this purpose the matrix of correlation was built, as shown in Fig. 4. Correlation was quantified based on the value of the Pearson's coefficient (ρ), calculated by the software R using the following equation (Mu et al., 2018):

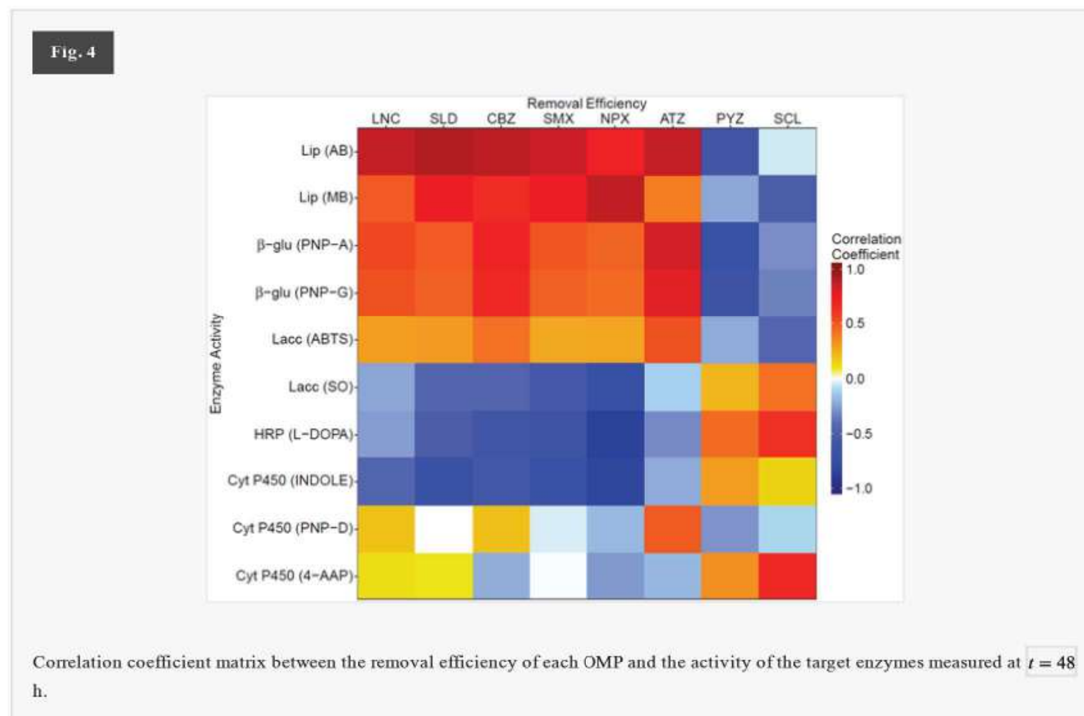
$$\rho = \frac{\sigma_{xy}}{\sigma_x \cdot \sigma_y} \tag{4}$$

where σ_{xy} is the covariance, σ_x and σ_y are the standard deviations of the two variables, i.e. the removal efficiency of each OMP (x) and the activity of the target enzymes (y) measured at the end of the test, respectively. HRP (ABTS) was excluded from the statistical evaluation because no activity was detected in all the tests.

Fig. 4 highlights a positive correlation for the activity of LiP, β-glu and Lacc (only with ABTS dye) and LNC, SLD, CBZ, SMX, NPX and ATZ removals. Particularly, a very strong correlation ($\rho \approx 1$) was observed with the LiP activity, thus highlighting that this enzyme plays a sensitive role in the degradation processes. The results are in accordance with Naghdi et al. (2018), who reported the Lip capability to degrade several recalcitrant aromatic pollutants (such as some pharmaceuticals like Diclofenac, Tetracycline, Oxytetracycline, endocrine-disrupting compounds and dyes). In the same paper, the mechanisms through which LiP attacks the recalcitrant compounds is well described. LiP has a high redox potential and therefore it can oxidize the compounds that are not oxidized by other enzymes. LiP follows a well-known peroxidase catalytic mechanism in which native enzyme is oxidized by hydrogen peroxide and forms LiP-I with two electron deficiencies. LiP-I oxidizes the target compound and reduces to one electron deficient LiP-II. When LiP-II oxidizes another target molecule, it returns to the native form of LiP (Naghdi et al., 2018). Degradation of NPX was observed due to Lac and the Cyt P450 using a liquid medium with white rot-fungi *Trametes versicolor* (TV) pellet (Marco-Urrea et al., 2010). However, Rodríguez-Rodríguez et al. (2010) studied the

removal of NPX in sewage sludge samples with TV and observed that NPX removal did not necessarily correlate with Cyt P450 or Lac.

Fig. 4



Correlation coefficient matrix between the removal efficiency of each OMP and the activity of the target enzymes measured at $t = 48$ h.

A study by Jelic et al. (2012) also showed an efficient removal of carbamazepine due to the presence of laccase, peroxidase and Cyt P450 enzymes.

β-glu showed a slightly less correlation coefficient value ($\rho \approx 0.5$) as compared to LiP for the same OMPs, also due to the really low values of its activity. Since β-glu is the only enzyme belonging to the class of hydrolase, therefore, the hydrolysis reaction plays also a role in the OMPs removal. Previous studies demonstrated that hydrolysis is a key step in the degradation of persistent micro-pollutants also under anaerobic conditions (Tiwari et al., 2017). Hydrolase was also found in both aerobic and anaerobic sewage treatment plants, since its activity do not depend on redox conditions (Gonzalez-Gil et al., 2019).

Regarding the other investigated enzymes, i.e. Lacc (SO), HRP (L-DOPA), Cyt P450 (INDOLE and 4-AAP), a positive correlation was only found with PYZ and SCL removals: however, these results must be considered are less reliable because not confirmed for both dyes used as a substrate for the enzyme activity determination.

It is reported that oxidative stress generated by high concentrations of intracellular reactive oxygen species (ROS), (e.g. superoxide, hydrogen peroxide and hydroxyl radicals) induces consequent synthesis of antioxidative enzymes such as oxidoreductases (peroxidases and cytochromes) to protect cells against such oxidative stress (Imlay, 2013). Since some of these enzymes are capable of degrading recalcitrant pollutants, such as polycyclic aromatic hydrocarbons and organophosphorus contaminants, it was expected they can also achieve a better degradation of OMPs (Rao et al., 2014; Torres et al., 2003). By comparing the enzymes activity observed at the different aeration frequencies and in the control tests, it can be deemed that the highest and lowest aeration frequencies applied in the present study were capable of enhancing the activity of some OMPs degrading enzymes such as LiP. In addition, the metal ~~centers~~ centres of the peroxidases and laccases have reactive affinities for various functional groups in OMPs (Gonzalez-Gil et al., 2017).

It is confirmed that exposing microbes to transitioning ~~aerobic-microaerobic-anoxic~~ aerobic–microaerobic–anoxic conditions can induce the activation of alternative cellular central metabolic pathways leading to differential enzyme synthesis (Partridge et al., 2007). Indeed, oxygen modulation between aerobic and anoxic conditions seems to reprogram central carbon metabolism, with final production of catalases and peroxidases.

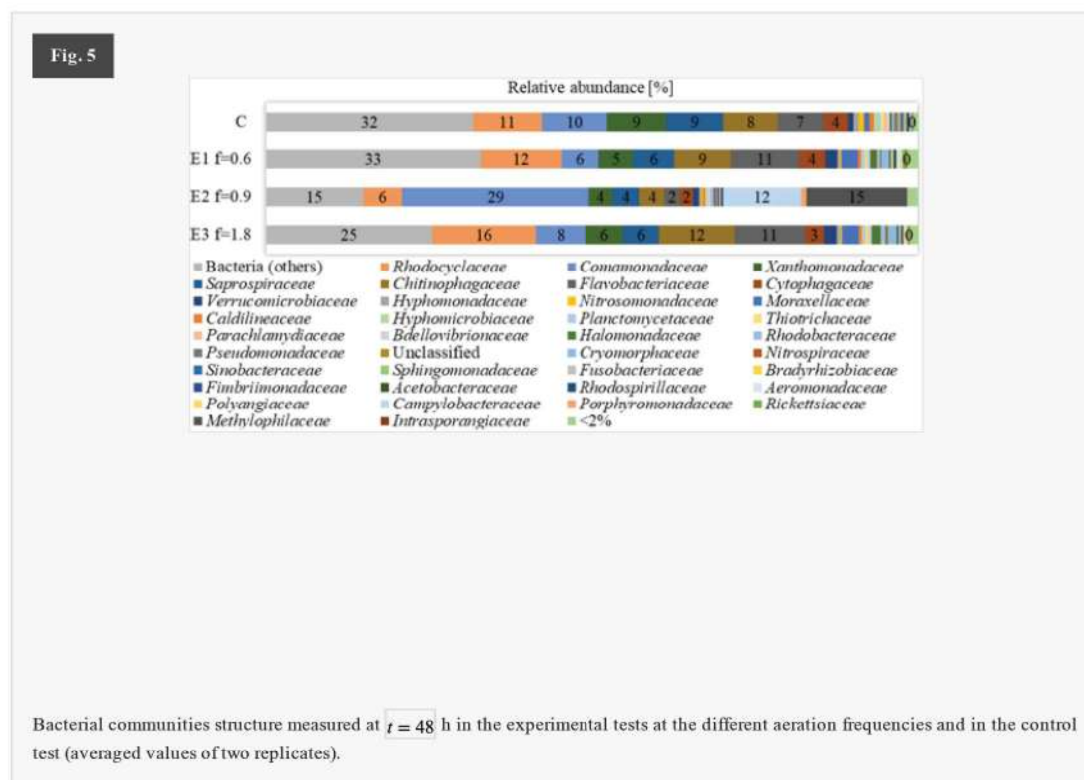
3.3 Microbial speciation

Next step of the study was to investigate if the different aeration frequencies could produce an effect on the composition of the microbial community and if the bacterial speciation was correlated with the observed enzyme activity and OMPs removal.

A total of 432 operational taxonomic units (97% OTUs) were identified in biomass samples at the end of each test ($t = 48$ h), of which 425 represented by bacteria and 7 by archaea. In general, as highlighted by Fig. 5, the aeration frequencies applied in the present study did not produce a significant effect on the activated sludge structure, and the bacteria composition between the control and the perturbed conditions remained very similar (p-value > 0.05 obtained through the application of the paired Student's t-test).

Particularly in C, E1 and E3, the group named as Bacteria (others) in Fig. 5 was found to be the most abundant; it includes bacteria which were not classified at the family level, and therefore grouped as “others”. It includes different bacterial phyla such as *Actinobacter*, *Bacteroides*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria* and *Verrumicrobia*. Since under E1 and E3 aeration frequencies the removals were higher as compared to the control, it can be deemed that this group included those species capable of ~~synthesising~~ synthesizing oxidoreductases, which promoted OMPs degradation. In general, the microbial speciation showed the presence of the bacterial families which are dominant in domestic wastewaters (Shchegolkova et al., 2016; Xu et al., 2018; Zhang et al., 2018, 2017). The most relevant families, under C, E1 and E2 cultures, were present in the following order: *Rhodocyclaceae* > *Comamonadaceae* > *Xanthomonadaceae* > *Saprospiraceae* > *Chitinophagaceae* > *Flavobacteriaceae* > *Cthophagaceae*. *Comamonadaceae* (29%), *Campylobacter* (12%) and *Methylophilaceae* (15%) were the dominant families found in E2 cultures compared to other frequencies. *Comamonadaceae* were in charge of aromatic degrading and denitrifying processes (Xu et al., 2018). *Methylophilaceae* synthesizes laccases, which can catalytically degrade CBZ and other OMPs, as reported above. However, cultures treated with E1, E3 and constant aeration (C) conditions showed similar relative abundance of *Rhodocyclaceae* (≈11%–16%), *Comamonadaceae* (≈8%–10%) and *Xanthomonadaceae* (≈4%–9%) bacterial families. Members of *Rhodocyclaceae* family are known to degrade SMX (Aissaoui et al., 2017). These bacterial families are also reported to be responsible of efficient degradation of other OMPs such as ibuprofen and CBZ (Fischer and Majewsky, 2014). Therefore, selection of these species was favoured by the intermediate frequency perturbation. It is worth of note that OMPs removal efficiency measured in the E2 test was the lowest one with respect to the values recorded in the other tests: this suggests that the dominant bacteria families under this aeration frequency were not relevant for the OMPs degradation.

Fig. 5



Bacterial communities structure measured at $t = 48$ h in the experimental tests at the different aeration frequencies and in the control test (averaged values of two replicates).

4 Conclusions

The present experimental work showed that alternating oxic/anoxic conditions in the bioreactor of a WWTP can affect OMPs removal. Lower and higher anoxic times (i.e. E1 and E3 aeration strategies, respectively) provided better results in terms of removal percentages for most of the OMPS, corresponding to an average increase of 30% as compared to the control tests conducted under continuous aeration. This indicates that a better degradation rate could be established at longer duration of either anoxic or aerobic conditions. Furthermore, lower anoxic time test was characterized by a reduced variability of the average OMPs removal as compared to the other tests.

These results are consistent with the findings of previous works (Grandclément et al., 2017). For instance, Bains et al. (2019) observed a better OMPs removal by using intermittent cyclic DO condition (corresponding to the longest duration of the anoxic phase) with respect to continuous cyclic DO condition.

Beside the better OMPs degradation, carbon and nitrogen were also efficiently removed at the lowest frequency of aeration, with promotion of simultaneous ~~nitrification-denitrification~~ nitrification–denitrification inside the same biological reactor (He et al., 2018).

A good relationship was found between the investigated variables (i.e. bacterial families, enzyme activity and OMPs degradation rate), and the changes in the applied variables (aeration frequencies). However, with respect to previous studies on the effects of changes of DO conditions (Bains et al., 2019), the investigated variables were less affected.

This might be due to the limited differences between the values of the applied variables considered in the present study and to the different seed as compared to Bains et al. (2019) (i.e. activated sludge from domestic sewage and dairy farm sludge, respectively). Therefore, it is suggested as future perspectives to test more stressing conditions to evaluate if they are capable of promoting a better enhancement of the OMPs removal.

The results of this experimental work suggest the opportunity to apply a reduction of the aeration rate in real scale WWTPs to promote a better OMPs removal efficiency, while carbon and nitrogen removal processes, which are the actual main goals of the treatment, remain highly efficient with a possible cost saving as well.

Uncited References

~~The European Parlament and the Council of the European Union (2020)~~

CRediT authorship contribution statement

Camilla Di Marcantonio: Investigation, Formal analysis, Writing - original draft. **Agostina Chiavola:** Writing - review & editing. **Amrita Bains:** Investigation. **Naresh Singhal:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2020.101161.

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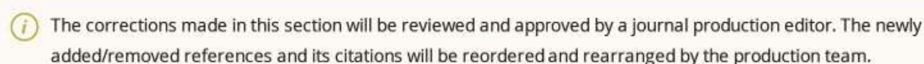

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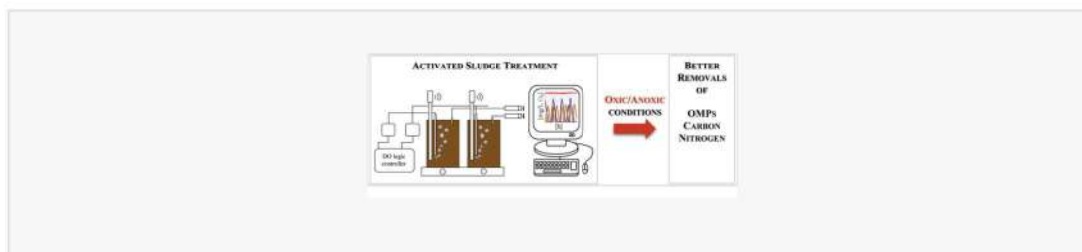
Graphical abstract



Highlights

- Alternating oxic/anoxic conditions in bioreactor can positively affect OMPs removal.
- High acetate and nitrogen removal were maintained in the same bioreactors.
- Increased OMPs removal was related to increased LiP and Cellulase enzyme activity.
- The best overall performance was observed for the longest anoxic conditions.

Appendix A Supplementary data

The following is the Supplementary material related to this article.