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RESEARCH ARTICLE

Effect of Powdered Activated Carbon as Advanced Step in Wastewater Treatments on Antibiotic Resistant Microorganisms

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Abstract: ***Background***: Conventional wastewater treatment plants discharge significant amounts of antibiotic resistant bacteria and antibiotic resistance genes into natural water bodies contributing to the spread of antibiotic resistance. Some advanced wastewater treatment technologies have been shown to effectively decrease the number of bacteria. Nevertheless, there is still a lack of knowledge about the effectiveness of these treatments on antibiotic resistant bacteria and antibiotic resistant genes. To the best of our knowledge, no specific studies have considered how powdered activated carbon (PAC) treatments can act on antibiotic resistant bacteria, although it is essential to assess the impact of this wastewater treatment on the spread of antibiotic resistant bacteria

Methods: To address this gap, we evaluated the fate and the distribution of fluorescent-tagged antibiotic/antimycotic resistant microorganisms in a laboratory-scale model simulating a process configuration involving powdered activated carbon as advanced wastewater treatment. Furthermore, we studied the possible increase of naturally existing antibiotic resistant bacteria during the treatment implementing PAC recycling.

Results: The analysis of fluorescent-tagged microorganisms demonstrated the efficacy of the PAC adsorption treatment in reducing the load of both susceptible and resistant fluorescent microorganisms in the treated water, reaching a removal efficiency of 99.70%. Moreover, PAC recycling did not increase the resistance characteristics of cultivable bacteria neither in the sludge nor in the treated effluent.

Conclusion: Results suggest that wastewater PAC treatment is a promising technology not only for the removal of micropollutants but also for its effect in decreasing antibiotic resistant bacteria release.

Keywords: Powdered Activated Carbon (PAC), antibiotic resistant microorganisms, wastewater treatment plant, fluorescent-tagged microorganisms, next-generation sequencing (NGS) analysis, Denaturing Gradient Gel Electrophoresis (DGGE).

1. INTRODUCTION

Today's municipal wastewater treatment plants (WWTPs) are designed to remove solids, degradable organic substances and nutrients (nitrogen and phosphorus compounds) from wastewater, contributing significantly to water protection and to the generally good quality of surface waters. Primary and secondary treatments remove the majority of the organic matter and suspended solids found in wastewaters as well as some potentially harmful substances present in trace concentrations. Nevertheless, many micropollutants, such as drugs, detergents, and personal care products, are not easily degradable. Their release from WWTPs is currently one of the biggest hazards for water bodies since harmful effects on aquatic organisms are possible and the whole ecosystem may be threatened [1, 2].

Microorganism concentration in sludge normally decreases during the wastewater treatments, but the biological tanks where the degradative processes are carried out represent a potentially suitable environment for the development and spread of antibiotic resistance. In fact, in this environment, which is characterized by the continuous mixing be-

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tween a large number of microorganisms and pollutants, in particular antibiotics that are present at sub-inhibitory concentrations, bacteria can mutate and can exchange resistance determinants *via* plasmid transfer, transduction by bacteriophages and transformation [2-4]. As a result, conventional WWTPs discharge important amounts of Antibiotic Resistant Bacteria (ARB) and antibiotic resistance genes (ARGs) into natural water bodies, in spite of their effectiveness in reducing both nutrients and the total bacterial concentration [5, 6]. Therefore, in order to limit the spread of antibiotic resistance, treatments able to eliminate or at least to reduce ARGs, ARB and pathogens from WWTP outlet have to be put in place.

Through recent improvements in environmental legislations, many WWTPs in Switzerland will be forced to implement advanced treatment steps to reduce the release of micropollutants into surface waters. These regulations will concern approximately 100 of the 700 existing WWTPs, which treat ca. 50 % of the wastewater [7, 8]. Currently, two main technologies are investigated since they have been shown to be suitable for implementation on an industrial scale in terms of efficiency, costs, and energy requirements: ozone oxidation and adsorption onto granular or Powdered Activated Carbon (PAC). Studies and pilot trials showed that both additional treatment processes significantly improve the quality of the treated wastewater in relation to micropollutants and their undesired effects [9-11]. Moreover, these treatments contribute to decreasing the number of bacteria to a level that fulfills the hygienic standards in force for bathing waters in lakes and rivers [8, 12, 13].

In the activated carbon process, PAC is mixed with the wastewater after the biological treatment so that residual organic matter and micropollutants adsorb onto the carbon particles. Once loaded, PAC is separated from the purified wastewater by filtration or by dissolved air flotation. It is then disposed of, along with the sludge, by incineration [12, 14]. In order to optimize the process and to decrease the treatment costs, PAC can be reused until its saturation by collecting and adding it back to the biological treatment step, increasing micropollutant elimination efficiency [15-17]. In this way, the amount of PAC can be reduced by 15-20% while maintaining a positive effect on sedimentation of the activated sludge flocs. On the other hand, used PAC presents adsorbed micropollutants such as antibiotics and a high concentration of bacteria, including antibiotic resistant ones [18, 19], that will be mixed to the activated sludge by recycling. This might lead to a selection and possibly to an increase of ARB in the sludge and in the depurated effluent waters. The effectiveness of activated carbon in reducing antibiotics from wastewater has been extensively proved [10-12], but only a few studies have considered how activated carbon treatments act on ARB and ARGs [1, 20, 21].

To address this knowledge gap, our study had the following aims: 1) to evaluate the fate and distribution of fluorescent-tagged antibiotic/antimycotic resistant microorganisms during the advanced wastewater treatment implementing a PAC adsorption step, and 2) to evaluate the occurrence of an increase in naturally-present antibiotic resistant bacteria in the sludge and in the depurated effluent waters during the same advanced treatment implementing PAC recycling. Measurements and analysis were carried out in a laboratory-scale model simulating a process configuration involving PAC and its recycling as a micropollutant removal phase.

2. MATERIALS AND METHODS

2.1. The Lab-scale Treatment Plant

The lab-scale installation consisted of a 7-L sequencing batch reactor (SBR) for the biological treatment of the wastewater connected to a 5-L dissolved air flotation unit (DAF). The saturated water injected at the bottom of the DAF unit was produced in an Air Dissolving Tube (ADT) with a maximum operation volume of 2 L and a working pressure of 1-6 bar (Fig. **1**).

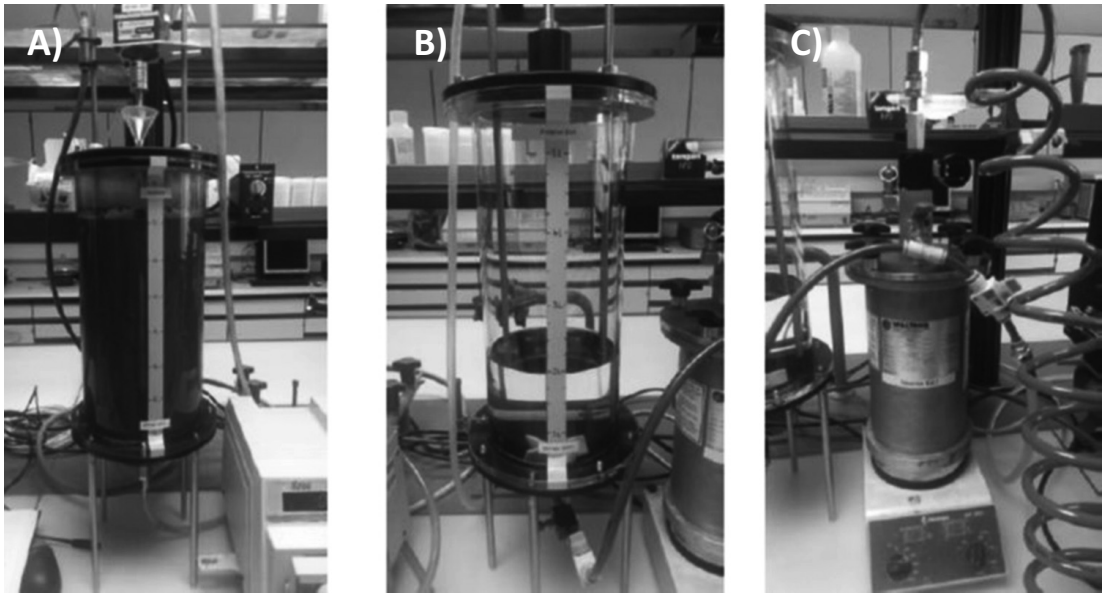

Fig. (1). The lab-scale wastewater treatment plant equipped with a powdered activated carbon/dissolved air flotation unit. **A**) the sequencing batch reactor (SBR) for the biological treatment; **B**) the dissolved air flotation unit (DAF) for powdered activated carbon treatment; **C**) the air dissolving tube (ADT) cell.

Activated sludge from the WWTP of Lugano was used to inoculate and feed the laboratory plant. This WWTP treats approx. 100'000 population equivalents and treats 1.0E+07 m3 of water year-1 [22]. Activated sludge was collected and characterized for its main parameters - ammonium, nitrate, nitrite, phosphate, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), Total Suspended Solid (TSS), and pH - at weekly intervals, and stored at 4°C until used. Five L of activated sludge was pumped into the SBR, mixed and aerated for two hours before the sludge was left to settle for 30 minutes. 1.7 L of the supernatant was transferred to the DAF unit where powdered activated carbon (PAC; SAE Super, Norit, concentration=15 mg L-1) was added and mixed (30 min). The next step consisted of the addition of the coagulant poly-aluminium chloride and, after 12 minutes, of the flocculant Superfloc C-82080. Finally, saturated water (6 bar saturated tap water with an injection ratio of 15 %) was injected into the DAF unit, initiating the separation step (12 min). The full process lasted for about four hours.

Before starting the laboratory tests, the performance of the SBR reactor was monitored during a period of 2.5 weeks of continuous running. Six L of raw wastewater collected from the WWTP of Lugano was inoculated into the SBR tank, which was further fed every day with the same wastewater. The biological process was checked by daily measurements in the inflow and in the effluent of the SBR reactor of BOD_5, COD, TSS, nutrients (ammonium, nitrate, nitrite, and phosphate), and pH for two weeks (data not shown). The elimination rates and parameters were compared to the conversion rates of the real scale plant.

For the continuous operation of the lab-scale plant with PAC recycling, the sludge age was controlled by regular sludge discharge and monitored by BOD_5 COD, TSS, nutrients and pH measurements. The effluent of the biological treatment unit was collected in 10-L tanks, and stored in the fridge (4°C). Once per week the stored effluent was treated with activated carbon (15 mg L-1 PAC), coagulated, flocculated and finally separated. The floated PAC layer was collected and recirculated into the SBR. The flotation efficiency was visually checked.

2.2. Scanning Electron Microscopy

The surface morphology of activated carbon (PAC SAE Super Norit) was evaluated by Scanning Electron Microscopy (SEM) (JSM-6010PLUS, Jeol, LTD Japan). Samples of PAC before and after its use were dehydrated, gold coated for 30 sec (Autosputter coater JFC 1300 JEOL, LTD Japan) and observed with the secondary electron detector at 10kV accelerating voltage, a spot size of 40 and x2000 magnifications.

2.3. Physico-chemical Parameters

Analyses of TSS, BOD_5 and pH were performed as described in [23]. Nitrogen and phosphorous compound concentrations, as well as COD were measured with a spectrophotometer DR2800 (Hach Lange, Germany) using the Hach-Lange cuvette test LCK 303 for ammonium, LCK 342 for nitrite, LCK 340 for nitrate, LCK349 for orthophosphate and LCK 514 for COD.

2.4. Microbiological Analysis

2.4.1. Analysis of Microbial Populations

In order to verify the compliance of the model to the real-scale plant regarding the microbial populations, Next-Generation Sequencing (NGS) analysis and Denaturing Gradient Gel Electrophoresis (DGGE) based on 16S rDNA were performed on corresponding samples taken from the WWTP of Lugano and the laboratory-scale model. Samples were collected during the first week of November 2014 in the WWTP of Lugano and in the laboratory-scale plant. Fig. (**2**) summarises the sampling points. The sample volumes were as follows: 10 ml for WWTP-PS, WWTP-AT and M-SBR; 200 mL for WWTP-CL; 48 mL for M-SBR-SN.

After filtration on 0.2 µm pore-size membrane filters (Sartorius Stedim Biotech, Goettingen, Germany), DNA was extracted using the PowerWater® DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) and quantified with a NanoDrop®

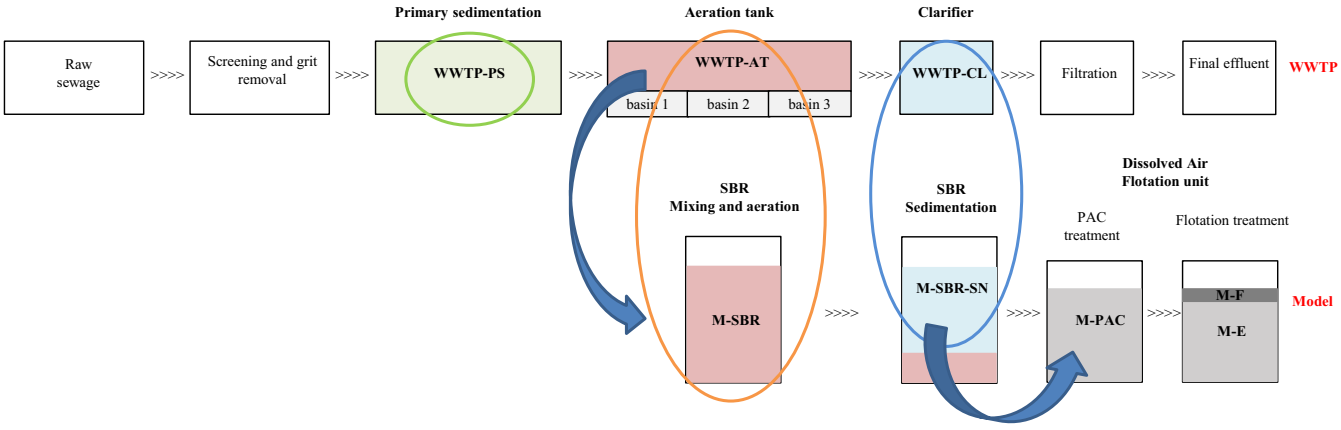

Fig. (2). Localizations of samples collected from the WWTP of Lugano and the lab-scale model. The purification steps that correspond in the two plants are circled while the arrows indicate the water flow. The samples in the WWTP of Lugano are: WWTP-PS: Primary Settling tank; WWTP-AT: Aeration Tank; WWTP-CL: Clarifier tank. The samples in the model are: M-SBR: Mixing and aeration phase; M-SBR-SN: Supernatant after sedimentation of activated sludge; M-SBR-SE: Sludge sediment; M-PAC: Water after adsorption on PAC; M-F: Floated PAC layer; M-E: Final effluent.

ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Next-generation sequencing was performed at the Research and Testing Laboratory (Lubbock, TX, USA) on an Illumina MiSeq platform targeting the bacterial 16S rRNA variable regions V1-V3. Reads were analysed for operational taxonomic units (OTUs) identification, diversity indices calculation and microbial community annotation.

For DGGE, bacterial 16S rDNA was obtained by PCR amplifications using the primer combination GC338f (GCTGCCTCCCGTAGGAGT)/518r (ATTACCGCGGCTGCTGG). A GC clamp [24] of 40 nucleotides was hanged at the 3' end of primer 338f. The length of the product (without GC clamp) was 180 bp. Amplifications were performed using a touchdown PCR protocol modified from Bottinelli [25] consisting in 10 cycles with annealing temperatures decreasing from 65 to 56°C in decrement of 1°C, and 20 additional cycles at an annealing temperature of 55°C. After the initial denaturation step at 94°C for 5 min, denaturation, annealing and polymerisation steps were 1 min, 30 secs, and 1 min, respectively. PCR amplification products were then separated on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Switzerland) during 5 h at a constant voltage of 150 V and at 60°C in a 20 to 60% vertical denaturing gradient (where 100% denaturant was 40% formamide and 7 M Urea). Gels with banding profiles were photographed with a Quantum ST4 System (Vilber Lourmat, Germany) using a UV transilluminator after staining with GelRed (Biotium, Hayward, CA, USA) for 25 min. Profiles were normalised and compared using Gelcompare software (Applied Math, Belgium).

2.4.2. Analysis of Fluorescent-tagged Bacteria and Yeasts

The *Escherichia coli* strains for fluorescence labelling, were isolated from the WWTP of Lugano. The strain *E. coli* 1-10 was sensitive to ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid, and streptomycin, whereas *E. coli* 3-12 was resistant to these antibiotics. Susceptibility was determined by the Kirby-Bauer disk diffusion susceptibility test.

E. coli 1-10 was transformed with plasmid pBK-miniTn7-gfp2 [26], which carried a green fluorescent protein (GFP) gene, whereas to transform *E. coli* 3-12, the plasmid pME9407 [27], carrying a mCherry protein (a variant of red fluorescent protein) gene, was used. Transformed bacterial cells, obtained using a standard electroporation technique [28], were selected by gentamicin resistance (10 µg mL-1) and fluorescence emission. Tagged strains exhibited the same growth rates as their non-transformed parental strains (data not shown).

Candida albicans ALY46 (ADH1::pADH-RFP-SAT1) has a chromosomally inserted gene for the red fluorescent protein (RFP), and *C. albicans* ALY47 (ADH1::pADH-GFP-SAT1) has a chromosomally inserted GFP. These strains were obtained by transformation of the laboratory reference *C. albicans* strain SC5314 [29]. To study the distribution of fluorescent-tagged microorganisms during the PAC-DAF treatment in the first phase of the study, a suspension of a single fluorescent-labelled microorganism was inoculated in the SBR to reach an initial density of 10^4-10^5 cells mL^{-1}. The laboratory scale model was then run for a complete cycle. Samples were collected (Fig. **3**), stored at 5°C and analysed within 24 hours.

SBR and DAF units were washed and disinfected between each trial, consisting in a separate cycle run for each tagged strain and repeated independently three times for each of the four fluorescent strains.

Samples M-SBR-SE (SBR sludge sediment) and M-F (floated PAC layer) were constituted of highly aggregated structures that hindered the quantification of the fluorescent-tagged microorganisms. To disrupt the aggregates and release the microorganisms, a sonication-based protocol [30, 31] was modified as follows: the sample (M-SBR-SE: 100 µl diluted in 900 µl 1x phosphate buffered saline (PBS) solution; M-F: 1 mL) was centrifuged at 8'000 g for 5 min, and washed twice in 1x PBS. The pellet was suspended in 1 mL of sterile detergent sodium hexametaphosphate (SHMP) 0.5% (w/v) solution to disrupt aggregates. After 10-min incubation, the suspension was sonicated 1 min at 65 W, 5 impulses sec^{-1}, in an ice bath. The sample was centrifuged again at 8'000 g for 5 min and the pellet suspended in 1 mL 1x PBS solution.

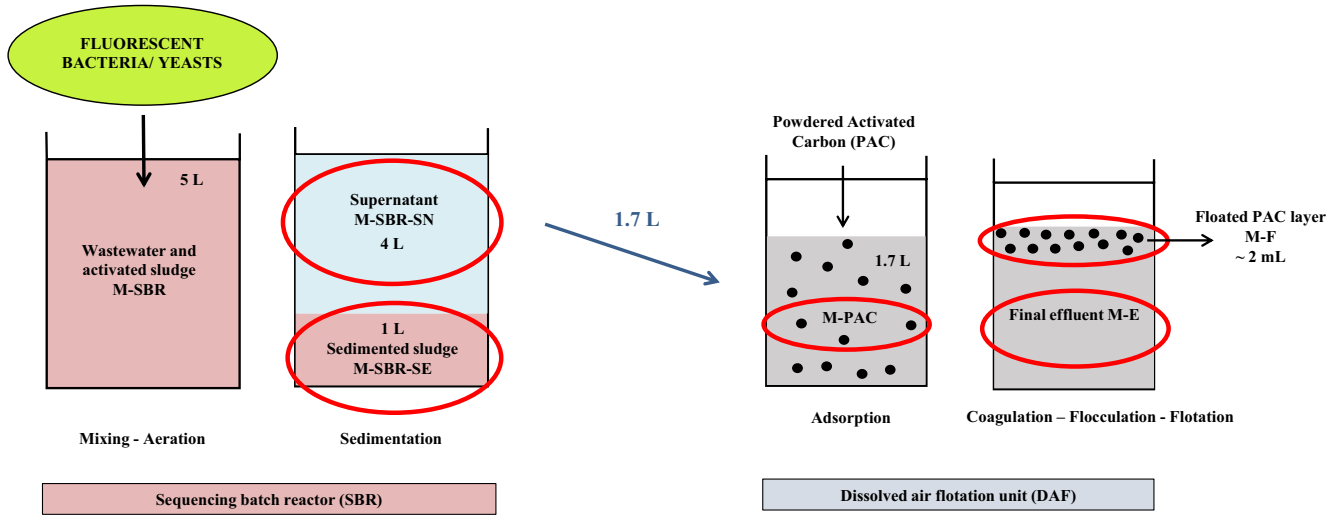

Fig. (3). Summary of the SBR/DAF process. Sampling points are circled (dashed line). Refer to Figure 2 for abbreviations.

Fluorescent-tagged microorganisms were enumerated in unstained subsamples in triplicates by epifluorescence microscopy and flow cytometry. To avoid clogging of the cytometer's flow cell, samples were filtered on 30-µm-pore-size nylon filters (Merck Millipore, Switzerland) before flow cytometry enumeration. For epifluorescence microscopy, subsamples were filtered onto 0.2-µm-pore-size polycarbonate membrane filters (Merck Millipore) that were examined immediately with an Axiolab epifluorescence microscope (Zeiss, Germany) equipped with filter sets for detecting GFP (470/40-nm excitation, 525/50-nm emission, beam splitter 495 nm) and RFP or mCherry (545/30-nm excitation, 605/70-nm emission, beam splitter 570 nm) (AF Analysentechnik, Germany). The average number of fluorescent cells was determined from 10 separate field counts, and the cell concentration (number of cells per sample) was then inferred taking into account the filter and the field areas. Flow cytometry was performed using an Accuri C6 Flow Cytometer Instrument equipped with a 14.7-mW laser excitation light source (488 nm) (BD Accuri, USA). The forward scatter (FSC-H) threshold was set to 10'000 for *E. coli* cells and 40'000 for *C. albicans* cells. Green fluorescence was detected using the FL1 channel (emission filter 533/30-nm) and red fluorescence was detected using the FL3 channel (emission filter 670-nm for mCherry). The samples were analysed setting the following parameters: flow rate 35 µl min-1, core size 16 µm, total number of events recorded per sample 50'000. The BD Accuri C6 Software (v.1.0.264.21) was used for data collection and analysis.

2.4.3. Analysis of Antibiotic Resistant Bacteria

In order to quantify naturally-present ARB in the second phase of the study implementing PAC recycling, samples were collected at intervals of 2 months during the 6-month operation time of the lab-scale model. Samples, stored at 4°C and analysed within 24 hours, were taken from the inflow to DAF unit (M-SBR-SN), the floated PAC (M-F), and the final effluent after the PAC treatment (M-E). Initial samples were collected at time 0, corresponding to the first time that the PAC was used, and were followed by those collected at time 1 (after 2 months of running the lab-scale model), time 2 (after 4 months), and time 3 (after 6 months, corresponding to the end of the trial).

One-mL serial dilutions of samples were filtered in triplicates on 0.45-µm pore size, 47-mm diameter cellulose nitrate membranes (Sartorius Stedim Biotech, Germany), and placed onto plate count agar (PCA, Oxoid, Switzerland) for total heterotrophs, C-EC Agar (Biolife, Italy) for faecal coliforms, and m-Enterococcus Agar (Difco, BD, Switzerland) for enterococci counts. After an incubation period of 24 h at 44°C (for faecal coliforms) and 48 h at 30°C (heterotrophs) or 35°C (enterococci), the number of colony forming units (CFU) was recorded. For each sample and each type of medium, 100 colonies (or all if the number of CFU was < 100) were isolated on Columbia agar with 5% sheep blood (BD). Heterotrophs and antibiotic resistant faecal coliforms were identified by MALDI-TOF mass spectrometry (Shimadzu-Biotech Corp., Japan), using the SARAMIS^{TM} v.4.09 database (Spectral Archive and Microbial Identification System, AnagnosTec, Germany).

To test antibiotic resistance, each isolate was cultured on Luria-Bertani (LB) agar (NaCl 10 g L-1, tryptone 10 g L-1, yeast extract 5 g L-1, agar 15 g L-1) supplemented with antibiotics, and on LB agar as growth control. Heterotrophs were grown on LB supplemented with 2 µg mL-1 of Clarithromycin, 1 µg mL-1 of Norfloxacin, and 4-76 µg mL-1, corresponding to a ratio of 1:19, of Trimethoprim-Sulfamethoxazole, respectively. Faecal coliforms were tested on LB agar containing 1 µg mL-1 of Norfloxacin, or 4-76 µg mL-1 of Trimethoprim-Sulfamethoxazole, whereas enterococci were tested on LB agar supplemented with 2 µg mL-1 Clarithromycin or 4-76 µg mL-1 of Trimethoprim-Sulfamethoxazole. Heterotrophs and enterococci were incubated for 48 h at 30°C and 35°C, respectively, while faecal coliforms were incubated for 24 h at 37°C.

Antibiotic inhibitory concentrations for faecal coliforms and enterococci were based on EUCAST tables [32]. For heterotrophs, the highest Minimum Inhibitory Concentration (MIC) found in EUCAST tables for a given antibiotic was selected, assuming that bacteria growing on these media were resistant to the respective antibiotic, independently of the clinical resistance definition.

2.5. Statistical Analysis

Statistical analysis was carried out with IBM SPSS Statistics Version 22.0 (IBM Corp, USA). Results of experiments with fluorescent-tagged microorganisms are the means of at least three tests. The coefficients of variation between all replicates were < 18%, except for one case where the coefficient was 33%. Differences between means were assessed by analysis of variance (ANOVA), where $p \leq 0.05$ was considered significant.

The results from the total bacterial load in the PAC-recycling experiments are the means of triplicates of the same sample. For the analysis of variance between sampling times in the six months operation process, the antibiotic-resistance results of the three samples for one sampling time were pooled (missing data were not taken into account in the statistical analysis). Removal efficiencies of the PAC treatment were calculated for total heterotrophs, faecal coliforms, and total enterococci [33, 34].

3. RESULTS AND DISCUSSION

3.1. Validation of the SBR Reactor

The performance of the SBR reactor was monitored during a period of 2.5 weeks of continuous running. The average removal rates obtained were of 94% for BOD_5, 95% for COD, and 94% for TSS. The pH values remained constant. The average remaining ammonium, nitrate and nitrite concentrations in the SBR effluent were lower than 0.1 mg L-1. The performance of the unit processes of the SBR was therefore comparable to that of the conventional activated sludge treatment of the WWTP of Lugano.

By next generation sequencing, an average of ca. 21'000 effective sequences per sample were obtained and used for taxonomic identification (Table **1**).

The most frequent bacterial phyla and classes found in samples are resumed in Table **2**.

Table 1. Number of raw reads, clean and identified sequences (percentage in brackets) obtained for each sample by MiSeq Illumina. Refer to Figure 2 for abbreviations.

Sample	Raw Reads	Clean Sequences	Identified Sequences
WWTP-PS	81,043	21,514	12,957 (60%)
WWTP-AT	61,858	17,423	13,272 (76%)
WWTP-CL	61,087	16,837	12,178 (72%)
M-SBR	85,378	23,636	17,867 (76%)
M-SBR-SN	90,900	24,877	18,112 (73%)

Table 2. Taxonomic distribution of the identified sequences (expressed in percentage) into the most predominant bacterial phyla and classes. Refer to Figure 2 for abbreviations.

	WWTP-PS	WWTP-AT	WWTP-CL	M-SBR	M-SBR-SN
Actinobacteria	0.6	8.1	11.6	7.3	8.6
Bacteroidetes	7.4	6.4	5.5	5.6	3.0
Firmicutes	15.4	2.4	10.3	2.2	5.5
Proteobacteria	73.4	70.4	63.4	61.7	46.6
Alpha-proteobacteria	0.5	8.9	7.6	15.2	10.2
Beta-proteobacteria	21.7	54.5	48.9	28.6	25.5
Epsilon-proteobacteria	43.3	0.3	0.3	0.4	0.3
Gamma-proteobacteria	7.6	3.2	4.3	13.9	6.3
No Hit	0.8	5.2	4.1	9.4	18.9

Gram-negative bacteria belonging to the *Proteobacteria* phylum represented the predominant community in all samples. With the exception of the sample from the WWTP primary sedimentation tank (WWTP-PS), *Beta-proteobacteria* were the most frequently retrieved members of this division, in agreement with results of other authors [35, 36]. The *Proteobacteria* in sample WWTP-PS were dominated by bacterial phylotypes belonging to the *Epsilon-proteobacteria* as already reported [37]. *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* represented other important community members in all the samples. The Bray-Curtis dendrogram based on the next-generation sequencing data (genus counts) highlighted the similarity between the microbial communities of the WWTP of Lugano and the laboratory-scale model (Fig. **4A**).

By DGGE analysis, each sample produced a complex fingerprint composed of a large number of bands. Fingerprints obtained for WWTP-AT and M-SBR samples were similar and differed from the fingerprints for WWTP-CL and M-SBR-SN, confirming the similarity of the bacterial populations of the WWTP and the model (Fig. **4B**).

Since the lab-scale treatment plant model was fully comparable to the real scale plant of Lugano considering the performance and the microbiological communities, the model was used to follow the fate of the microorganisms throughout the treatment processes.

3.2. Fate of Fluorescent-tagged *E. coli* and *C. albicans* in the Laboratory-scale Model

The distribution of the fluorescent-tagged microorganisms in the laboratory-scale model was established by epifluorescence microscopy and flow cytometry (Fig. **5**).

Cell counts obtained with flow cytometry were in general higher than those obtained with epifluorescence microscopy, probably because cytometry detected also other autofluorescent particles and organisms. The distributions of the four labelled microorganisms in the lab-scale model showed a similar trend. After the initial inoculation and three hours of incubation in the biological treatment unit of the model, the cell densities in the SBR supernatant after sedimentation (M-SBR-SN) and in the sedimented portion (M-SBR-SE) showed only minor differences, being slightly higher in the sediment. As expected, the cell density M-PAC was very similar to the cell density in the SBR supernatant (M-SBR-SN), which was used to feed the PAC/DAF unit. On the contrary, the concentration of all the tagged cells decreased dramatically after the PAC and dissolved air flotation treatment in the effluent water (M-E), and tagged microorganisms concentrated in the floated layer (M-F). Log transformed absolute cell counts were analysed with analysis of variance (ANOVA) for significant differences between means. There was no statistically significant difference between the cell

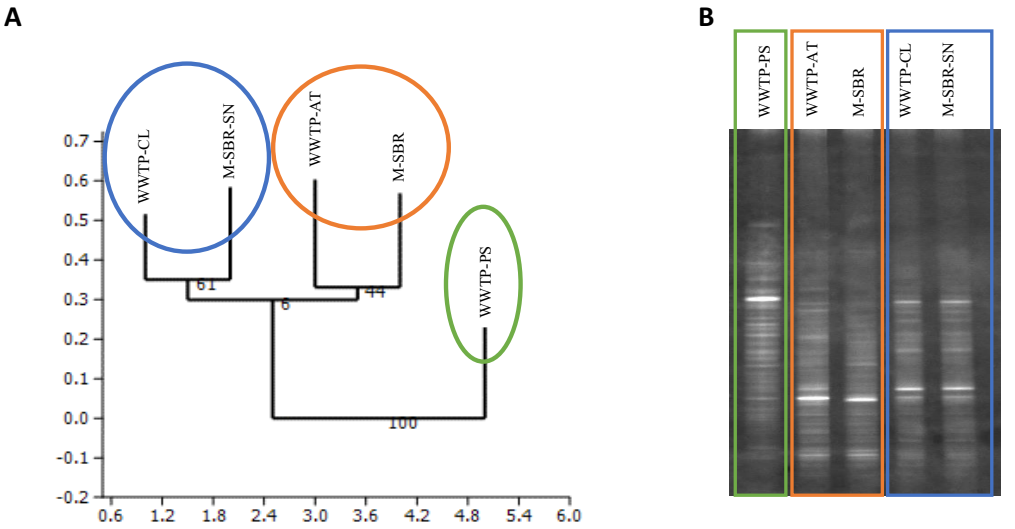

Fig. (4). Comparison of the microbial populations identified in samples from the WWTP of Lugano and the lab-scale model. **A**) Bray-Curtis similarity dendrogram based on the total OTU abundance (97% identity); **B**) denaturing gradient gel electrophoresis (DGGE). Refer to Figure 2 for abbreviations.

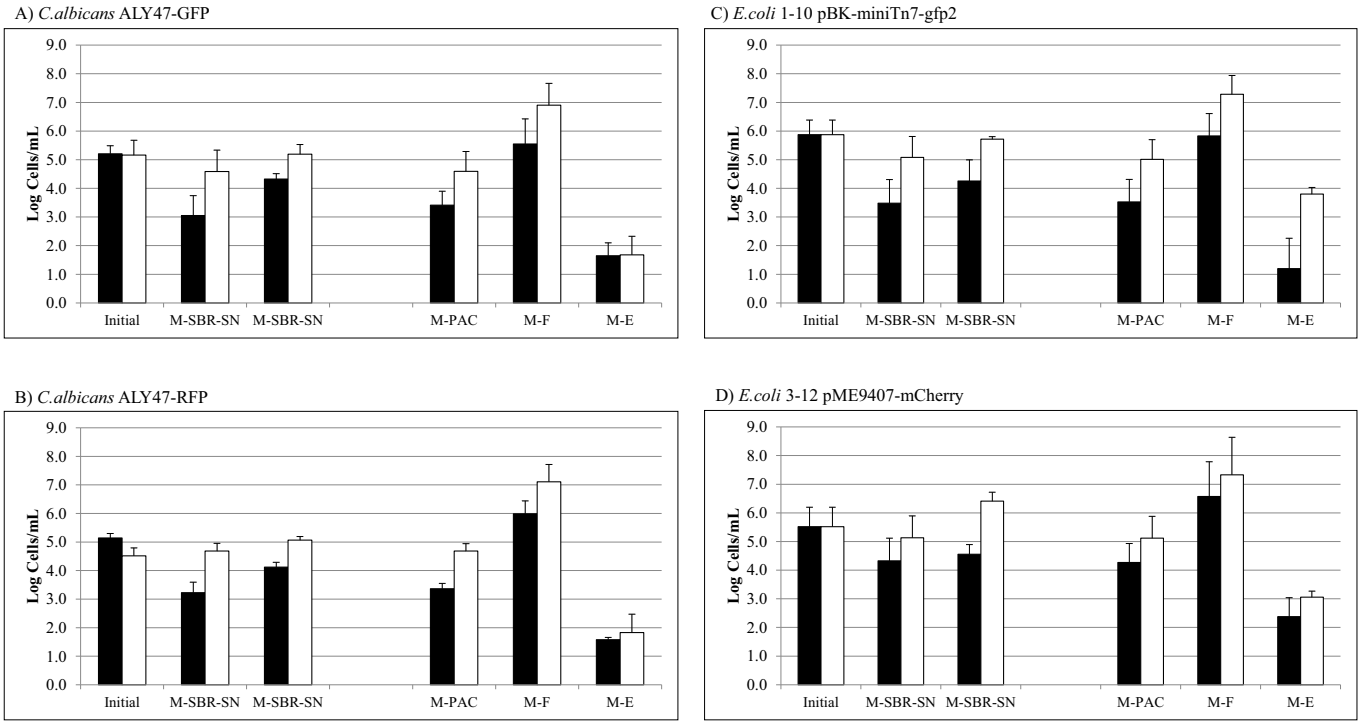

Fig. (5). Distribution of fluorescent-tagged *E. coli* (**A**, **B**) and *C. albicans* (**C**, **D**) in the laboratory-scale model. Concentrations (Log cells mL-1) were calculated by epifluorescence microscopy (black bars) and flow cytometry (white bars) and represent the mean of three independent tests. Refer to Figure 2 for abbreviations.

distribution in sediment or supernatant in SBR whereas the number of cells remaining in the floated PAC (M-F) was significantly higher than the number of cells released in the final effluent ($p < 0.01$).

The biological treatment (mixing and aeration phase) and the subsequent separation of sludge from the effluent water in the reactor reduced the cell count number, based on epifluorescence microscopy of tagged microorganisms, of approx. 1.8 log-units on average, reaching a nearly 98 % efficiency of removal similar to what reported in real scale WWTPs [38, 39]. The PAC-DAF treatment reduced the number of tagged microorganisms of 3.8 log-units on average, allowing an additional removal of about 99.70% of fluorescent-tagged microorganisms from the biological treatment effluent.

The addition of PAC to the DAF unit did not substantially change the number of fluorescent bacterial cells (Fig. **5**). The main mechanism of PAC action is based on sorption and electrostatic repulsion, depending on the contaminant [1, 40]. Therefore, bacterial cells initially adsorb to the surface of

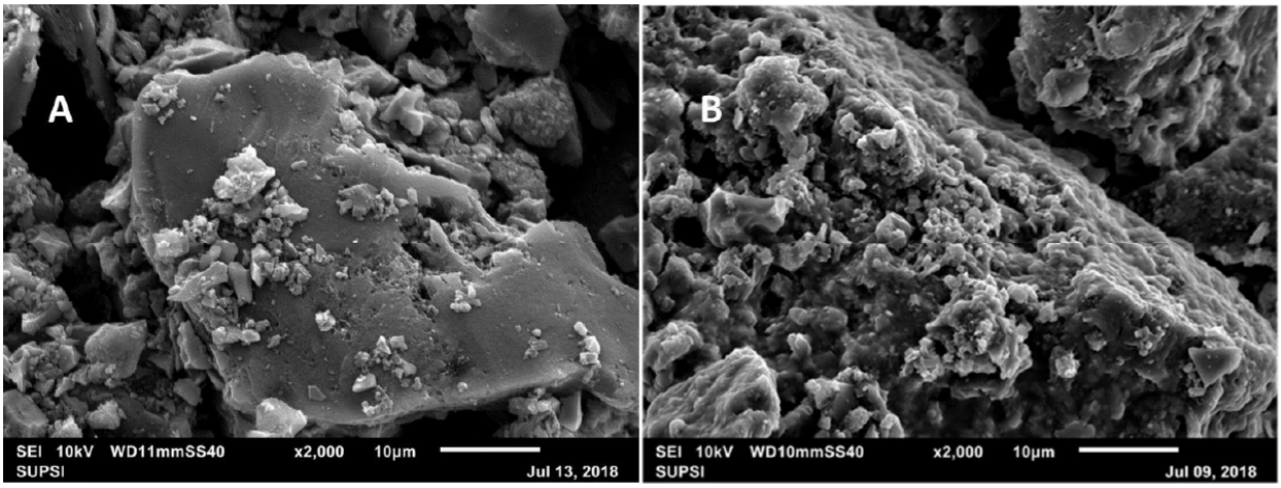

Fig. (6). Scanning electron micrograph of the PAC surface (PAC SAE Super Norit). **A**) before use; **B**) after 30 min in the DAF unit. x2000.

PAC mainly because of charge attractions and only with time, bacteria can colonize its surface and form an active biofilm [41, 42]. As confirmed by SEM images of PAC surface before its use and after 30 min in the DAF unit (Fig. **6**), the short time of contact probably allowed the preferential adsorption of the smaller negative molecules present in water rather than the largest negatively charged bacterial cells [43, 44].

On the other hand, the coagulation/flocculation process seems to effectively remove bacterial cells *via* interparticle bridging and charge neutralization [45-47]. Coagulation alone allowed the removal even of resistance genes from WWTP effluents because of its ability to remove whole bacterial cells, as well as extra-cellular genes [1, 48]. Whatever the reason, the combination of the advanced PAC-DAF treatment applied in our laboratory-scale model demonstrated its efficacy in the removal of fluorescent-tagged microorganisms, regardless of their resistance to antibiotics/ antimycotics.

3.3. Antibiotic Resistant Bacteria in the Lab-scale Wastewater Treatment Plant Model Implementing Powdered Activated Carbon (PAC) Recycling

To explore the occurrence of an increment of ARB in the sludge and/or in the depurated effluent waters due to the PAC recycling, the laboratory-scale model of WWTP was completed with a recycling phase, and was run for 6 months.

The model reached a nitrification efficiency of 92.4%, thus comparable with the real-scale treatment plant of Lugano (between 85-95%), and, as this latter, it did not completely remove nitrates. The average COD elimination was 80.4%. The inflow COD concentration was varying between 20 and 80 mg L-1 and the outflow between 0 and 30 mg L-1. The fluctuations of the values in the inflow water reflected the fluctuation observed in the aeration tank of the real-scale plant, and were due to the moment of sampling and to the weather conditions. The COD concentrations in the SBR effluent were, after three months of operation, stable and values lower than 15 mg L-1 could be measured. Again, these values were comparable to those measured in the secondary settling tank of the real-scale treatment plant. The inflow pH was constantly measured between 7.5 and 8.4, whereas after biological degradation (mixing and aeration phase), values were settled around 7.8, showing that activated sludge had sufficient alkalinity to buffer the nitrification reaction as observed in the real-scale plant.

The distribution of bacteria (heterotrophs, faecal coliforms, and enterococci) in the dissolved air flotation unit during the six-month period is shown in Fig. (**7**).

Heterotrophs, faecal coliforms, and enterococci counts in the inflow and the effluent of the DAF unit and in the floated PAC varied significantly ($p < 0.01$) in each sample. However, bacterial counts in the floated PAC were always significantly higher ($p < 0.05$) than those in the final effluent from DAF, reaching a mean removal efficiency of more than 85 %. In samples taken after four months of operation, the removal efficiencies were the lowest (88% for heterotrophs, 82% for faecal coliforms and 52% for enterococci), affecting consequently the counts of viable cells in the effluent (Fig. **7**). These samples were collected in August 2016. No changes in the laboratory procedures were introduced and no evident differences were noticeable in the microbiological quality of the inflow waters. Only the concentration of nitrate in the DAF inflow reached a peak of 19 mg L-1. The drop in the removal efficiency might therefore be explained by a decreased adsorption capacity during the PAC/coagulation/ flocculation steps because the smaller negative ions present in water could have been preferentially adsorbed than larger negatively charged particles [44, 49]. Therefore, Gram negative and positive bacteria, which are charged negatively due to the presence on their surface of lipopolysaccharides and of teichoid acids, might actually have been prevented from adsorption.

The total number of heterotrophs in the inflow to the DAF unit (corresponding to the water after the biological treatment) was of ca. 50'000 CFU mL-1; faecal coliforms and enterococci reached on average approx. 60’000 and 9’000 CFU 100 mL-1, respectively. For comparison, in the real-scale pilot assay carried out in WWTP of Lausanne [18], the corresponding microbiological charges were the double

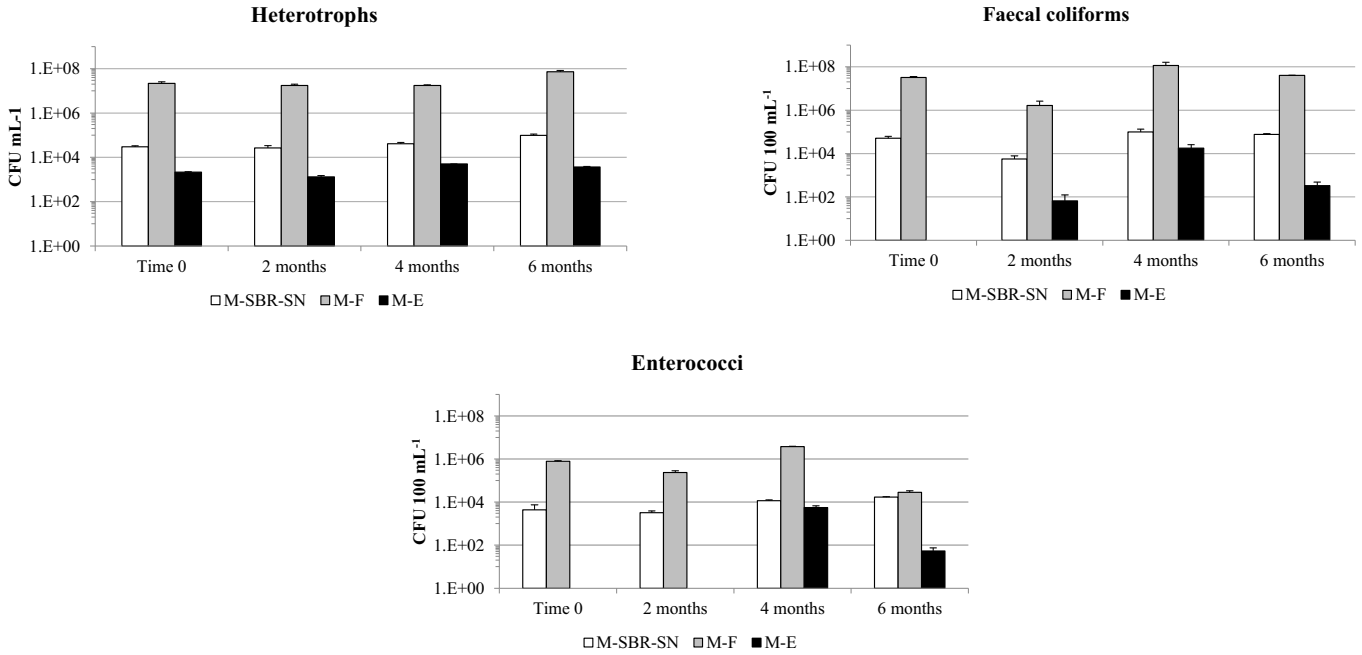

Fig. (7). Total viable counts for heterotrophs, faecal coliforms, and enterococci in the DAF unit before and after flotation. Error bars indicate standard deviation on triplicates of the same sample. Refer to Figure 2 for abbreviations.

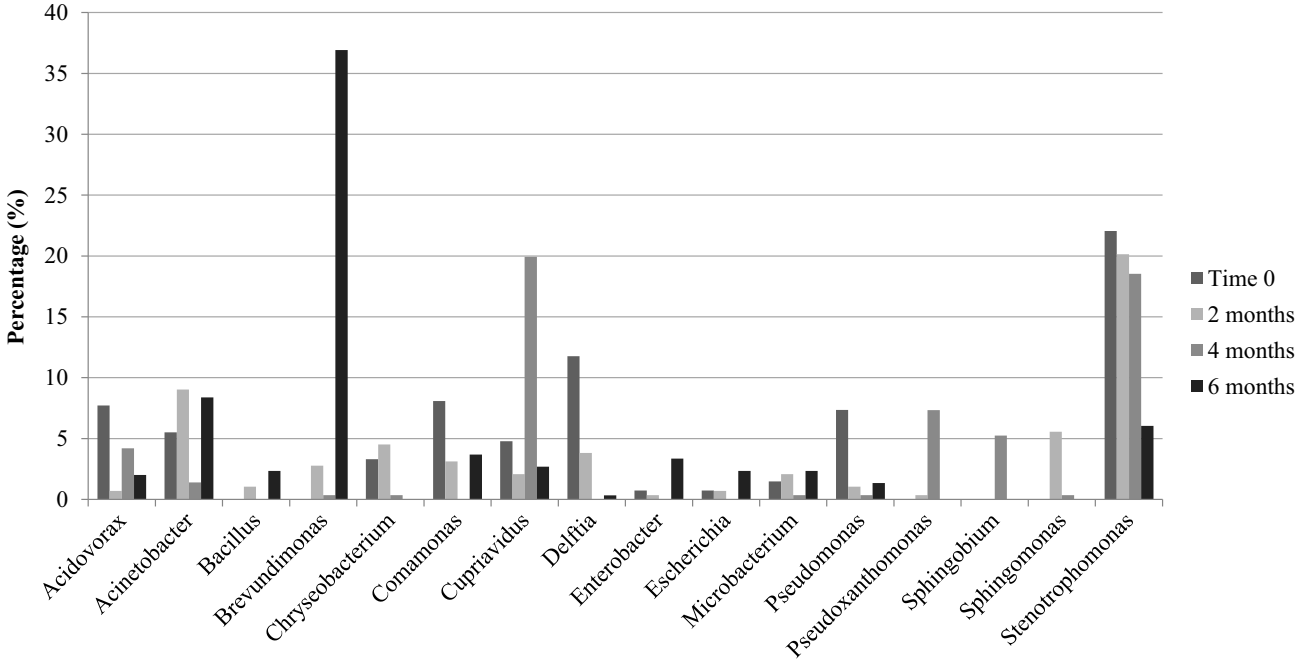

Fig. (8). Percentages of the main heterotrophic bacterial genera identified by MALDI-TOF mass spectrometry.

(approx. 100'000 CFU mL-1) for heterotrophs but similar for faecal coliforms and enterococci. In the study of Margot and coworkers, the PAC treatment was followed by an ultrafiltration step. This treatment allowed the elimination of 100% of *E. coli* and enterococci, and 98% of total heterotrophic bacteria [18]. Therefore, the effluent after PAC and ultrafiltration treatments fulfilled the hygienic standards in force for bathing waters in lakes and rivers (<1'000 CFU 100 mL-1 for *E. coli*, <400 CFU 100 mL-1 for faecal enterococci). In our lab-scale model lacking the ultrafiltration step, PAC-DAF treatment decreased the number of viable faecal coliforms to ca. 4'500 CFU 100 mL-1 and that of enterococci to 1'400 CFU 100 mL-1, on average. However, if data of the samples collected in August 2016 are not considered, the average total number of faecal coliforms and enterococci decreases to ca. 150 CFU 100 mL-1 and ca. 20 CFU 100 mL-1, respectively, dropping therefore within the mentioned limits even without the filtration step.

Dominant bacterial genera varied across the sampling campaigns (Fig. **8**).

Table 3. Concentrations of total and resistant heterotrophs, faecal coliforms, and enterococci in the floated PAC and in the effluent of the DAF unit.

-	-	Heterotrophic Bacteria (CFU/mL)				Faecal Coliforms (CFU/mL)			Enterococci (CFU/mL)		
-	-	Total	CLR Resistants	NOR Resistants	SXT Resistants	Total	NOR Resistants	SXT Resistants	Total	CLR Resistants	SXT Resistants
-	Time 0	2.16E+07	1.61E+07	8.84E+06	8.53E+06	3.22E+05	2.30E+04	5.55E+03	7.88E+03	2.37E+03	5.13E+03
M-F	2 months	1.76E+07	6.10E+06	1.26E+07	5.02E+06	1.63E+04	2.82E+02	2.82E+03	2.33E+03	7.37E+02	1.69E+03
	4 months	1.75E+07	3.78E+06	1.26E+07	7.40E+06	1.15E+06	4.37E+04	1.09E+05	3.73E+04	1.32E+04	2.95E+04
-	6 months	7.32E+07	2.87E+07	5.95E+07	ND	4.00E+05	2.67E+04	4.57E+04	2.83E+02	1.27E+02	2.40E+02
-	Time 0	2.14E+03	1.79E+03	1.54E+03	2.29E+02	ND	ND	ND	ND	ND	ND
M-E	2 months	1.31E+03	8.01E+02	1.08E+03	4.50E+02	6.67E+01	ND	ND	ND	ND	ND
	4 months	5.00E+03	4.07E+03	4.96E+03	1.44E+03	1.77E+02	2.97E+00	1.76E+01	5.57E+01	1.38E+01	4.67E+01
-	6 months	3.69E+03	1.85E+03	2.59E+03	7.85E+01	3.33E+00	ND	3.33E-01	5.33E-01	1.42E-01	5.33E-01

Numbers were derived from the percentages of resistant strains detected. CFU: Colony-forming unit; M-F: Floated PAC layer; M-E: Final effluent. ND: Not detected; CLR: Clarithromycin; NOR: Norfloxacin; SXT: Trimethoprim-Sulfamethoxazole

As an example, the bacteria belonging to the genus *Brevundimonas* were more common in the final sampling campaign, after six months of operation, compared to previous samplings, while bacteria belonging to the genus *Cupriavidus* were more present after four months of the recycling process. *Stenotrophomonas*, which comprises Gram negative bacilli that can be extremely resistant to antibiotics, was the only genus constantly identified throughout the entire trial duration, even if its relative number decreased from time 0 to time 6-months.

The recycling of PAC in biological reactors enhances the removal of micropollutants such as antibiotics but it can be expected that also a high concentration of bacteria, including antibiotic resistant ones, will be mixed to the activated sludge by this procedure. Moreover, it can be expected that the adsorbed antibiotics on PAC may affect the resistance of bacteria. Based on the mean elimination rates reported by Abegglen and Siegrist [8], we chose to investigate the effect on resistance of three antibiotics that are differently eliminated by PAC treatment, namely clarithromycin, eliminated at a rate of 84-92%, norfloxacin, eliminated at least at 79% and sulfamethoxazole, which is eliminated only at approx. 50%.

The concentrations of resistant heterotrophs, faecal coliforms, and enterococci in the effluent of the DAF unit and in the floated PAC during the six months period of the test are resumed in Table **3**.

The distribution of viable resistant bacteria between floated PAC and effluent water from the DAF unit followed the partitioning already discussed for viable and fluorescent bacterial counts, being higher in the floated PAC than in the effluent. The highest number of viable and resistant cells per mL were detected, except for heterotrophic and resistant heterotrophic bacteria, in the samples taken after 4 months of operation, when we observed a drop in the removal efficiency of the model.

Heterotrophs were commonly less resistant to trimethoprim-sulfamethoxazole than to the other two antibiotics tested. The percentage of heterotrophs resistant to clarithromycin and norfloxacin was generally higher in the effluent after PAC treatment compared to the percentage of resistant bacteria in the floated PAC (Fig. **9**).

The analysis of variance, for which the antibiotic-resistance results of the three samples for each sampling time were pooled, did not show any significant increase or decrease in antibiotic resistance to clarithromycin and norfloxacin during the PAC recycling operation. On the contrary, we observed a significant ($p < 0.05$) decrease in resistance to trimethoprim-sulfamethoxazole in the heterotrophs after six months of operation.

The percentages of faecal coliforms resistant to norfloxacin and trimethoprim-sulfamethoxazole were much lower than those found for heterotrophs. Faecal coliforms did not show any significant increase or decrease in antibiotic resistance for the two antibiotics tested between the different time phases of the recycling operation. More than 70% of the faecal coliforms resistant to norfloxacin and trimethoprim-sulfamethoxazole were identified as *Escherichia coli*. The other resistant species identified were *Citrobacter* sp. (13%), *Klebsiella* sp. (6%), and *Enterobacter* sp. (4%).

The percentage of resistant enterococci to trimethoprim-sulfamethoxazole over the 6 months period showed a slightly significant increase ($p=0.049$). On the contrary, no significant differences were observed for the antibiotic clarithromycin. As for faecal coliforms, no resistant colonies could be detected in the effluent samples taken at times 0 and 2 months.

Our survey over a period of six months of the PAC-DAF treatment and PAC recycling does not show any increase in the resistance characteristics of cultivable heterotrophs, fae-

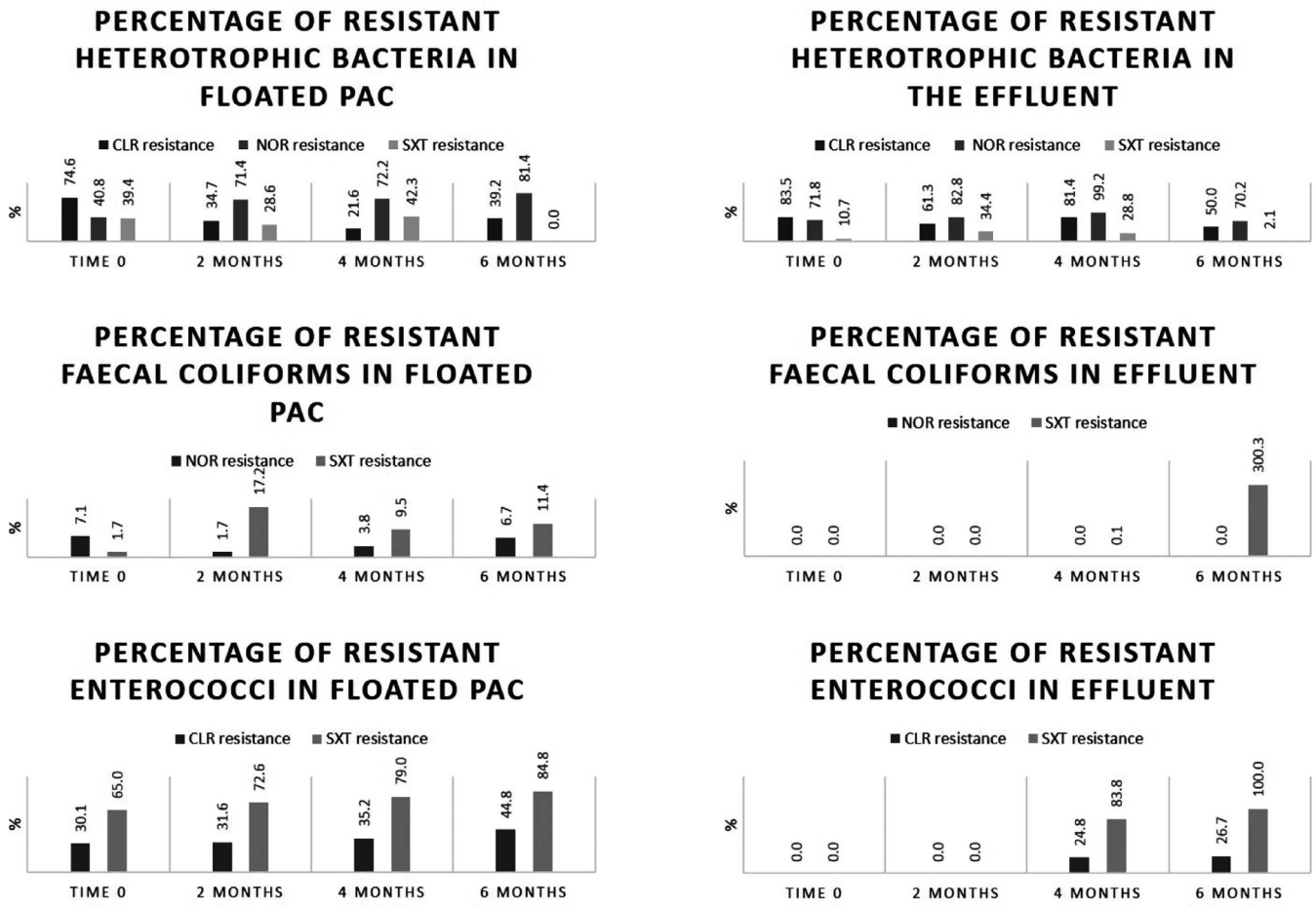

Fig. (9). Percentages of resistant heterotrophic bacteria, faecal coliforms and enterococci in the dissolved air flotation unit of the model. CLR: Clarithromycin; NOR: Norfloxacin; SXT: Trimethoprim-sulfamethoxazole.

cal coliforms and enterococci, at least for the antibiotics taken into consideration.

CONCLUSION

To study the fate of antibiotic resistant microorganisms present in wastewater during an advanced treatment procedure, we developed and validated a lab-scale wastewater treatment plant equipped with a powdered activated carbon - dissolved air flotation unit.

The depurative efficiency as well as the microbial populations present in each treatment step, analyzed by next-generation sequencing and DGGE, certified the compliance of the model to the real wastewater treatment plant of Lugano, which collects approx. 100'000 population equivalents.

The analyses of the fluorescent-tagged microorganisms demonstrated the efficacy of the advanced PAC-DAF treatment in reducing the microbial load in treated water. Moreover, we could verify that this treatment did not differentiate between susceptible or resistant microorganisms since both were eliminated with the same efficiency. In the lab-scale plant, we obtained a microbiological quality of the effluent water respecting the Swiss and European Union hygienic standards.

The study shows that the treatments implementing a powdered activated carbon adsorption step are efficient in reducing substantially bacteria and yeasts released in the effluent of water treatment plants, and that PAC use, even when recycled, does not increase the resistance characteristics of cultivable heterotrophs, faecal coliforms and enterococci.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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