

EVOLUTION OF THE ACTIVATED SLUDGE COMMUNITY OF A WASTEWATER TREATMENT PLANT WITH INDUSTRIAL DISCHARGES

ÁNGELA BAEZA-SERRANO¹, FELIU SEMPERE¹, NURIA OLIVER¹,
PILAR GUTIÉRREZ² & GLORIA FAYOS¹

¹Global Omnium Medioambiente, S.L., Spain

²Explotaciones Hidricas del Cinca, SA, Spain

ABSTRACT

Stability and high microbial activity of the biological processes in wastewater treatment plants (WWTPs) based on activated sludge processes, is a key factor for the correct treatment of wastewater prior to its discharge into the environment. This can be a challenge, as WWTPs are obliged to treat all the wastewater they receive, including possible uncontrolled discharges, some containing toxic substances or poorly balanced loads to the microorganisms in charge of carrying out the wastewater treatment. In fact, the effects of these pollutants on biological treatment by activated sludge include inhibition of bacteria in the removal of organic compounds and nutrients, reduction of the efficiency of solids separation and modification of the compaction properties of the sludge, effects that can have a negative impact on the treatment processes and on the quality of WWTP effluents. The aim of this study is to describe the composition and changes of the bacterial community in a Spanish full-scale activated sludge WWTP which suffers seasonal discharges from the vegetable canning industry during a year.

Keywords: activated sludge, metagenomic analysis, industrial discharges, bacterial community.

1 INTRODUCTION

There are more than 30,000 urban wastewater treatment plants (WWTPs) in the EU [1], including 80% of them secondary treatment using activated sludge. In countries from south east Europe, such as Romania and Bulgaria, this percentage is much lower, due to the problems associated to maintaining the biological process. The activated sludge is a biological process consisting in a bacterial culture growing in an aerated reactor. This culture is able to metabolize organic compounds, nutrients (nitrogen and phosphorus) and other substances present in wastewater at given design loads. The produced sludge sediments and approximately 90% is recirculated back to the bioreactor to maintain the process biomass. The remaining 10% is separated in a settler and removed from the system to maintain the solids concentration constant.

Even though there are important economic investments for the creation and renovation of urban WWTP [2], the increase of the urban wastewater toxic load hinders the treatment. During the year, the entry of spills greater the load which cannot be eliminated by the bacterial culture. Uncontrolled discharges containing harmful substances or high loads can damage the biological units, reducing the cleaning capacity of the bacterial culture or creating nutritional imbalances that favors the growth of filamentous morphotypes that cause sludge separation problems such as bulking or foaming. These discharges are often seasonal and industrials coincide with campaigns such as the pouring of wine or canning companies.

In the vegetable canning sector, approximately 70%–80% of the water consumption is discharged as wastewater and the remaining 20%–30% is incorporated into the product or lost in evaporation. In most cases, work is done on a seasonal basis, taking advantage of the availability of different raw materials throughout the year. In each season, different vegetables are used, sometimes requiring different manufacturing stages and therefore producing a significant change in the levels of water consumption as well as in the



characteristics of the wastewater generated. Regarding the pollutant load of these discharges, it is basically composed of organic matter (biochemical oxygen demand at 5 days, BOD5 and chemical oxygen demand, COD) and suspended solids (SS), and sometimes there are also discharges with high conductivity and variable pHs depending on the cleaning processes or if alkaline peeling is used. Contamination levels increase significantly in operations such as blanching and cleaning of facilities. In blanching, the water is loaded with organic matter, COD, due to the dissolution of substances such as sugars, starches and soluble organic products from the vegetables. Depending on the raw material used, these are the levels of contamination that can be found in the discharge are between 600–12,000 mg/l COD and 100–3,000 mg/l SS [3].

The wastewater from the municipality of Binaced (Fig. 1) is a double-stage system treating 1,445 p.e. with an average flow of 600 m³/day. Wastewater is treated in a 190 m³ integrated fixed-film activated sludge (IFAS) system equipped with a reagent dosing system. The water then passes into the biological reactor with a volume of 711 m³, from where it is distributed to the secondary settling tanks.



Figure 1: Binaced WWTP (Huesca, Spain).

A percentage of the sludge extracted from the secondary settling tanks is recirculated to the IFAS system, while the remaining portion is pumped to the thickeners, from where it is finally dewatered and destined for agricultural application.

The influent received by the Binaced WWTP is largely conditioned by the campaign of the vegetable cannery industry in the municipality, since during the period July–December

both the organic load and the flow received by the facility increase. These industrial inputs vary depending on the year and the evolution of the harvests, but they tend to follow a similar pattern every year: July–September canned peach, September–October canned fruit cocktail and November–December canned pear.

This kind of discharges are characterized by a high COD load, causing a nutritional imbalance because sufficient nutrients are required to degrade organic matter and satisfy bacterial requirements both for growth and good floc formation. Moreover, nutrient limitation has been linked to the filamentous microorganisms growth causing poor settling [4].

Binaced WWTP influent commonly presents imbalances between organic matter and nutrients during these incidents, with the addition of urea and/or phosphoric acid being necessary to achieve the desirable ratio of degradable organic matter to available nutrients, BOD:N:P ratio, of 100:5:1 [5].

In this paper, a study of the discharges effects in mixed liquor microbiome of biological reactor of Binaced WWTP is presented. Full-scale studies are important since the selective pressures are likely very different compared to in small-scale systems.

2 MATERIALS AND METHODS

Monthly samples of 5 ml were taken in three different points of biological reactor and were immediately frozen for their transport on dry ice to ADM-Lifesequencing S.L. laboratory (Paterna, Valencia, Spain). During the month of April, it was not possible to take samples because the biological reactor had to be emptied for maintenance work.

Genomic DNA was extracted from 2 mL of the mixed activated sludge samples with Qiaamp Power Fecal Mini kit (Qiagen) with enzymatic lysis and mechanic disruption. DNA were amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). In brief, the first amplification step, primers were designed containing: a universal linker sequence allowing amplicons for incorporation indexes and sequencing primers by Nextera XT Index kit (Illumina); and 16S rRNA gene universal primers [6] and in the second and last amplification indexes were included. Libraries were quantified by fluorimetry using Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher) and pooled before to sequencing on the MiSeq platform (Illumina), configuration 300 cycles paired reads. 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 hours. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument.

For massive sequencing, the hypervariable region V3–V4 of the bacterial 16s rRNA gene was amplified using key-tagged eubacterial primers [7] and sequenced with a MiSeq Illumina Platform, following the Illumina recommendations.

The resulting sequences were split taking into account the barcode introduced during the PCR reaction, while R1 and R2 reads were overlapped using PEAR program version 0.9.1 [8] providing a single FASTQ file for each of the samples. Quality control of the sequences was performed in different steps, (i) quality filtering (with a minimum threshold of Q20) was performed using fastx tool kit version 0.013, (ii) primer (16s rRNA primers) trimming and length selection (reads over 300 nts) was done with cutadapt version 1.4.1 [9]. These FASTQ files were converted to FASTA files and UCHIME program version 7.0.1001 was used in order to remove chimeras that could arise during the amplification and sequencing step. Those clean FASTA files were BLAST [10] against NCBI 16s rRNA database 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) in order to annotate each sequence at different phylogenetic levels (Phylum, Family, Genus and Species).

3 RESULTS AND DISCUSSION

3.1 Bacterial community structure

In total, 215,501 effective sequences of the 16S rRNA gene were generated from 11 samples, similar that those found by Hu et al. [11] (202,968 in 16 samples) that widely represented the diversity of the microbial communities.

The calculated species richness Chao1 and Shannon index are shown in Table 1. Gonzalez-Martínez et al. [12] studied 10 different wastewater treatment systems from Spain and Netherlands and found Chao 1 and Shannon index between 1395,003 and 441,150 and 5.137 and 2.831 respectively, lower than those found in our study. High Shannon index values obtained point that activated sludge ecosystems are very diverse.

Table 1: Chao 1 Richness Estimation and diversity index.

Month	Shannon value	CHAO1
February	5,429	2012
March	5,366	2001
May	5,321	2647
June	5,435	2497
July	5,299	2104
August	4,989	2641
September	5,145	2724
October	5,378	2542
November	5,135	2465
December	5,443	2967
January	5,466	2886

Fig. 2 shows the heat map of core genera relative abundance in activated sludge samples during a year. Taxa represented occurred in a great than 1% relative abundance in at least one sample. Between 4.79% and 15.39% of the sequencing reads could not be assigned to any taxa at genera level. These “no hit” are lower values comparing with the 30% found by Wang et al. [13] or the 32%–34% described in Zhang et al. [14].

OTU genera with relative abundance in less than 1% were between 59.57% and 47.56%.

Significant values of the denitrifying *Flavobacterium* were found at the first half of the year. *Flavobacterium* was previously described as a part of community core in sewage plants and reported as floc-forming microorganisms [15]. Zhang et al. [14] also reported *Flavobacterium* as a dominant genus in three WWTP from North America of the 14 samples studied. However, a drop in its abundance is observed coinciding with the start of the peach canning season in July, and with discharges with high pH. A Similar drop is observed for *Thermomonas*, which has been described as part of core members of the denitrifying communities in full-scale WWTPs too [16], and the denitrifying *Rhodoferrax* and *Ferruginibacter*, coinciding also with a decrease in COD elimination efficiency detected after a high Conductivity discharge on 18 July (Fig. 3).



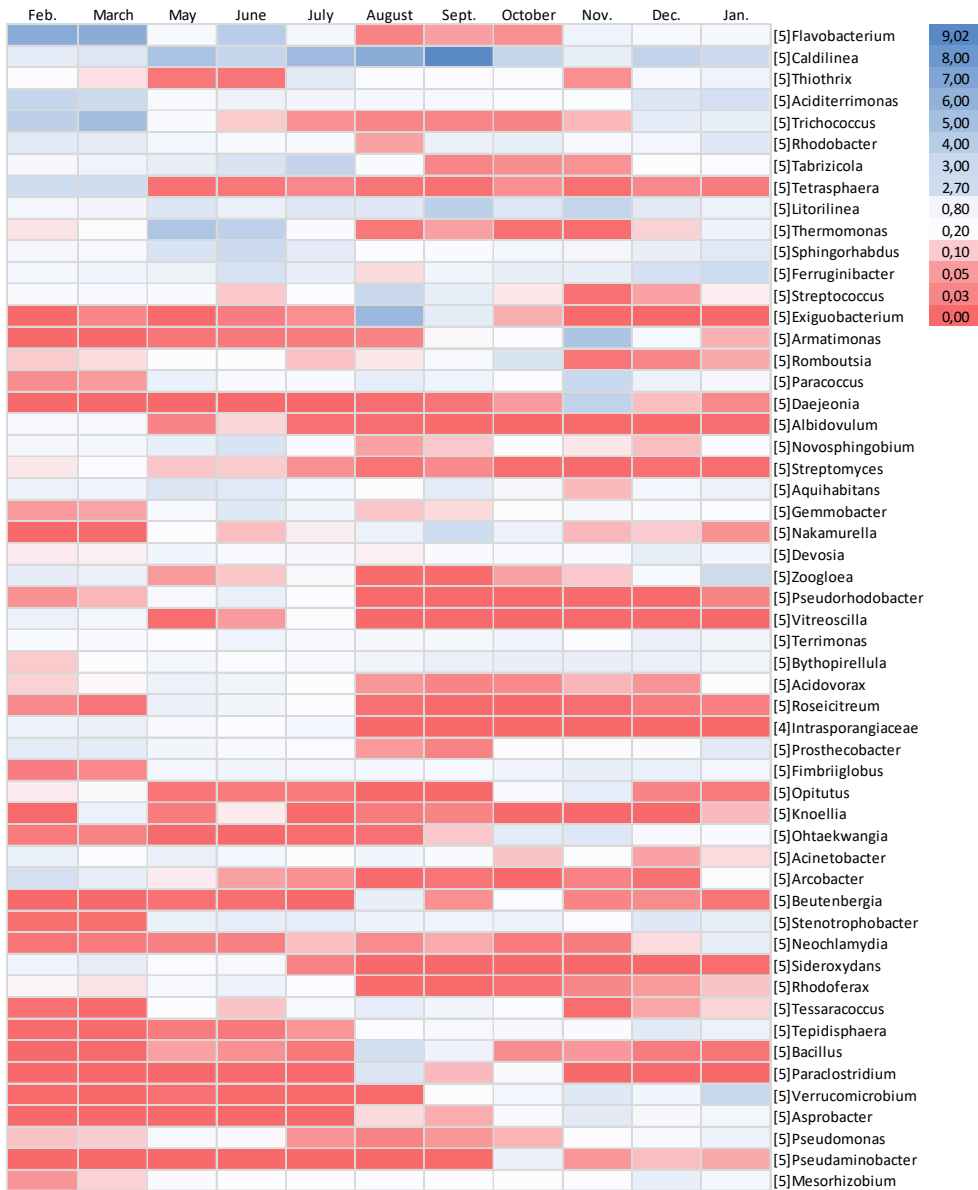


Figure 2: Heat map of abundance of genera OTUs > 1% in at least one sample, presented in terms of percentage from the total number of bacterial sequences in each sample. Confidence threshold of taxonomical identification was 99%.

On the other hand, the relative abundance of *Exiguobacterium* genus increased in August, after the start of the campaign mentioned above. *Exiguobacterium* has been described as thermotolerant, also, capable of growing within a wide range of pH values (5–11) and

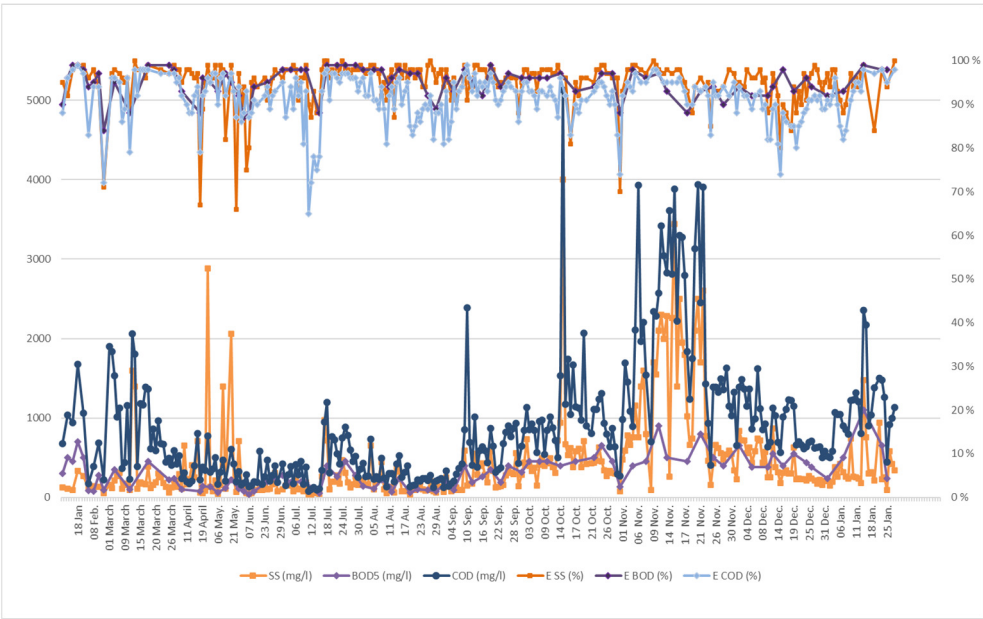


Figure 3: Evolution of organic matter and SS input and removal efficiency.

alkaliphilic, tolerant to high levels of UV radiation, and heavy metal stress (including arsenic) [17]. Some genera like *Stenotrophobacter* (represented by the only specie *S. terrae*), *Fimbrioglobus* and the denitrifying *Tepidisphaera* seem to be increasing their relative abundance coinciding with rising temperatures in May (Fig. 4).

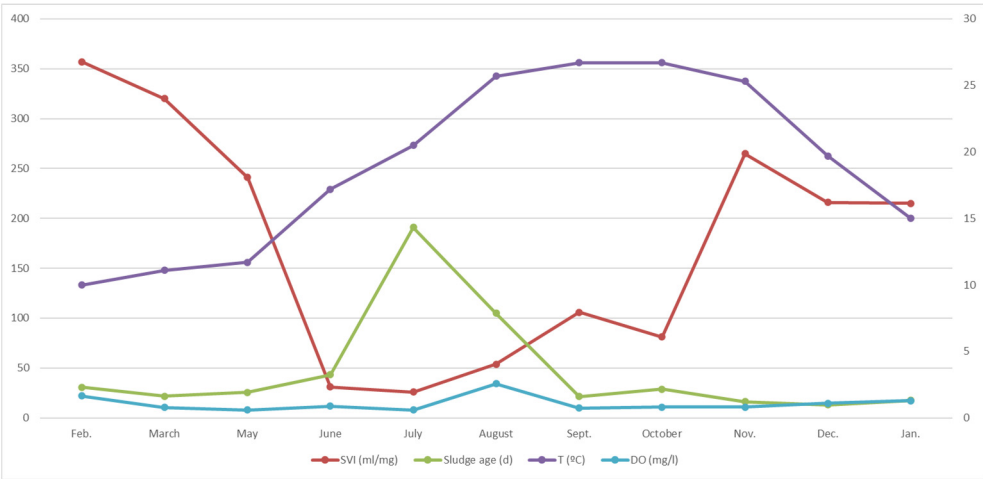


Figure 4: Evolution of temperature, sludge volume index, sludge age and dissolved oxygen in biological reactor.



With increasing COD in November (Fig. 3), fermentative acidogenic bacteria such as *Opiritatus*, which is associated with the use of moderately recalcitrant heteropolysaccharides such as xylan and pectin [18], both present in fruit, also increase. *Armatimonas*, on the other hand, also increased its relative abundance. They have been described in carbon removing plants, typically of high load with a large input of industrial wastewater. They grow on a limited range of substrates, such as sucrose, raffinose, gentiobiose (present in peach stones) and pectin [19].

According to Wang et al. [13] there were 60 genera of bacterial populations commonly shared by all samples, including *Ferruginibacter*, *Prostheco bacter*, *Zoogloea*, Subdivision 3 genera incertae sedis, Gp4, and Gp6, indicating that there is a core microbial community in the microbial populations of WWTPs at different geographic locations. Both, *Ferruginibacter* and *Zoogloea*, as well as *Prostheco bacter* were found at abundances greater than 1% (Fig. 2).

3.2 Evolution of nitrifying genera

High biodiversity of nitrifying bacteria was detected. Ten species of Ammonia Oxidizing Bacterium (AOB) were found, including the genera *Nitrosomonas*, *Nitrosococcus* and *Nitrospira*, being *Nitrosomonas oligotropha* the most abundant (with relative abundances between 0.641% and 0.039%). Regarding Nitrite oxidizing bacteria (NOB), seven species were detected, *Nitrospira moscoviensis* was the most abundant (0.348%–0.007%), but also representatives of *Nitrobacter*, *Nitrolancea* and nitrite reductor *Rhizobium* [13] were found.

AOB bacteria represented between 0.200% and 0.006% of samples relative abundance and NOB between 0.523% and 0.122%. Zhang et al. [14] indicated that AOB occupied 0%–0.64% of the total pyrosequencing sequences from six municipal WWTPs, and *Nitrosomonas* dominated in the WWTPs. Kim et. al. [20] described similar NOB amount (0.06% of *Nitrospira*-like nitrite-oxidizing bacteria) in suspended activated sludge of an IFAS system, and suggested that the suspended biomass contributed more to nitrification than did the attached biomass in a IFAS system.

Fig. 5 shows nitrifying bacteria evolution during the year. An increase in relative abundance is observed in July, with a subsequent decline in abundance, interestingly coinciding with the nitrogen removal efficiency loss (Fig. 6). The increase of abundance in May and June could be related to higher temperatures and sludge ages (Fig. 4).

3.3 Evolution of microorganisms causing bulking and/or foaming

High SVI were detected during February, March and again in November (Fig. 4). In the first months, two genera related with *Nostocoida limicola*-like filamentous morphotype were found: *Trichococcus* and *Tetrasphaera*, both related with low temperatures [19].

Caldilinea is a gram-negative and facultatively aerobic bacteria which is able to grow both aerobically and anaerobically (fermentatively), and is phenotypically recognized as Eikelboom's morphotype 0803 in activated sludge [21]. This genus was detected at high relative abundances from May to October, probably related to high temperatures (Fig. 4), and then increase again in December, maybe influenced by the numerous discharges recorded during November.

Also during November, the biodegradability of the sewage decreased by the presence of pear cannery discharges. This could be related with the drop of bulking forming bacteria *Thiothrix*, which is described as a consumer of rapidly biodegradable substrates such as volatile fatty acids.



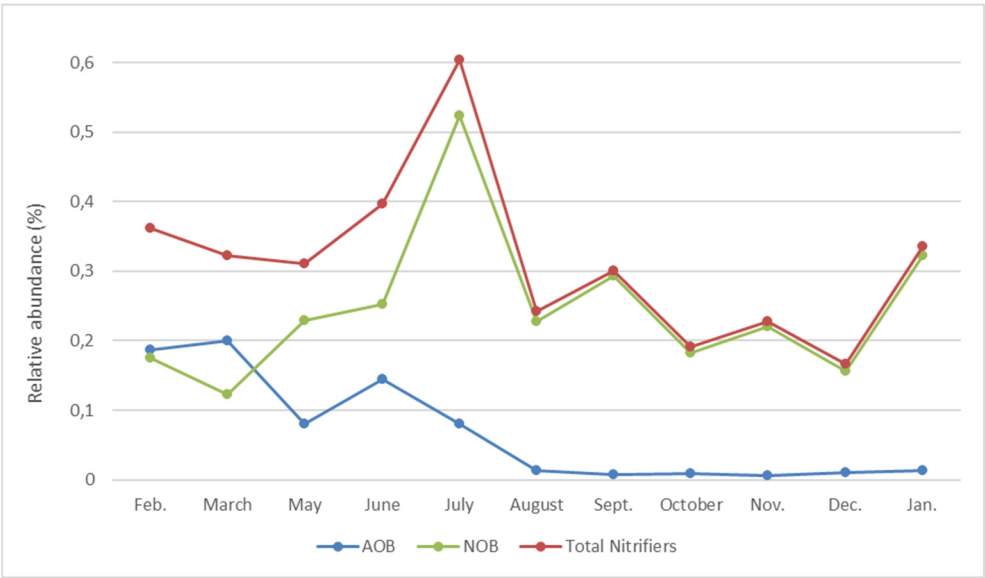


Figure 5: Abundance of nitrifying genera OTUs presented in terms of percentage from the total number of bacterial sequences in each sample. Confidence threshold of taxonomical identification was 99%.



Figure 6: Nitrogen and phosphorus removal efficiency.

4 CONCLUSIONS

This study describes the composition and changes of the bacterial community in a full-scale activated sludge WWTP suffering seasonal discharges from the vegetable canning industry during a year.

Fifty-four genera were found in high relative abundance at least one sample, accounting between 40% and 27% of total genera.

Certain denitrifying genera like *Flavobacterium*, *Thermomonas*, *Rhodoferrax* and *Ferruginibacter*, decreased coinciding with the start of the peach canning season and with discharges with high pH.

Certain bacteria related to the use of sugars present in the fruit and its stones, such as *Opitutus* and *Armatimonas*, significantly increase their relative abundance during the canning seasons.

On the other hand, the relative abundance of the alkaliphilic genera *Exiguobacterium* increased after the start of the peach cannery campaign.

Some genera like *Stenotrophobacter* and the denitrifying *Tepidisphaera* increased their relative abundance coinciding with rising temperatures.

Nitrifying bacteria do not seem to be affected by fruit cannery campaigns but are affected by discharges with high pH and conductivity.

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