

# Ozonation effects for excess sludge reduction on bacterial communities composition in a full-scale activated sludge plant for domestic wastewater treatment

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## Abstract

Activated sludge process is the most widely diffused system to treat wastewater to control the discharge of pollutants into the environment. Microorganisms are responsible for the removal of organic matter, nitrogen, phosphorous and other emerging contaminants. The environmental conditions of biological reactors significantly affects the ecology of the microbial community and, therefore, the performance of the treatment process.

In the last years, ozone has been used to reduce excess sludge production by wastewater treatment plants (WWTPs), whose disposal represents one of the most relevant operational costs. The ozonation process has demonstrated to be a viable method to allow a consistent reduction in excess sludge. This study was carried out in a full-scale plant treating municipal wastewater in two parallel lines, one ozonated in the digestion tank and another used as a control. Bacterial communities of samples collected from both lines of digestion tanks were then compared to assess differences related to the ozonation treatment. Data were then analysed with terminal restriction fragment length polymorphism (T-RFLP) analysis on 16S rRNA gene. Differences between bacterial communities of both treated and untreated line appeared 2 weeks after the beginning of the treatment. Results demonstrated that ozonation treatment significantly affected the activated sludge in WWTP.

**Keywords:** ozonation; wastewater treatment; T-RFLP; NMDS; 16S rRNA

## Introduction

Biological treatments are well-known practices to remove pollutants from civil and industrial wastewater using microbial metabolism. The biological processes are based on the transformation of dissolved and suspended nutrients into new biomass (sludge), soluble residuals and gaseous products. The excess sludge, before its final disposal or reuse, has to be regularly removed and treated. This process is performed by the wastewater treatment plant (WWTP) and represents more than 50% of the total operating costs for wastewater treatment.[1,2] The very high costs required for the reduction of the excess sludge demand for the development of new strategies.[3,4] Ozonation is one of the most studied and viable methods.[5–7]

Ozone (O<sub>3</sub>) is a strong oxidant ( $E=2.07$  V) commonly used in tertiary treatment of industrial wastewater [8] (e.g. for oxidation of recalcitrant compounds [9]) as well as in disinfection treatment of drinking water,[10] organic compounds oxidation and also for municipal wastewater treatment.[11] At low dosage, ozone destroys the cell wall of bacteria both in suspension or on the surface of flocks, causing their lysis.[12–15] At higher dosage, ozone causes the destruction of flocks, directly acting on the bacterial extracellular polymeric matrices that plays a crucial role in bacteria aggregation.[16] Moreover, ozone contributes to the oxidation of the dissolved

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organic material after bacterial cell lysis, and it improves sludge sedimentation,[17] allowing the control of bulking and foaming phenomena.[18] Up to now, two different approaches have been proposed for excess sludge reduction through ozonation: (1) reduction of bacterial growth and (2) induction of bacterial cell lysis processes.[13,14] Ozonation treatment can be applied at different steps of the treatment process, for instance, in the sludge recirculation line or in the sludge digestion tank.[19] Despite the effect of ozone treatment for the reduction of biological sludge has been already widely described in literature, no study has yet been carried out on the monitoring of the effects of ozonation on bacterial communities present in the WWTP.

The aim of this study is to assess the effect of ozonation treatment on bacterial community in a sludge digestion tank of a full-scale WWTP. This study was carried out by terminal restriction fragment length polymorphism analysis (T-RFLP). The effects on the bacteria are analysed in the recirculation loop of a full-scale aerobic digestion unit in the presence and in the absence of the ozonation treatment.

## Materials and methods

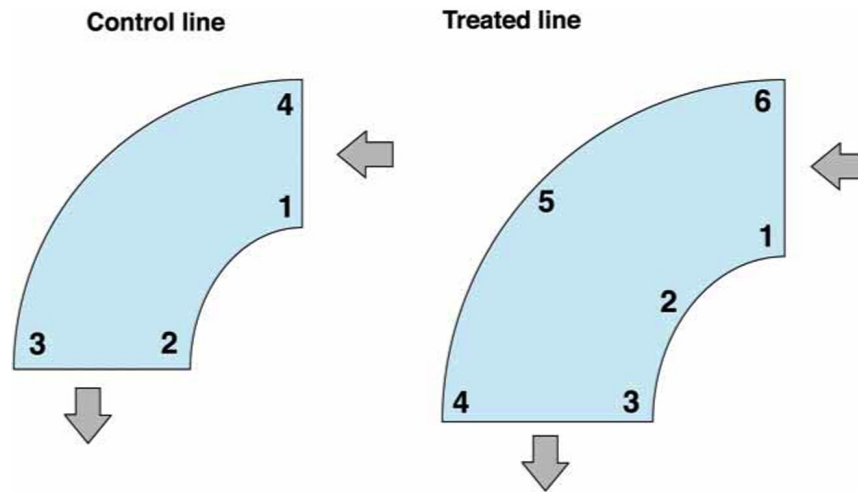
### Description of the study area

The municipal WWTP, managed by Acqualatina S.p.A. and located in Sabaudia (Latina, Italy), is a conventional activated sludge plant consisting of pre-treatments (fine bar screen, sand removal and degrease), pre-denitrification, oxidation–nitrification, secondary settling, aerobic digestion, sludge thickening and belt-press dryer. Only in case of emergency, disinfection occurs by chlorination. The plant is divided into two parallel lines, respectively, characterized by 10,000 population equivalent (PE) in ‘line 1’ and 20,000 PE in ‘line 2’. The municipal WWTP receives wastewater from the touristic area of Sabaudia, whose population varies from a minimum of 10,000 inhabitants during the winter season to a maximum of 30,000 inhabitants during the summer season. The touristic nature of this area is responsible for wide variations in the influent flow rate, ranging from 3500 to 4500 m<sup>3</sup>/d in winter and up to 6000 m<sup>3</sup>/d during summer. The description of the treatment of excess sludge by ozonation has been already described.[20] In this paper, we report the ozone dosage both to the concentration of total suspended solids (TSS) in the WAS, which corresponds to the content in the influent to the digester unit (TSS<sub>in</sub>), or to the TSS concentration measured inside the digester unit (TSS<sub>0</sub>). For this reason, TSS concentration variation could determine increase or decrease in specific dosage concentration even if the applied ozone flow rate was the same.

### Collection of samples

Samples were collected from February 2010 to July 2010 in the aerobic sludge digestion tank both in 10,000 PE line (representing the negative control with no ozone treatment) and in 20,000 PE line (the ozone treated line). We made four samples collection in the following days: 22nd February, 9th March, 25th May and 20th July.

Figure 1 represents the scheme followed to collect samples from the two tanks: four samples were collected from the control line at each sampling day described above and six samples from the line with ozone treatment, for a total of twenty samples during the whole study period. Samples were collected at 1-m depth in sterile plastic bottles (2-L capacity).



**Figure 1.** Sample collection scheme in the two tanks.

#### DNA extraction and T-RFLP analysis

Total DNA extraction was performed from all samples using a Soil master<sup>™</sup> DNA extraction kit (Epicentre Biotechnologies, WI, USA). 16S rRNA genes were directly amplified from extracted DNA, using polymerase chain reaction conditions as already described.[21] Universal bacterial primers were used: 8F (5'-AGA GTT TGA T(CT)(AC) TGG CTC AG-3') and reverse 1492R (5'-GG(AGCT)(AT)AC CTT GTT ACG ACT T-3').[22] Primer 8F was labelled with two different dyes (Applied Biosystems, CA, USA): 6-FAM and VIC. The template was digested either with the restriction endonuclease *Bsu*RI (GG<sup>^</sup>CC, 0.2 u/μl, Fermentas, Canada) or with the restriction endonuclease *Rsa*I (GT<sup>^</sup>AC, 0.2 u/μl, Fermentas). The use of more than one restriction endonuclease in T-RFLP approach is recommended for a better resolution of the experiment.[23] After restriction, DNA was precipitated by centrifugation with cold 100% ethanol in a 5424 R refrigerated centrifuge (Eppendorf, Italy) at 4°C and 10,000 RCF, to eliminate salts. T-RFLP was performed as previously described.[24] Each reaction was performed in a mixture containing 1.2 μl of loading buffer (GeneScan<sup>™</sup> 600 LIZ, Applied Biosystems), a maximum of 5.5 μl of sample at a concentration range of 0.2–1 μg/μl (the volume of samples was calculated on the basis of its final concentration after cold ethanol precipitation) and 13.3 μl of deionized formamide (Applichem, Germany). Capillary electrophoresis was performed with Abi Prism 310 Genetic Analyser (Applied Biosystems); T-RFLP profiles were analysed using GeneScan<sup>™</sup> analysis software (Applied Biosystems). T-RFLP data matrix was transformed for statistical analysis as described.[25]

#### Statistical analysis on T-RFLP data set

All the statistical analyses were performed using PAST software v.2.15.[26] A cluster analysis (CA) using Ward's method and 100 bootstrap value was performed to evaluate which groups of samples clustered together. Non-metric multidimensional scaling (NMDS) was also performed on the whole data set using the Bray–Curtis coefficient, which represent the best statistical analysis to evidence non-homogeneous data.[27] The quality of the data set was assessed through the Shepard plot (where ideally, all points should be placed on a straight ascending line,  $x=y$ ) and through the stress value calculation. A stress value  $>0.2$  indicates that the plot is close to random, a stress value lower than 0.2 indicates a useful two-dimensional picture and a stress value lower than 0.1 corresponds to an ideal ordination.[28] Two different diversity indices were also calculated to compare species diversity among samples on the normalized T-RFLP data matrix, containing profiles from both the restriction enzymes and assuming that each T-RF corresponds to one operational taxonomic unit [29]:

- Simpson's diversity index [30]  $D_s = 1 - \sum p_i$ , with  $p_i$  representing the population of the  $i$  species.
- Shannon's diversity index [31]  $H = -\sum p_i \ln p_i$ , with  $p_i$  being the proportion of the  $i$  species relative to the total number of species.

The Simpson's index gives the probability that two randomly selected individuals drawn from a population  $p_i$  may belong to the same species  $i$ . It measures the 'evenness' of a community ranging from 0 to 1.

The Shannon index varies from 0 in communities with only a single taxon, to higher values in communities with more taxa, each one having a few individuals.

## Results

### Ozonation effect on sludge reduction

Profiles of TSS and volatile suspended solids (VSS) measured in the influent and effluent of the line 2 digester as a function of time and of the dosed ozone flow rate were previously reported by Chiavola *et al.* [20; Figure 1, p. 659]. The concentration of both TSS and VSS in the influent of line 2 digestion tank was fairly constant both during the first and second period of operation, whereas a significant increase was registered in summer, probably as a consequence of the higher influent organic load. The VSS/TSS ratio in the outlet from the ozonated digestion tank decreased continuously from about 0.79 to about 0.74.

For both TSS and VSS, the reduction efficiency measured in the ozonated digester was very low in the first period, but then increased appreciably when the ozone dosage was increased.[20]

Table 1 reports the average TSS and VSS removal (indicated with  $\Delta$ ) measured in both the digesters during the experimental periods. It can be noted that in the line 1 digester (control), the percentage reductions were always very low. The low values observed during the first period were nevertheless higher than the efficiency measured in the line 1 in the same period: in this case, the specific ozone dosage was still too low to determine a significant improvement in cell oxidation process, but it enabled to provide the additional oxygen that was required to promote sludge stabilization. In the third operational period, the value decreased.

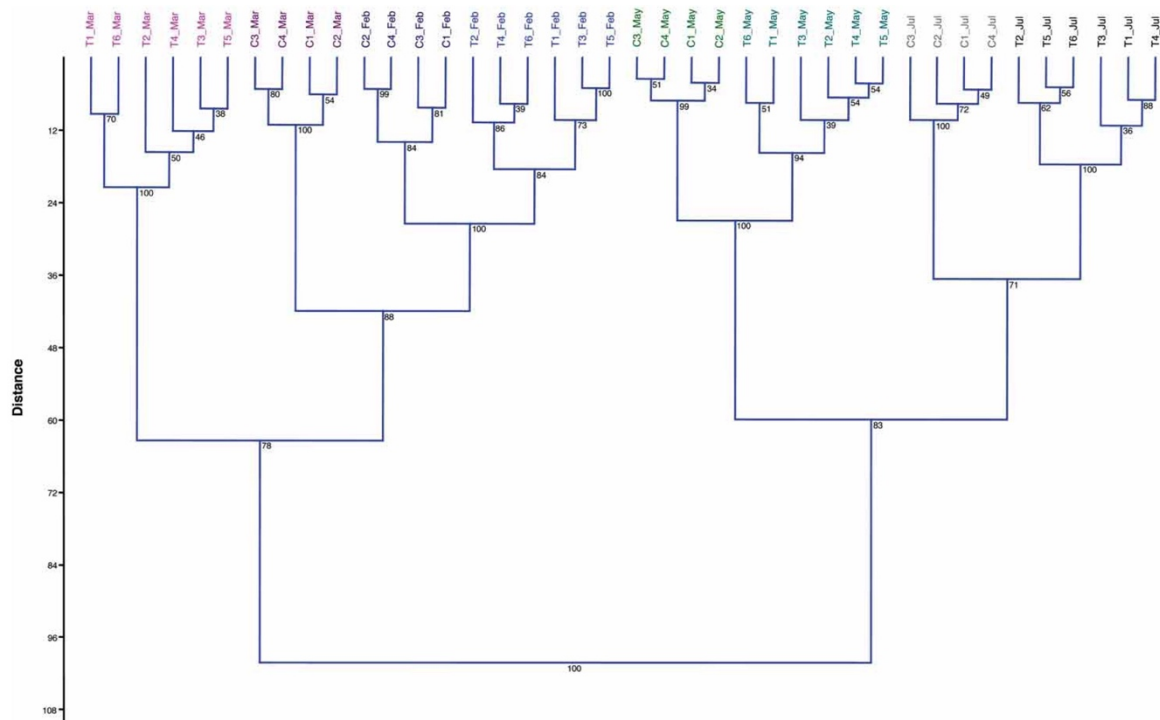
**Table 1.** Average monthly TSS and VSS percentage reductions in the digesters of line 1 (control) and line 2 (ozonated).

Period	O <sub>3</sub> Dosage (gO <sub>3</sub> kg <sup>-1</sup> TSS <sub>0</sub> )	Line 1		Line 2	
		$\Delta$ TSS (%)	$\Delta$ VSS (%)	$\Delta$ TSS (%)	$\Delta$ VSS (%)
1 (February to March)	0.15	0	0	0.2	1.4
2 (April to June)	1.4	0	0	19.5	22.6
3 (July to August)	1.23	1.1	1.4	9.5	12.4

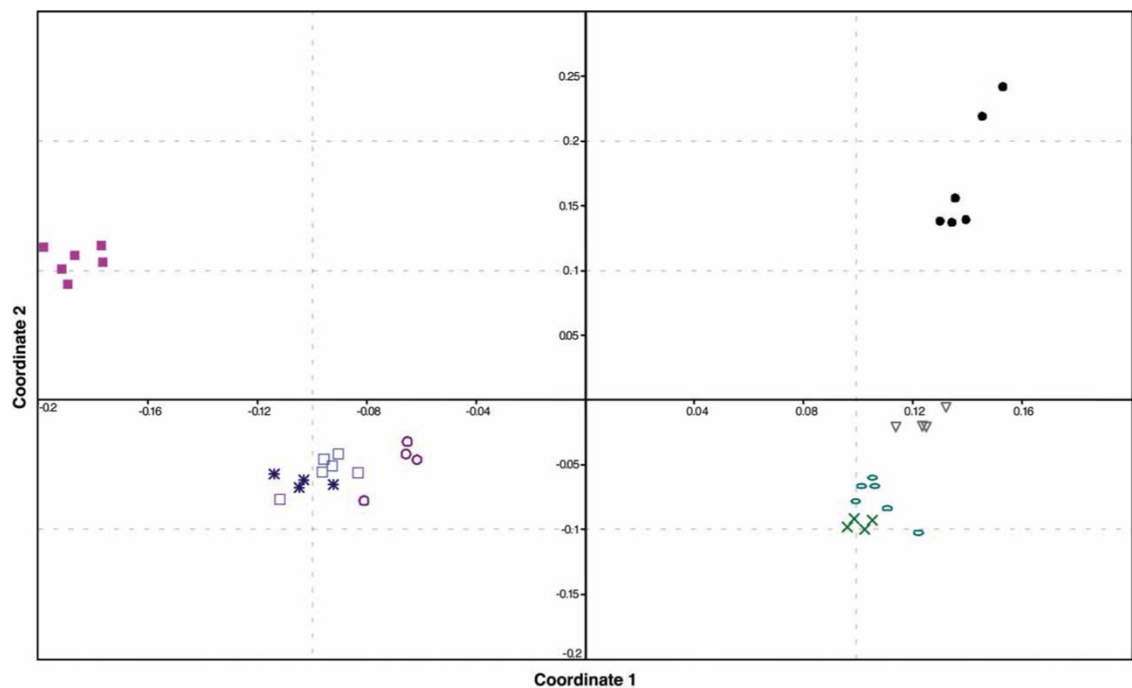
### Ozonation effect on microbial community

A cluster analysis (CA) made with Ward's method and 100 bootstrap on the whole T-RFLP data set is shown in Figure 2. The replicates of samples collected in the same tank during each sampling time clustered together in all cases with high bootstrap value support (84–100). Samples of the treated line (indicated as T in Figure 2) collected in February, before the onset of the ozonation treatment, clustered with samples of the not treated control line (indicated as C in Figure 2) collected in the same sampling time, with 100 bootstrap support. This clade, composed by control and treated samples collected in February, grouped with samples collected in the

control line in March (88 bootstrap support). Samples collected in the treated line in March were instead different from the control line collected at the same time, and they were placed in a basal position in the clad, with 78 bootstrap support. Samples collected in May showed a strong association between the control line and the treated one (100 bootstrap), whereas the association between the control and the treated line of July was supported by a low bootstrap value (71).



**Figure 2.** Cluster analysis made with Ward's method and 100 bootstraps on the whole T-RFLP data set. In the first row, T = treated; C = control.



**Figure 3.** NMDS scaling plot based on Bray-Curtis similarities of the T-RFLP data plot on T-RFLP data set: hollow squares: February Treatment; asterisk: February Control; closed squares: March Treatment; hollow circles: March Control; hollow ovals: May Treatment; crosses: May Control; closed circles: July Treatment; hollow triangle: July Control.

Figure 3 shows the NMDS plot based on Bray–Curtis similarity measure on T-RFLP data set. To discriminate between samples collected in both tanks and samples collected in different sampling dates, different colours have been used. The stress level was 0.109 that is very close to an ideal ordination.[28] In Figure 4 is reported the Shepard Plot that shows that all the points were placed close to the straight ascending line. In particular, the points corresponding to the samples collected in February were grouped to each other, and they were closely related to samples collected in the control line of March. Furthermore, samples collected in May were all similar between control and treated lines. The July control samples were placed close to May samples, whereas the March treatment samples were separated to all other samples in the left side of the plot, as well as July treatment samples that were placed in the right side of the plot. This analysis confirmed the quality of our results.

Table 2 resumes the diversity indices calculated for all samples. In general, the values of the indices were homogeneous among the replicates of each tank in each sampling time. For this reason, the average values with the standard deviation have been calculated for each group of samples. Values were also similar considering all samples collected during the whole experimentation time, with the exception of samples collected in the treated tank of the last sampling time (July 2010), showing a significant difference in the values of all the three indices. As a matter of fact, Simpson and Shannon diversity indices showed lower values in treated samples of July 2010.

**Table 2.** Diversity indices calculated as average and standard deviation for each group of samples collected in different sampling dates.

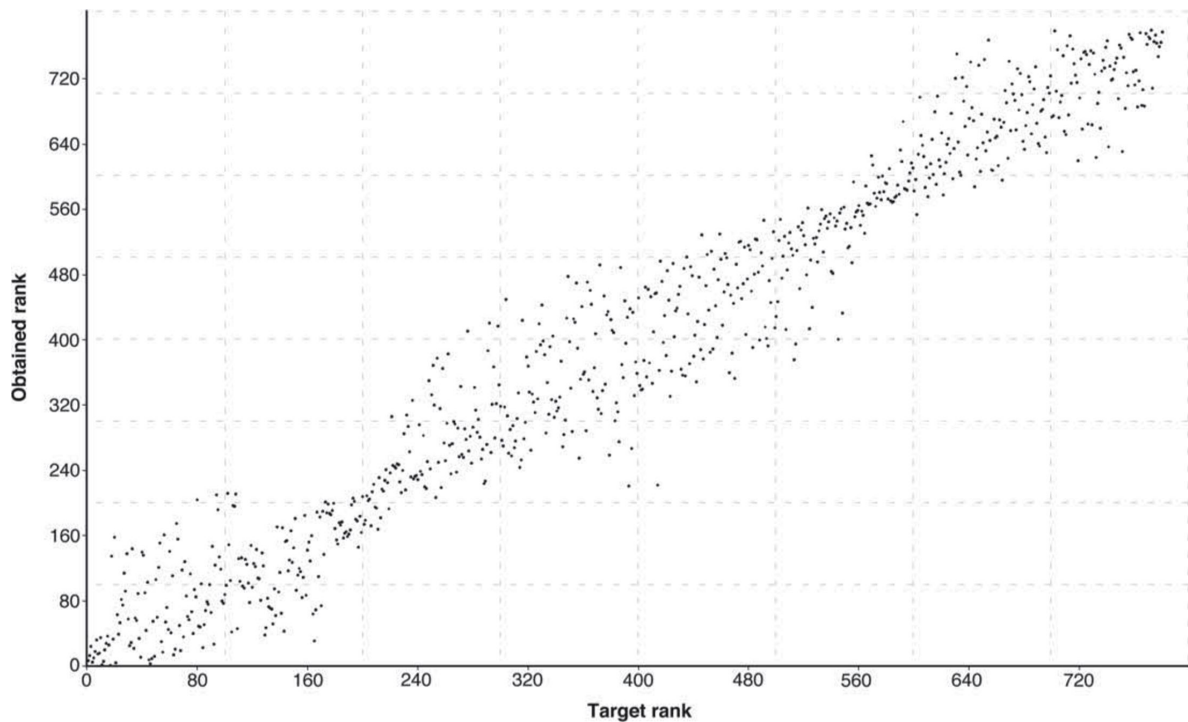
	Simpson Ds	Shannon-H
T_February	0.981 ± 0.001	4.337 ± 0.052
C_February	0.980 ± 0.002	4.230 ± 0.085
T_March	0.980 ± 0.003	4.404 ± 0.089
C_March	0.979 ± 0.001	4.209 ± 0.018
T_May	0.979 ± 0.001	4.229 ± 0.063
C_May	0.981 ± 0.001	4.338 ± 0.022
T_July	0.897 ± 0.023	3.335 ± 0.053
C_July	0.974 ± 0.001	4.119 ± 0.030

Note: Ds, Simpson diversity index; H, Shannon diversity index. In the first column: T, treated; C, control.

## Discussion

During the past decades, the effects of ozone on bacterial cells have been extensively studied. In detail, ozone treatment promotes protein denaturation and impairs enzymatic activities.[32–35] Furthermore, ozone may cause degradation of the unsaturated lipids present on the bacterial cell envelop.[36] As a consequence, ozone treatment may increase cytoplasmic membrane permeability with the release of cytoplasmic compounds including ATP, proteins and nucleic acids.[32,35,37] The application of ozonation treatment in WWTPs has been widely explored focusing on the sludge reduction efficiency,[20] or rather testing the efficiency of microorganisms degradation.[38,39] To the authors' best knowledge, no data are available about the effect of ozonation on bacterial communities composition in WWTPs. This paper represents the first study in which a molecular fingerprint technique is used to highlight the changes in wastewater bacterial community structure after the ozonation treatment. As a result of this study, we have actually observed the effects of ozone treatment on the bacterial community structure during the time of the experimentation.

Our study confirmed the results previously reported.[20] The continuous decrease in the VSS/TSS ratio in the outlet from the ozonated digestion tank can be regarded as an increase in the organic components in the sludge likely due to the release of inorganics from cell lysis into the medium as a consequence of ozone oxidation.



**Figure 4.** Shepard plot showing the quality of T-RFLP results.

As a matter of fact, this phenomenon was not observed in the control digestion tank. The fact that for both TSS and VSS, the reduction efficiency measured in the ozonated digester was very low in the first period and increased appreciably when the ozone dosage was increased, may be attributed to the biological stabilization process that took place in the digester and/or to the disintegration effect by ozonation. However, by comparing these data with the TSS and VSS variations in the line 1 digester, which was operated without ozonation, it is evident that the effects due to ozone oxidation appear. Concerning the monthly average TSS and VSS percentage reductions in the digesters of lines 1 and 2 (Table 1), the zero values reported for the first two periods of operation indicate that the solid concentration in the effluent from the digester of line 1 was approximately equal to that measured in the influent. The lack of an efficient sludge stabilization was likely due to a limited availability of oxygen supply in the tank. The more significant abatement of TSS and VSS observed in the line 2 digester was likely due to the disintegration effect caused by ozonation. This is confirmed by the higher reduction measured as the ozone dosage increased: in this particular case, a linear correlation was found between TSS removals and the applied specific ozone dosages. The oxidation treatment gave, thus, an important contribution to the sludge reduction achieved in the stabilization tank. Decay of some biomass determined the release of compounds that were then partly used for the synthesis process by other microorganisms. Salsabil *et al.* [40] observed similar VSS removal yields due to disintegration (about 20%) at 0.1 g O<sub>3</sub>/g TSS<sub>0</sub> in lab-scale batch conditions. At full-scale, Sievers *et al.* [41] measured a mass reduction of 20–35% for the aerobic stabilization with a specific ozone consumption of about 0.05 kg O<sub>3</sub>/kg TSS to be treated. It is worth noting that all these reduction efficiency values were achieved at significantly higher dosages than those used in this study. The decrease of the values in the third operational period (Table 1) might indicate that the higher TSS concentration in the digester determined a lower specific oxidant dosage, which limited the ozone disintegration effect.

Focusing on the biomolecular analysis performed on bacterial community, it is clear from both the CA and NMDS that replicates collected in each tank were homogeneous at each sampling time (Figures 2 and 3). This result is in agreement with previous studies that tested the homogeneity of bacterial community in replicates collected from the same tank.[42] It is also



evident from the CA that the association between samples collected from the two different tanks is well supported (100 bootstraps) in all the samples collected at the onset of experimentation (February 2010), when the ozonation treatment was not yet active. These samples are all grouped together also in NMDS plot (Figure 3), demonstrating that at the onset of experimentation, the bacterial community composition of the two tanks was substantially similar. The Shepard plot (Figure 4) and the value of the stress level (0.1089) support the robustness of our NMDS analysis on T-RFLP data set.[28]

Samples collected in March, after a few weeks of treatment, are different in the two tanks (Figures 2 and 3). It is likely that after 2 weeks of treatment, the bacterial community composition has been already modified by the effect of ozonation. March control samples associates with February control samples, both in CA (Figure 2) and in NMDS (Figure 3). This demonstrates that the bacterial community of the control line remains substantially stable during the first months of experimentation, whereas the bacterial community of the treated line starts to differentiate, as shown in NMDS plot (Figure 3). In May, almost after 3 months from the starting of the treatment (period 2), the two tanks appear similar concerning the bacterial communities composition (Figures 2 and 3). The situation of the sampling dated in July (period 3 of ozone treatment) is similar to the one performed in March, highlighting the differences among treated and control samples especially in NMDS (Figure 3). The estimation of diversity indices based on molecular data obtained for bacterial community in WWTPs is a commonly used approach.[43] In this study, data concerning diversity indices (Table 2) highlight significant differences that are present in samples collected in the treated tank in July 2010. The lower values of Shannon index indicates that the treated July 2010 samples have a lower number of bacterial taxa. At the same time, lower values of Simpson index indicate that these samples show a lower number of taxa that are dominant with respect to all other samples, which instead present more bacterial taxa with comparable richness. This is in agreement with the consideration that the ozonation treatment affects the bacterial community composition, probably reducing the number of taxa, allowing the development of lower species number that may be more resistant to the treatment or exhibit a higher division rate. On the contrary, control and treated samples collected in May 2010 are substantially similar in all performed analyses.

Considering NMDS and diversity indices data (Figure 3 and Table 2) in correlation with the three periods of ozonation treatment,[20] it is worth noting that during period 1 of treatment, in which the ozone dosage was the lowest, some differences in bacterial community composition between treated and control lines were highlighted (samples collected in March); during period 2, in which the ozone dosage was the highest, bacterial community composition did not show significant differences between treated and control lines. During the third period, when the ozone treatment was decreased, differences between control and treated bacterial communities were again evidenced as observed in period 1. Probably, the lower ozone dosage damaged only few bacterial species and influenced selectively the composition of bacterial community in the treated line. On the contrary, highest dosages of ozone destroys all bacterial cells that are present in the treated tank and, for this reason, it does not contribute to the selection of a peculiar bacterial community. From a practical point of view, the lower ozone dosages of period 1 significantly influence the bacterial community composition but did not induce a reduction in sludge production. On the contrary, high ozone dosages (period 2) seem to have a less strong effect on the bacterial community composition but can positively affect the reduction of sludge. At period 3 of ozone treatment, it is possible to observe an intermediate situation. From the point of view of the management of the plant, the results shown in this paper suggest that the effects on sludge production are obtained through a massive releasing of nutrients caused by the bacterial cell lysis that occurred after the ozonation process, rather than through the selection of more 'efficient' bacterial communities.



# Conclusions

In this paper, the effects of ozonation treatment on the reduction of excess sludge and on bacterial community composition of a full-scale plant for domestic wastewater treatment are reported.

The ozonation treatment was able to significantly reduce excess sludge production when ozone specific dosage was in the range 2.1–2.3 g O<sub>3</sub>/kg TSS<sub>0</sub> while negligible reduction was observed in the case of the lowest specific ozone dose experienced in this study (0.3 g O<sub>3</sub>/kg TSS<sub>0</sub>).

Samples from two different tanks (control and treated) were compared to verify the homogeneity of replicate samples in each tank, the homogeneity of samples between control and treated tanks before the starting of the ozonation process, and the effect of ozonation treatment on bacterial communities during the whole study period. What the study highlighted was homogeneity in composition of bacterial community among replicates collected in each tank during the whole experimental period, and a homogeneity between the control and the treated tank at the moment of the onset of the study, before the dosage of ozone. Our results demonstrated an overall differentiation on the bacterial community when low or moderate ozonation levels were applied, probably as a consequence of the reduction in the number of taxa present in the samples, which allowed the development of a few dominant species. By contrast, high level of ozonation apparently did not influence the composition of the bacterial community.

## Acknowledgments

S. Gabrielli is gratefully acknowledged for his help with photographic artwork; A.M. Pulina is gratefully acknowledged for her critical revision of English. The authors thank Acqualatina SpA who supported the research, Praxair for providing the Lyso™ sludge ozonation process and Rivoira S.p.A for providing the oxygen storage system.

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