MICROBIOME AND RESPIROMETRY ANALYSIS OF THE BACTERIAL COMMUNITY IN A BENCH-SCALE ACTIVATED SLUDGE REACTOR EXPOSED TO INSECTICIDE

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ABSTRACT

Wastewater treatment plants (WWTPs) have to treat sewage 24 h/365 days a year. Uncontrolled toxic spills can affect the microbial community in activated sludge and thus the biological treatment performance. Water below the quality requirements, established in the legislation could be, therefore released to the receiving environment, contributing to the loss of biological diversity, degradation of water resources and generating public health threats.

This study aims to analyse the community changes that occurred in an activated sludge as consequence of an insecticide spill in a laboratory-scale reactor. Next-generation sequencing was applied to identify genera that could serve as key indicators of a negative biological process affection.

The bench-scale system consisted of a 10-litre biological reactor with intermittent aeration cycles to remove organic matter and nutrients, which was fed with primary settled sewage, follow by a secondary clarifier with manual purging and an external recirculation to the biological reactor. Both settled sewage and mixed liquor were obtained from the same WWTP.

The effect of an artificial spill of insecticide containing D-tetramethrin, cyphenothrin and pyriproxyfen on the activated sludge biomass was studied through both, respirometry and microbiome analysis.

The toxic effect on the activated sludge community demonstrated to be significant, completely inhibiting nitrifying activity and changing the distribution of the microbiome. Results show that relative abundance of certain groups increased after the spill, being then the new conditions favourable for the development of these groups. By contrast, others groups are shown to be extremely sensitive to the toxic effect, such that their disappearance can act as a key indicator of an insecticide spill occurrence in a WWTP. However, the abundance of nitrifying bacteria was not affected.

Keywords: activated sludge, metagenomic analysis, respirometry, toxic spills, bacterial community.

1 INTRODUCTION

Activated sludge is the most widespread biological technology to treat domestic and industrial wastewater [1]. It consists of a biological culture growing in a biological reactor mainly in aerobic conditions by means of blowers or turbines. This culture is capable of metabolizing organic matter, nutrients (nitrogen and phosphorus) and other compounds contained in the sewage.

Knowing the biological community responsible of the organic matter and nutrients removal in activated sludge is key to the implementation of operational strategies that ensure the right wastewater processing and, thus, guarantee the environment protection.

In the last years, metagenomic approaches have replaced classical methods to detect microorganisms in environmental samples, like microscopy or culture-depending methods. High throughput sequencing targeting conserved regions in microbial genomes is currently considered as most reliable and cost-efficient method for taxonomical identification and population analysis of environmental samples [2].

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Uncontrolled spills containing harmful substances can damage the biological units, since an increase in the toxic load of urban wastewater reduces the degradation capacity of the bacterial culture. The occurrence of these uncontrolled spills is frequently seasonal and of industrial origin, resulting in non-compliance to quality requirements at certain times of the year. Consequently, improperly treated water can be released, contributing to the loss of biological diversity and degradation of water bodies.

All wastewater treatment plant (WWTP) annually suffer some minor incident due to activities related to hygiene and health standards (i.e. rat poisoning in wastewater pipes system), and a considerable number also suffer serious incidents involving some uncontrolled industry spills.

Late detection of the impact on the biomass is very common. When a decrease in purification performance is detected by the WWTP operator, many of the microorganisms are inevitably damaged. To restore the normal functioning of the process, different approaches could be used, like increasing the aeration, using coagulants, or, worst-case scenario, reseeding the biomass. The most common procedure to recover the biological process is to increment the air supply to the activated sludge tank which induces an increase in energy consumption, and, hence, an increase in the operational cost.

In this context, autotrophic nitrification in biological nitrogen removal systems has been shown to be sensitive to any environmental changes as well as many organic and inorganic substances [3].

Over the last decade there has been a great development of culture-independent techniques for exploring microbial communities, which have led to new insights into their structure and function in both natural environments and engineered systems [2]. Next-generation sequencing data regarding microorganisms diversity can be used in order to improve bioaugmentation and to stimulate wastewater purification, or for improving the biodegradation of specific compounds [4].

Against this backdrop, the LIFE16 ENV/ES/000390 BACTIWATER project proposed the use of microorganism cultures to boost growth for recovering the activated sludge process after the entry of uncontrolled spills to sewage plants, in order to stimulate the recovery of the microbiome [5]. Likewise, within the project the implementation of an early detection system based in metagenomic techniques to early diagnosis of dysfunctions in the biological process by microbial communities disturbances will be carried out.

In this study, a first approach to the spill effects in mixed liquor microbiome at bench-scale is presented.

2 MATERIALS AND METHODS

2.1 Pilot scale assay

Figure 1 shows the bench-scale system. A 10-litre biological reactor was fed continuously with agitated primary settled wastewater (BDO/CDO = 2.6). Organic matter and nutrients were removed in the reactor thanks to intermittent aeration cycles (20 min of aerobic cycle and 9 min of anoxic cycle). The system was completed by a secondary clarifier and an external recirculation to the biological reactor (100% of inlet flow). The purge was carried out manually once a day to maintain the optimal cellular retention time of 14 days. Parameters of laboratory scaled plant are shown in Table 1. Both primary settled sewage and mixed liquor were obtained from the Quart-Benàger plant (Valencia, Spain), property of the Public Entity

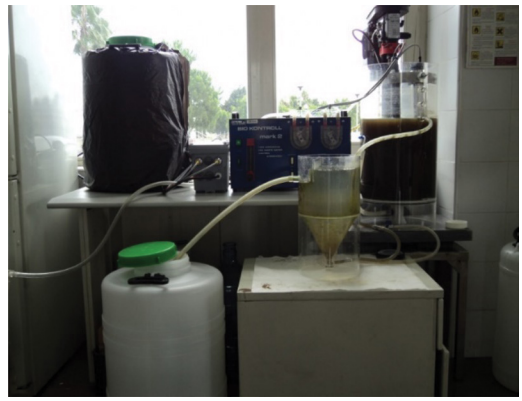

Figure 1: Bench-scale system.

Table 1: Parameters of laboratory scaled plant used during the assay.

Parameter	Value	Unit
Inlet flow	0.6	l/h
SS demo plant	1.356	mg/l
BOD_5	110	mg/l
	0.002	kg/d
COD	283	mg/l
TSS	0.014	kg
Reactor volume	0.01	m^3
F/M ratio	0.12	kg BOD_5/kg MLSS /d

for Wastewater Sanitation of the Valencian Community (EPSAR), attached to the Conselleria de Agricultura, Desarrollo Rural, Emergencia Climática y Transición Ecológica.

The effect of an artificial spill of insecticide containing D-tetramethrin, cyphenothrin and pyriproxyfen on the activated sludge biomass was studied, analysing microbiome in samples collected 2 h after the spill, and on days 1, 2, 3, 6 and 7 after that. Mixed liquor samples were frozen at 20°C and analysed in Lifesequencing S.L.-ADM laboratory (Paterna, Valencia, Spain).

2.2 Respirometry tests

Respirometry test were carried out before and after the spill using a BMT plus (SURCIS) respirometer to evaluate the impact of this spill on the nitrification activity of the activated sludge biomass. To get the maximum nitrification rate, a R test was carried out to 1,000 ml of endogenous activated sludge with: ammonium chloride at saturation concentration (40 ml in 1,000 ml of activated sludge), oxygen concentration at saturation level and controlled temperature during the assay until reaching the maximum exogenous respiration rate. Then, allyl thiourea was added to inhibit the nitrifying bacteria and to verify that the oxygen consumption was due to nitrification.

2.3 Microbiome analysis

The extraction of genomic DNA was carried out from 2 mL of mixed liquor samples with Qiamp Power Fecal Mini kit (Qiagen) with enzymatic lysis and mechanic disruption. A total of 50 ng of DNA was amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (ILLUMINA). In summary, primers were designed containing: (1) a universal linker sequence allowing amplicons for incorporation indexes and sequencing primers by Nextera XT Index kit (ILLUMINA) and (2) 16S rRNA gene universal primers [6].

In the last assay we included amplification indexes. 16S based libraries were quantified by fluorimetry using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher).

Libraries were pooled prior to sequencing on the MiSeq platform (Illumina), with a 300 cycles paired reads configuration. The size and quantity of the pool were assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 20%).

Sequencing data were available within approximately 56 h. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument (MiSeq Control Software (MCS v3.1).

Raw sequences, forward and reverse, were merged in order to obtain the complete sequence using the BBMerge package of BBMap V.38 software. With this approach, the ends of the sequences can be overlapped to obtain complete sequences.

The amplification primers from the sequences obtained in the sequencing step were trimmed to reduce the bias in the annotation step, with 'Cutadapt v 1.8.1' and parameters by default.

Once the primers have been removed, sequences lower than 200 nts were removed from the analysis; short sequences have a higher chance of generating erroneous taxonomical group associations. After obtaining the clean complete sequences, a quality filter was applied to them to delete poor quality sequences. Those bases in extreme positions that did not reach Q20 (99% well incorporated base in the sequencing step) or a greater phred score were removed. Subsequently, sequences whose average quality did not surpass the Q20 threshold, as a mean quality of the whole sequence, were also deleted. These FASTQ files were converted to FASTA files and in order to remove chimeras CD-hit program version 4.8.1 was used. NCBI 16S rRNA database was used to BLAST [7] the FASTA files without chimeras, using blastn version 2.2.29+. The resulting XML files were processed using a python script developed by ADM-Lifesequencing S.L. (Paterna, Valencia, Spain) for annotating each sequence at different phylogenetic levels. The Specaccum program was used to calculate Shannon and Chao Biodiversity indexes, implemented for R version 3.2.3.

3 RESULTS AND DISCUSSION

3.1 Respirometry assays

Respirometry assays results showed a decrease in the nitrification rate of 53% the first day and of 100% the 6th day with respect to the initial value (Fig. 2). Despite the decline in nitrifying activity, values of ammonia uptake rate (AUR) and nitrifying rate (Rn) of day 1 were in the range of reference values for nitrifying biomass described in Young and Cowan [18] (Table 2).

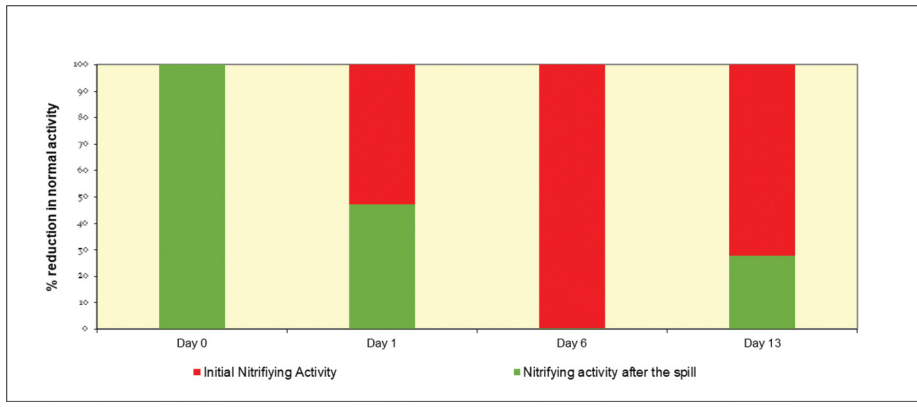

Figure 2: Reduction of the percentage of nitrifying activity.

Table 2: Respirometry assay results.

	Day 0 (before spill)	Day 1	Day 6	Day 13	Optimal range [18]
AUR ($mgNH_4$/g VSS/h)	10.27	4.83	0.03	4.81	2-8
Rn ($mgNH_4$/l/h)	20.03	9.46	0.03	5.58	1.5-6

The addition of ammonium chloride at the beginning of the assay causes a reach in oxygen uptake in days 0, 1 and 13 but no in day 6 after the insecticide addition (green arrow in Fig. 3). After obtaining the Rsmax, allylthiourea was added to inhibit the nitrifying activity as is shown by red arrows in the figure. In day 1 test, the inhibition of nitrifying bacteria does not show a modification in the slope of the respirogram, so we can conclude that ammonia removal is not due to nitrification, but to its use as nutrient. Therefore, the 100% of nitrifying activity disappeared in the first day. After 6 days, the drop of nitrification activity was still the 100%, being AUR and Rn almost 0.03.

After a week, respirometry analysis was repeated, showing a recovery in nitrifying activity and being AUR and Rn values in the optimal range, but still lower than the initial values (Table 2).

3.2 Bacterial community structure

Phylum abundance in mixed liquor samples is shown in Fig. 4 (only the Operational Taxonomic Unit, OTU, with >1% abundance in at least one sample are included). 'No hit' represents the sequencing reads that could not be assigned to any taxa, in this case, to any phylum (4.4–5.1%).

Proteobacteria, Actinobacteria and Bacteroidetes dominated the activated sludge microbiome at the beginning of the study, comprising 51% of the total sequences. Meerbergen et al. [8] and Liang et al. [9] found the same dominant phyla, being Proteobacteria predominant in urban wastewater. Nevertheless, after 7 days of the sludge exposition to the insecticide, the third phylum in relative abundance switched to Firmicutes. Yang et al. [10] described

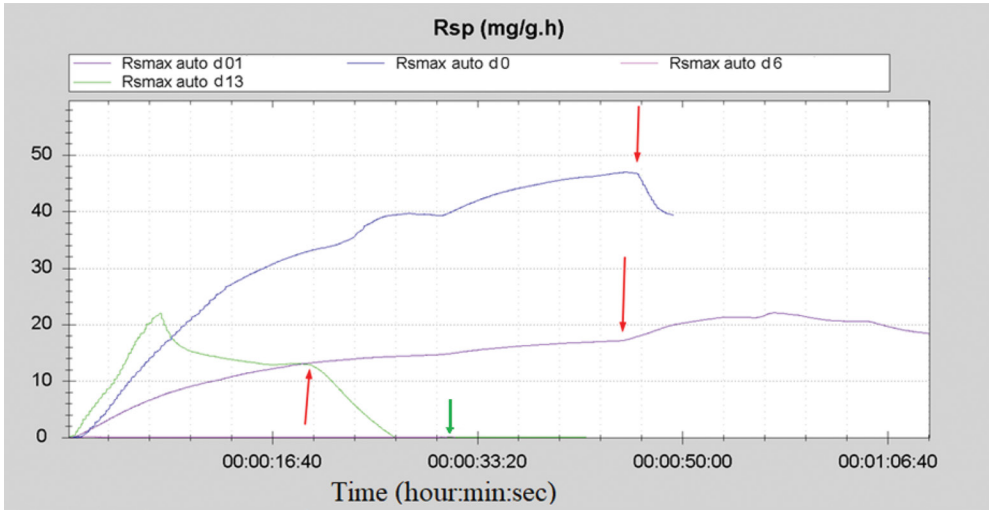

Figure 3: Autotrophic respiration rate. Red arrows indicate allylthiourea addition.

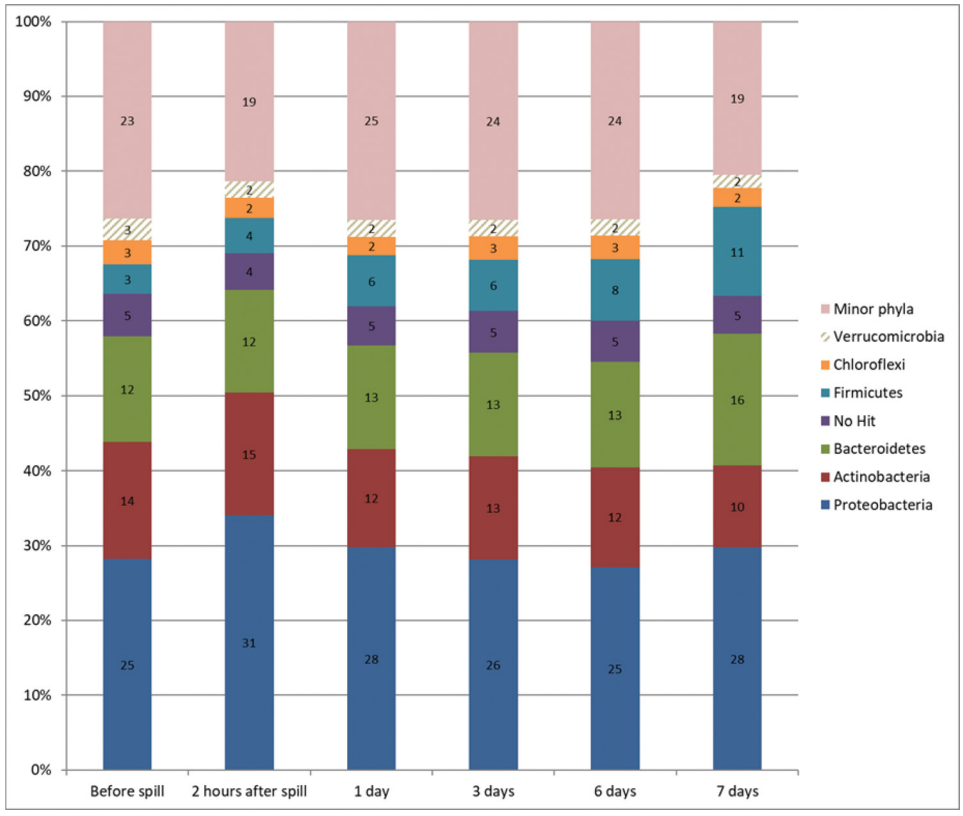

Figure 4: Abundance of OTUs at phylum level excluding taxa with abundance <1%. The abundance is presented in terms of percentage from the total number of bacterial sequences in each sample. Confidence threshold of taxonomical identification was 99%.

Firmicutes as widely distributed in anaerobic sludge treatment systems and, Liu et al. as versatile in degrading a big range of environmental substrates [11]. Also, Bacteroidetes, frequently reported as proteolytic bacteria, involved in degrading proteins [12], increased their relative abundance.

Figure 5 shows the main genera abundance in the samples (taxa represented occurred at >1% abundance in at least one sample). At genus level, between 14% and 15% of the sequencing reads could not be assigned to any taxa ('No hit' in figure), these are significantly lower values comparing with the 30% found by Wang et al. [13] or the 32–34% described in Zhang et al. [14].

The number of OTU detected and calculated species richness Chao1 and Shannon indexes are shown in Table 3. The high Shannon Index values obtained indicate that activated sludge samples have a rich biodiversity. Gonzalez-Martínez et al. [15] described lower Chao 1 and Shannon indexes values in the ten different WWTP studied (Chao 1 1,395,003–441,150 and Shannon index 5.137–2.831). After 2 h of the insecticide contact, values showed a richness decrease. This might be attributable to spill harmful effect.

The denitrifying Flavobacterium were found as a part of community core in sewage plants and have been reported as extracellular polymers producer, so they can act as floc-forming microorganisms [16]. Zhang et al.[14] also reported Flavobacterium as a dominant genus in three WWTP from North America of the 14 samples studied.

Aciditerrimonas and Caldilinea are the following genera in abundance, respectively. Aciditerrimonas is an iron-reducing bacterium as Sideroxydans [17], which is part of the microbiome studied in abundance greater than 1%. Mixed liquor and settled effluent used in the assay come from a WWTP that uses ferric chloride in primary treatment, so that could explain the considerable presence of both genera.

Zhang et al. [14] observed that genera Zoogloea, Dechloromonas, Prosthecobacter, Caldilinea and Tricoccus are present in all the studied WWTPs, nevertheless, the species composition of biomass changed with geographic location. In this study, the same distribution was found, except Dechloromonas, who presented a relative abundance smaller than 1%.

3.3 Genera variation after the spill

In Fig. 6, the genera that raised at least 40% of their relative abundance after the spill are represented. An increase in the relative abundance of a given group may be due to a decrease in predator or competitor groups, or to the growth of that group, or both.

Both, Tetrasphaera and Arcobacter experienced the strongest growth 2 h after the insecticide addition. Arcobacter has been described as dominant genus in influent wastewater of several WWTP [19,20], but they are efficiently removed by activated sludge system [20]. Furthermore, Saunders et al. [21] described Trichococcus and Acinetobacter between the ten

Table 3: Chao 1 and diversity index.

	Before spill	2 h after spill	1 day	3 days	6 days	7 days
Chao 1	2009	1838	2000	1900	1995	1948
Shannon	5,295	5,281	5,414	5,324	5,362	5,240
OTU	954	824	954	891	965	913

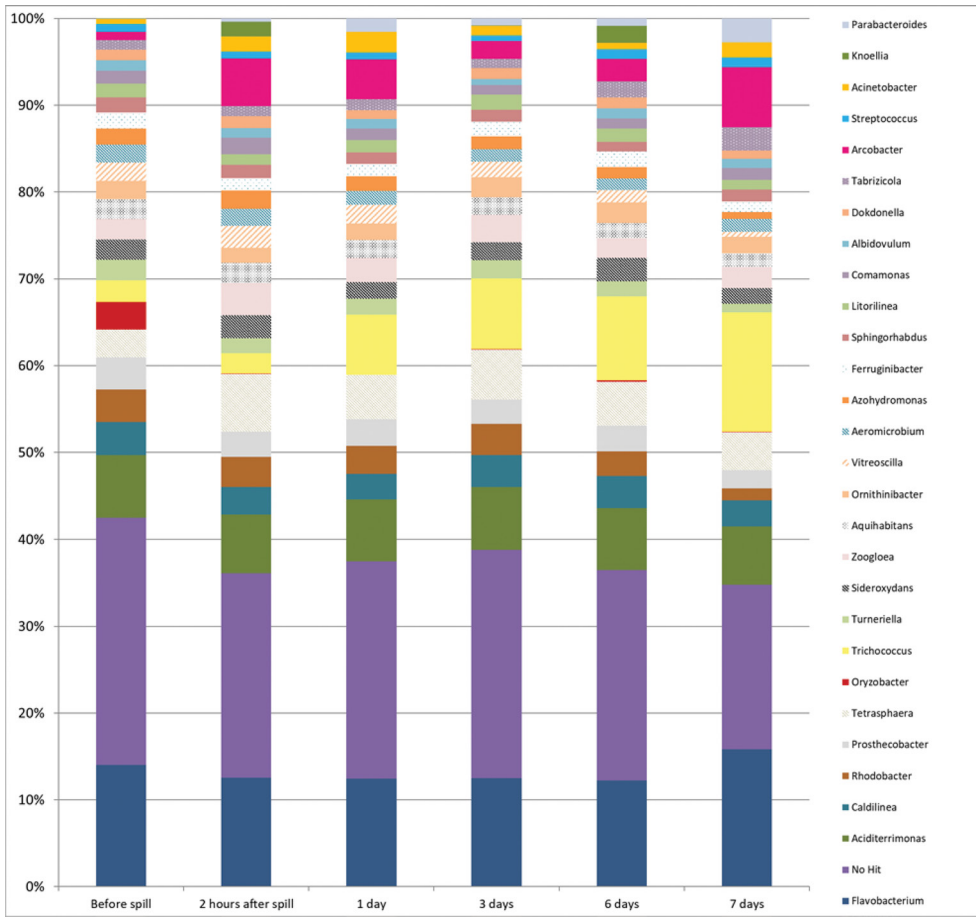

Figure 5: Abundance of genera OTUs >1%, presented in terms of percentage from the total number of bacterial sequences in each sample. Confidence threshold of taxonomical identification was 99%.

genera most abundant in the sewage influent of three WWTP. Growth of Arcobacter, Trichococcus and Acinetobacter could indicate that activated sludge efficiency is being affected by the insecticide, inasmuch as their relative abundance increased quickly at the start of the test, and also after six days from the spill, when a cumulative toxic effect could take place.

Saunders et al. [21] highlighted the high net growth rate of Trichococcus in activated sludge, and its high presence in the influent. In our case, Trichococcus is the most growing genus, specifically represented by the specie *Trichococcus pasteurii*. This genus was described by Zhang et al. [14] as psychrotolerant mesophile at high abundances in the 14 plants studied (between 1.55% and 5.53%) reaching in our case the 8% in the 7th day from the insecticide inoculation.

Both, *Trichococcus pasteurii* and Tetrasphaera genus (mainly *Tetrasphaera elongata* and also *T. vanveenii* and *T. jenkinsii*) have been related with Eikelboom filamentous morphotype *Nostocoida limicola,* causing foaming problems in WWTP [22,23], respectively.

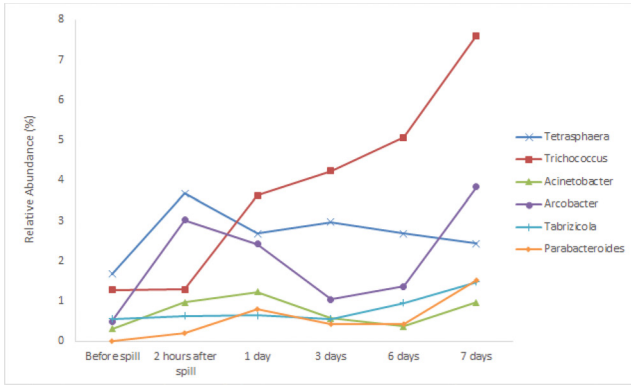

Figure 6: Relative abundance of genera OTUs that increase at least a 40%.

Nostocoida limicola morphotype has been described in industrial plants [24,25,26], indicating that it might be able to adapt to the presence of toxic compounds.

Tabrizicola (represented by the aerobic specie *Tabrizicola aquatica*) increased its relative abundance [27]. Interestingly, Parabacteroides genus, represented by the only specie *Parabacteroides chartae* which is defined as obligatory anaerobic [28], also increased its relative abundance. This may be related to the fact that the increase in relative abundance is mainly due to the lack of predators or competitors, and not to a real growth of the group.

Figure 7 represents genera with at least a 40% of relative abundance decrease. Oryzobacter genera experienced a significant decline only 2 h after the insecticide addition, almost disappearing in the next days (relative abundance <0.016 in day 7). The specie *Oryzobacter terrae* was the only specie of the genus present in the samples. It has been described as a rod-shaped bacterium isolated from rice soil [29]. *O. terrae* seems to be very sensible to the toxic effect of the insecticide.

3.4 Nitrifying genera

Nitrifying activity decreased a 100% after the insecticide addition (Fig. 2). Despite this, respirometry assays results were maintained until the end of the assay, remaining ammonia oxidizing bacteria (AOA) and nitrate oxidizing bacteria (NOB) without noticeable changes (Fig. 7). Only Rhizobium shows a slight variation after 2 h of the spill. According to Wang et al. [13], Rhizobium genera could perform nitrite reduction. All these results point that inhibition could be the cause of nitrifying activity decrease, as nitrifying biomass is maintained despite insecticide occurrence.

Nitrifying bacteria showed low values in all microbiome samples, being usually nitrifying genera abundance below 1%. These low values were previously described by Wang et al. [13].

Nitrosomonas and Nitrosospira are the dominant genera of AOB in WWTPs [30,31]. Nitrosomonas were the dominant AOB in the studied samples, accounting between 0.16% and 0.21% of the total sequences. However, the genus Nitrosospira only accounted 0.004–0.007% of the total sequences.

AOB species most commonly found in activated sludge are *Nitrosomonas europaea, N. eutropha, N. mobilis* and *N. oligotropha* [31]. At the specie level in the present study,

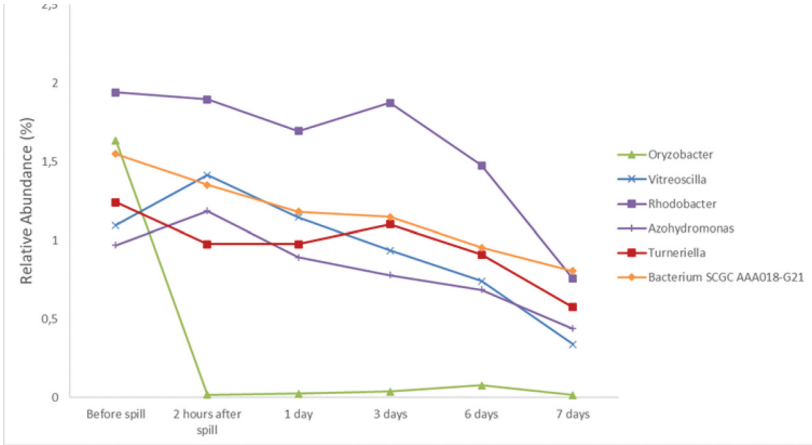

Figure 7: Relative abundance of genera OTUs that decrease at least a 40%. Identification was at a confidence threshold of 99%.

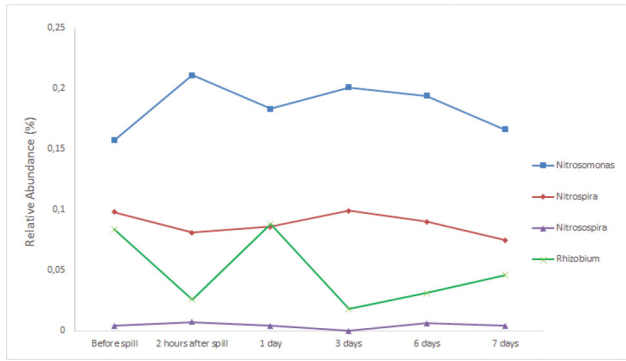

Figure 8: Relative abundance of nitrifying genera OTUs variation after spill (relative abundance <1%).

Nitrosomonas oligotropha was the dominant one, followed by *N. moscoviensis*. Limpiyakorn et al. [32] also reported *N. oligotropha* as dominant AOB in five sewage treatment systems. *Nitrosospira multiformis* was also present.

Dominant NOB were Nitrospira genus, represented by *Nitrospira moscoviensis*. Neither representative of the genus Nitrobacter nor Nitrotoga were found.

4 CONCLUSIONS

This study analysed changes in the microbial community structure of an activated sludge system at bench-scale caused by an artificial insecticide spill by means of metagenomic analysis.

Initially, Proteobacteria, Actinobacteria and Bacteroidetes were dominant at phyla level, but the spill switched the distribution of the sludge community, replacing Firmicutes to

Actinobacteria as the 3rd most abundant phylum. Species richness decreased in the first 2 h but only in short term.

Respirometry analysis shown a 100% decline of nitrifying activity. However, nitrifying genera OTUs did not change substantially. These results point that although certain genera are present in activated sludge, they could be inhibited, so it becomes necessary to use techniques like the respirometry to diagnosticate the process status. On the other hand, microbiome analysis also provides useful information. Knowing about the existence of nitrifying species in the community whose abundance has not been affected, can help to recover the process (by increasing oxygen levels or sludge age) without resorting to reseeding.

The increase in relative abundance of Arcobacter, Trichococcus and Acinetobacter genera in activated sludge after the contact with the toxic, could evidence a disorder in the purification performance capacity of the activated sludge, since all these genera are described as abundant in wastewater influents. In the case of Trichococcus, this increase may also be related to its ability to grow in active sludge. Furthermore, Oryzobacter genus, represented by *Oryzobacter terrae*, appears as very sensitive to the toxic effect of spill, almost disappearing after 2 h of the insecticide exposition. These results indicate that Arcobacter, Acinetobacter and Oryzobacter could be indicators of a negative affection in the biological process studied.

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