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Aerobic treatment of dairy wastewater in an industrial three-reactor plant: Effect of aeration regime on performances and on protozoan and bacterial communities

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ABSTRACT

An industrial three-reactor plant treating 45 m^3 d^{-1} of dairy wastewater was monitored to investigate the effect of different aeration regimes on performance efficiency and to find relationships with bacterial and protozoan communities in the activated sludge. During the study, the plant was maintained at six different "on/off" cycles of the blower (45/15, 15/15, 15/45, 30/30, 30/45 and 30/60 min), providing between 30.2 and 90.6 kg O_2 d^{-1}, and the main chemical/biochemical parameters (COD, BOD, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, etc.) were determined. When at least 45.4 kg O_2 d^{-1} (30/45) were provided, COD removal efficiencies were always in the range 88–94% but decreased to about 70% under aeration regimes 15/45 and 30/60. Ammonium ion degradation performance was compromised only in the lowest aeration regime (15/45). Total number of protozoa and their species richness, and bacterial viable counts and denaturing gradient gel electrophoresis (DGGE) profiles were used to characterize the microbiota of the activated sludge. Cell abundances and community structures of protozoa and bacteria were very similar in the three aerated reactors but changed with the aeration regimes. In particular, the 15/45 and 30/60 regimes led to low protozoan diversity with prevalence of flagellates of the genus *Trepomonas* at the expense of the mobile and sessile forms and, thus, to a less efficient activated sludge as indicated by Sludge Biotic Index values (3 and 4.5 for the two regimes, respectively). The structure of the bacterial community strongly changed when the aeration regimes varied, as indicated by the low similarity values between the DGGE profiles. On the contrary, number of viable bacteria and values of the biodiversity index remained stable throughout the whole experimentation. Taken together, the results of the present study clearly indicate that aeration regime variations strongly influence the structure of both protozoan and bacterial communities and, above all, that a high biodiversity among protozoan populations in the activated sludge is prerequisite for high performances in dairy wastewater treatment.

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1. Introduction

Nowadays, the dairy industry is considered the largest source of food processing wastewater. The effluent, that includes wasted milk and water from cleaning, sanitization, heating, cooling and floor washing, but excludes whey that is generally separated and differently treated or upgraded, is characterized by variable volumes, flow rates and organic matter content, which ranges approx. from 0.8 to 7.0 g/l COD (Britz et al., 2006). Dairy wastewaters require, therefore, specialized treatments (EU Directive 2000/60/EC) to meet effluent discharge standards and to reduce the risk of environmental problems such as eutrophication in rivers, lakes and coastal waters.

Conventional dairy wastewater treatment plants (WTPs) are mainly based on activated sludge processes that involve the aerobic microbial metabolism of fats, lactose and proteins. The anaerobic treatment, which is often inhibited by the presence of fats causing poor nutrient removal (Vidal et al., 2000), is generally considered more suitable for high organic loads (e.g., effluents that include whey) (Britz et al., 2006; Kushwaha et al., 2011). Treatment based on intermittently aerated reactors consisting of alternate anoxic/anaerobic and aerobic phases has been proved to be the best way to achieve carbon, as well as nitrogen and phosphorus removals (Gutierrez et al., 2007; Kushwaha et al., 2011). In this respect, the control of the aeration regimes represents a key issue since anaerobic under aeration may lead to partially treated effluent, while over-aeration results in higher than necessary oxygen that may cause destabilization of the sludge and, definitely, in higher electricity and maintenance costs (Britz et al., 2006).

Activated sludge systems consist of a complex mixture of bacteria and protozoa that remove organic substances and nutrient contaminants from wastewaters. Thus, a better understanding of the microbial communities of activated sludge, and particularly of the correlation between microbial diversity and ecosystem function, is necessary to rapidly monitor and assess process performances and to optimize the biological processes occurring in wastewater treatment plants (Sanz and Kochling, 2007).

Protozoa populations play a major role in the microbial food webs during the biological treatment in WTPs and their abundance and diversity are commonly used as an indicator of activated sludge plant performance (Seviour and Nielsen, 2010). In this respect, Madoni (1994) has introduced an objective index, the Sludge Biotic Index (SBI), based on the presence and abundance of certain key protozoan groups, that provides a numerical value that enables the operator to monitor the prevalent plant operating conditions and performances on a daily basis. In the last decade, several studies have been aimed at demonstrating the applicability of the SBI as a useful monitoring tool to assess the activated sludge health by using different WTP typologies and/or wastewaters added of possible toxic substances (e.g., chromium VI, cupper, phenol and cyanide) (Papadimitriou et al., 2007; Drzewicki and Kulikowska, 2011). Although the majority of the studies have reported direct correlations between high SBIs and good plant treatment performances, the index does not appear to be always reliable (Arevalo et al., 2009; Drzewicki and Kulikowska, 2011).

Further, and in spite of their importance in the activated sludge, the information on the ecological role of the bacterial populations in wastewater treatment systems is quite limited. Conventional microbiological techniques based on cultivation-dependent approaches have, in fact, proven inadequate since cultivable bacteria represent only a minor fraction of the whole community of such complex ecosystems. On the contrary, molecular methods based on polymerase chain reaction (PCR) amplification of 16S ribosomal RNA (rRNA) genes allow the profiling of complex bacterial communities on the basis of sequence diversity, thus avoiding the biases associated with laboratory culturing. Among the genetic fingerprinting methods, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes permits direct visualization and rapid comparison of the structure of bacterial communities, thus showing useful in investigating the microbial ecology of the activated sludge (Liu et al., 2007; Sanz and Kochling, 2007).

In this context it is worth noting that, to the best of our knowledge, no studies have been reported on the combined monitoring of both bacterial and protozoan populations of the activated sludge in order to assess possible relationships between microbial communities and treatment performances. With these points in mind, objective of the present study was to assess the effect of six different aeration regimes on both biotreatment performances and activated sludge microbiota in a dairy WTP. To this end, an industrial plant characterized by three aerated reactors working in series (namely, R1, R2 and R3) was operated at six different “on/off” cycles of the blower with consequent various extents of aerobiosis and anoxia. Under these aeration regimes, relationships between removal efficiency of the main chemical/biochemical parameters and the structures of both protozoan population and bacterial community have also been investigated.

2. Materials and methods

2.1. Dairy wastewater

The “Buona Tavola Sini” dairy (Monterosi, Viterbo, Italy) processes 15,000–20,000 L of milk per day and produces about 45 m^3 d^{-1} of wastewater. The dairy mostly treats sheep milk and, in much lesser amount, bovine milk. The wastewater mainly comes from the cleaning of the equipment in contact with milk or milk derivatives; whey is disposed of separately. The wastewater characteristics are reported in Table 1.

2.2. Wastewater treatment plant (WTP) and operative conditions

The WTP, designed and manufactured by Manzi Aurelio Srl (Montefiascone, Italy), is constituted of a primary section for sedimentation of 200 m^3, three aerated reactors (R1, R2 and R3) connected in series and of 18 m^3 capacity each and a secondary section for sedimentation of 18 m^3. Also, there is

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Table 1 – Chemical characteristics of the dairy wastewater used in the this study.

	Unit	Range
Chemical oxygen demand (COD)	mg l^{-1}	662–1293
Biological oxygen demand (BOD_5)	mg l^{-1}	380–702
Total nitrogen (Kjeldahl)	mg l^{-1}	8.1–38.8
Ammonia nitrogen ($N–NH_4$)	mg l^{-1}	5.9–36.7
Nitrous nitrogen ($N–NO_2$)	mg l^{-1}	0–0.34
Nitric nitrogen ($N–NO_3$)	mg l^{-1}	0–0.5
Phosphate ($P–PO_4$)	mg l^{-1}	0.79–6.84
Bismute Active Substances (BiAS)	mg l^{-1}	0.36–4.20
Methylen Blue Active Substances (MBAS)	mg l^{-1}	0.42–5.6
Suspended Solid	mg l^{-1}	275–450
Chloride (Cl^-)	mg l^{-1}	1265–6852
pH		5.3–7.0

a digestion sector of 18 m^3 connected with three drying beds (Fig. 1). The oxygen is provided by two blowers that operate alternately. The air is diffused into the reactor by means of membrane tubular diffusers (model TMF 750 S, ITT Water & Wastewater Italia Srl, Lainate, Italy). The blowers (model SCLK06MS, FPZ spa, Concorrezzo, Italy) have an air flow of 180 m^3 h^{-1}. Based on technical specifications, the diffuser performance is estimated to be ca. 10% for the hydraulic head of the plant (2 m), so that the biological reactors receive ca. 0.028 kg of O_2 h^{-1}.

During the study, the WTP worked at a hydraulic load of about 45 m^3 d^{-1} with a hydraulic retention time of approximately 8 h for each aerated reactor. The recirculation ratio was kept constant at 150%. The excess sludge was 3 m^3 d^{-1}.

2.3. *Research plan and sampling procedures*

Six different aeration regimes (Table 2) were tested by varying the on/off cycle of the blower as follows (on/off minutes): 45/15 (corresponding to 90.6 kg O_2 d^{-1}); 15/15 (60.4 kg O_2 d^{-1}); 15/45 (30.2 kg O_2 d^{-1}); 30/30 (60.4 kg O_2 d^{-1}); 30/45 (45.4 kg O_2 d^{-1}); 30/60 (40.2 kg O_2 d^{-1}). Each regime was run for at least two weeks with samples of wastewater influent, mixed liquor of the three aerated reactors, liquid effluent and recirculation sludge taken every week in duplicate. Unless indicated otherwise, data reported in Table 2 and figures refer to samples taken after two weeks from the change of the aeration regime.

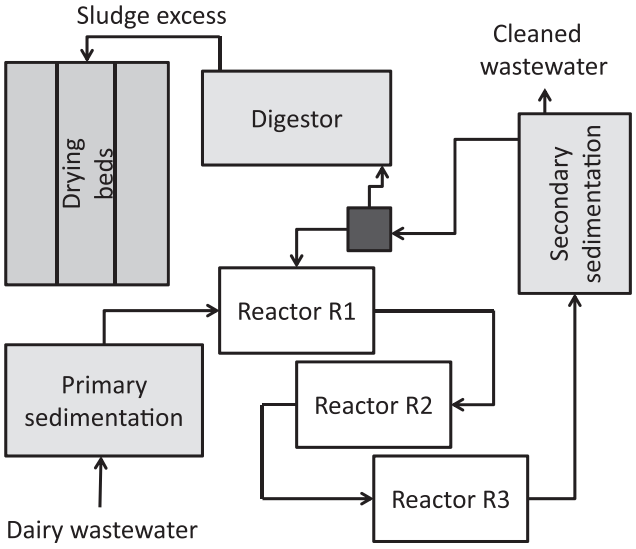

Fig. 1 – Flow diagram of the treatment plant for dairy wastewater operating in cheese factory "Sini", Monterosi (VT).

2.4. *Physico-chemical analysis*

The following parameters were measured directly on-site: dissolved oxygen (DO) and temperature using a Hach Lange meter (mod. LQ20, Lainate, Italy); redox potential (ORP) and pH by means of a portable meter (mod. HI 83140, Hanna Instruments).

Samples of influent, settled mixed liquor of the three aerated reactors and effluent were analyzed for BOD_5, COD, $N–NH_4$, $N–NO_2$, $N–NO_3$, Total-N (Kjeldahl), $P–PO_4$, Bismute Active Substances (BiAS), Methylen Blue Active Substances (MBAS), suspended solid, mixed liquor suspended solids (MLSS), sludge volume index (SVI) and chloride ions (Cl^-). All the procedures were performed according to the Standard Methods (APHA, 2005). The BOD_5 was measured respirometrically using an apparatus System 6 (VELP Scientifica srl, Milan, Italy) and suppressing nitrification by the addition of 0.5 mg l^{-1} of allylthiourea.

In order to quantify the treatment performance of the reactors, removal efficiencies (RE%) were calculated for chemical parameters (COD, BOD_5, $N–NH_4$ and $P–PO_4$) using the following equation:

Table 2 – Different aeration regime, expressed as cycle on/off of the air-blow, and related operative conditions in the three aerated reactors (named R1, R2 and R3) connected in series.

Aeration regime ON/OFF (min)	Supplied oxygen (kg O_2 d^{-1})	Dissolved oxygen (mg l^{-1})			Redox potential (mV)			pH			Temperature (°C)		
		R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
45/15	90.6	3.1	8.9	8.1	5.5	92.5	145.7	7.0	7.2	7.3	21.6	21.8	22.1
15/15	60.4	6.2	8.0	8.2	59.3	73.2	86.0	7.3	7.5	7.6	23.0	22.6	22.6
15/45	30.2	0.2	0.2	0.2	−182.8	−190.5	−200.2	7.0	7.2	7.3	24.8	25.1	25.3
30/30	60.4	6.1	9.1	9.4	75.8	147	204.7	6.7	7	7.2	21.7	21.6	21.6
30/45	45.4	5.5	7.0	7.8	18.3	84.8	130.6	6.7	6.9	7.0	21.9	22.0	21.9
30/60	40.2	0.5	0.8	1.0	−41.3	6.6	7.1	6.2	6.6	7.0	18.4	18.0	18.2

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$$RE(\%) = \frac{Cin - Ceff}{Ceff} \cdot 100$$

where *Cin* and *Ceff* are the concentrations in the influent (IN) and the effluent (after settling) of reactor R3, respectively. Data from the two set of samples (duplicates) were mediated.

2.5. Microscopic analysis of the protozoan community

Protozoa enumeration and identification were carried out on 25 μl of mixed liquor samples (each in duplicate) from the three aerated reactors *via* contrast microscopy (Labolux 11, Leitz) at 100× or 400× magnification depending on the species size and using the identification keys of Foissner et al. (1991, 1992, 1994, 1995) and Curds et al. (2008). For the count of small flagellates a Fuchs–Rosenthal camera was used, following guidelines by Madoni (1994). In addition to the protists, small metazoa were also counted, if and when present. Calculations of the Sludge Biotic Index (SBI) were undertaken according to the guidelines by Madoni (1994). The sludge samples were maintained under aeration conditions and analyzed within 4 h from sampling.

Protozoan diversity indices were also calculated: Richness (*S*) was determined from the number of taxa detected while the Shannon–Weaver index (*H*) was calculated using the Past Software (version 1.94b). Data were means of duplicate samples.

2.6. DNA extraction, PCR amplification and denaturing gradient gel electrophoresis (DGGE) analyses of the bacterial populations

In order to analyze the bacterial community, sludge samples (each in duplicate) from the three biological reactors were subjected to both viable cell counts and DGGE analysis.

Cultivable heterotrophic bacteria were enumerated according to the most probable number (MPN) count technique (Wrenn and Venosa, 1996).

Total community DNA was extracted from 250 mg of sludge using the Power Soil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instruction. The variable V3 region of 16S rDNA was amplified by PCR using primers targeted to conserved regions of the 16S rRNA genes: 341F (ATTACCGCGGCTGCTGG) and 534R (ATTACCGCGGCTGCTGG) (Muyzer et al., 1993). Primer 341F had at its 5′ end an additional 40-nucleotide GC-rich sequence (GC clamp) to facilitate separation by DGGE. The 16S rRNA gene was amplified from 10 ng of DNA in a PCR reaction with 0.4M of each primer, using the illustraTM HotStart Master Mix (GE Healthcare, UK). PCR amplification was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) as previously reported (Federici et al., 2011). PCR products from 3 parallel amplifications were pooled, concentrated with a Microcon filter (Millipore, Bedford, MA), separated in 1.5% (w/v) agarose gel and then stained with ethidium bromide.

The INGENYphorU-2 system for DGGE (Ingeny International BV, Goes, NL) was used following the protocol of analysis as already reported (Federici et al., 2011). DGGE banding patterns were digitized and processed using the Quantity-one analysis software (Bio-Rad Laboratories).

Richness (*S*) was determined from the number of bands in each lane while the Shannon-Weaver index (*H*) was calculated from $H = \sum(n_i/N)\log(n_i/N)$, where n_i is the peak height of a band and *N* is the sum of all peak heights in a lane. An unweighed pair group method with arithmetic means dendrogram was generated from a similarity matrix based on common band positions between lanes and calculated using the Dice's coefficient (Li and Moe, 2004).

3. Results and discussion

3.1. Influent characteristics and operational conditions

With only occasional variations, the influent's COD was low (<1200 mg l^{-1}) and rather stable throughout the whole experimentation (Table 1) with a BOD/COD ratio higher than 0.5. The nitrogen fraction was almost exclusively made up of ammoniacal nitrogen. The phosphate content was rather variable throughout the whole experimentation (from 0.79 to 6.84 mg l^{-1}); its concentration, however, was generally low for this kind of wastewater (Britz et al., 2006). High was the surfactants content (up to 4.2 and 5.6 mg l^{-1} for BiAS and MBAS, respectively) since the wastewater was mostly made up of washing waters rich of detergents. Finally, noticeable was the chloride ions content (1265–6852 mg l^{-1}) deriving from the curing procedures.

The influent's COD load was stable with values constantly in the range 39.8–58.2 kg COD d^{-1}; significant differences were only recorded for the loads at the aeration regimes 15/15 and 30/60 as compared to that at 30/30 (Fig. 2A). Conversely, the ammonium daily load varied more being high when the aeration condition was 15/45 (1.85 kg d^{-1}) and very low when it was 30/60 (0.32 kg d^{-1}) (Fig. 2B). During the whole experimentation, the MLSS varied from 1170 to 2515 mg l^{-1}, but, regardless of the aeration regimes, no significant differences ($P \leq 0.05$) were observed comparing the MLSS values of the three reactors working in series (data not reported).

3.2. Effect of the aeration regime on the biotreatment performances

Varying the aeration regimes, both DO and ORP varied in the three reactors R1, R2 and R3 (Table 2) affecting, as a consequence, the degradation performances (Fig. 2A and B).

With oxygen amounts of 40.2 and 30.2 kg O_2 d^{-1} (aeration regimes 30/60 and 15/45, respectively) both DO and ORP were rather low. In particular, in the case of 15/45 DO and ORP were 0.2 mg l^{-1} and −180 mV, respectively, values typical of serious anoxic conditions (Dubber and Gray, 2011); clearly, 15 min of aeration were not enough to counterbalance the long anoxic phase (45 min). The biodegradable organic fraction, mainly made up of fatty acids (Ndegwa et al., 2007), was scarcely consumed in the first reactor due to low DO availability; in R2 and R3 the organic matter content was still too high for the little available DO that was rapidly and completely consumed. On the contrary, in the case of the aeration regimes 45/15, 15/15, 30/30 and 30/45, in which the amount of oxygen provided was always higher than 45.4 kg O_2 d^{-1}, the values of ORP and DO increased passing from R1 to R2 and, subsequently, to R3

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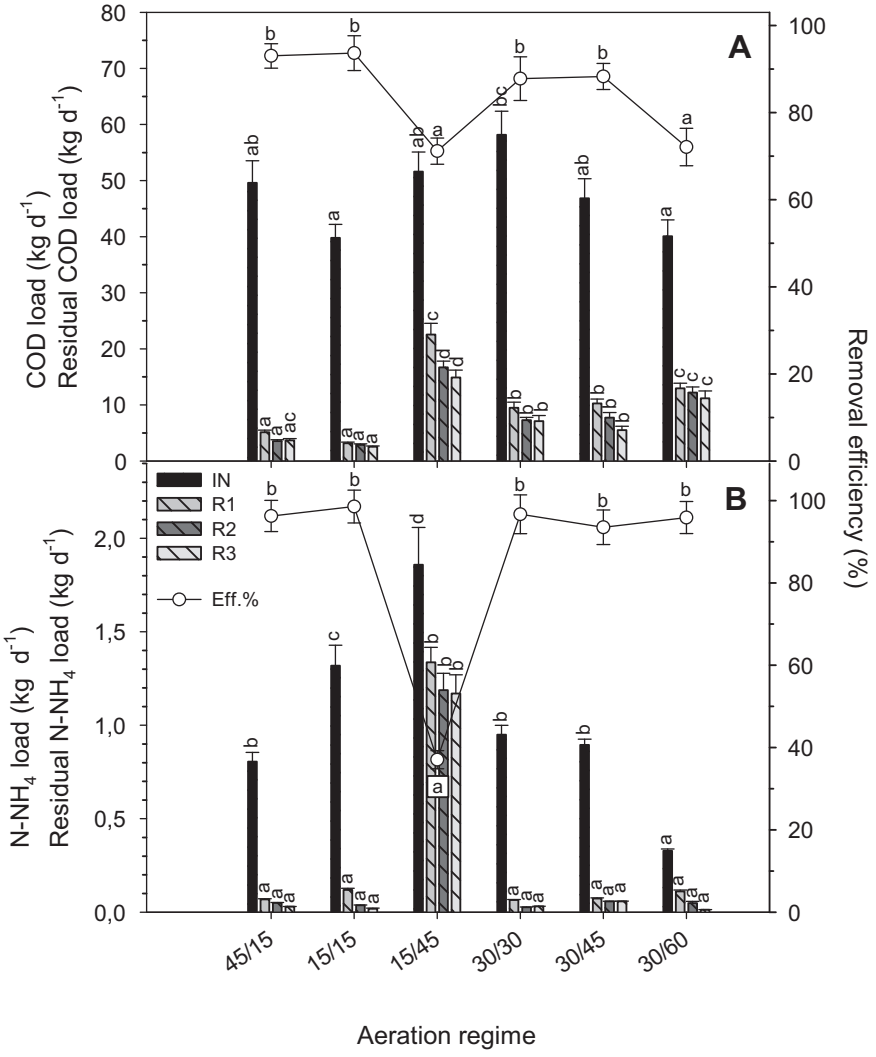

Fig. 2 – A, B - COD load, residual COD load and related removal efficiency (A) and N–NH_4, load residual N–NH_4 load and related removal efficiency (B) in the three aerated reactors in series (R1, R2, R3) at different aeration regimes (On/Off cycles). Values are the means of two replicates and error bars indicate standard deviations. Pairwise comparisons were performed by the Tukey test ($P \leq 0.05$): same lower case letters denote absence of statistical significance between different aeration regimes within the same group (i.e., IN, R1, R2, R3 or Eff.%).

(Table 2). At these more favorable aeration regimes, the degradation of the rapidly biodegradable organic fraction took place in R1; as a consequence, an excess of available oxygen was present in the reactors R2 and R3 in respect to the oxygen needed for the aerobic degradation of the still available organic fraction (Ndegwa et al., 2007).

With the only exceptions of the regimes 15/45 and 30/60, the COD removal efficiencies were always in the range 88–94%, similar to those that Carta-Escobar et al. (2004) obtained using synthetic dairy effluents treated in a three-sequential oxidation phases pilot plant the configuration of which was similar to that of the full-scale plant of the present study. According with these authors (Carta-Escobar et al., 2004), the pH of the mixed liquor increased passing through the three reactors (Table 2). At these aeration regimes, the COD load was mostly removed in R1 while in R2 and R3 small was the further COD reduction (Fig. 2A); in any case, the final effluent's COD (out of reactor R3) was always less than 160 mg l^{-1}. On the contrary, at the low aeration regimes 15/45 and 30/60, corresponding to oxygen supplies of 30.2 and 40.2 kg O_2 d^{-1}, respectively, the COD removal rate decreased to about 70% with less COD reduction in R1 (Fig. 2A). As already mentioned, at these regimes of aeration, but particularly in the case of 15/45, both the DO and ORP values were low (Table 2) thus indicating anoxic conditions and, as a consequence, reduced removal performances (Metcalf and Eddy Inc., 2003; Li and Bishop, 2004). The effect of the various aeration regimes on BOD_5 removal appeared to be quite similar to that on the COD (figure not shown).

Similar was the ammonium ion depletion; only under the aeration regime 15/45, the removal efficiency went down to about 37%. In the same way, Zhanping and Jingli (2010) found important decreases in the nitrification process with a critical DO concentration of 0.5–0.2 mg l^{-1}, the same found in this

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study. Guo et al. (2009) report that the nitrification process can take place also at low levels of DO; it must be noticed, however, that in their study the COD level was 215 mg l^{-1} and, therefore, much lower than that (1147 mg l^{-1}) recorded when the aeration regime was 15/45. Very likely, in our case the little oxygen available was mostly used to oxidize the carbon substrate to the detriment of the nitrogen fraction which requires for the oxidative process an amount of O_2 (4.57 kg O_2 per kg NH_4) higher than that needed for the depletion of the organic fraction (Metcalf and Eddy Inc., 2003). Consequently, the amount of N–NH_4 removed (0.69 kg N–NH_4) could be utilized by the effluent biomass through an assimilative process; several studies, in fact, have reported an active role for the protozoa in the removal of the nitrogenous substrate (Petropoulos and Gilbride, 2005; Akpor et al., 2008). Also, it is worthwhile to mention that the concentrations of nitrites (NO_2–N) and nitrates (NO_3–N) in the mixed liquor under conditions of low oxygenation (aeration regimes 15/45 and 30/60) were extremely low in all three reactors (data not shown). This phenomenon might be partly explained with a scarce depletion of the ammonium ion, particularly when the aeration regime was 15/45, and, partly, with denitrification processes that, according to Yuan and Gao (2010), might take place also in the anaerobic micro-zones within the activated sludge flocs. Under all other aeration regimes, the removal levels of the ammonia nitrogen were high, ranging from 93 to 98%. Differently from what observed in the case of the COD, also at the aeration regime 30/60 the removal efficiency was high; it must be noted, however, that the daily load of the influent N–NH_4 (0.33 kg N–NH_4 d^{-1}) was significantly lower than that at the aeration regime 15/45 (1.86 kg N–NH_4 d^{-1}) which might have favored the better removal performance. Similarly to the COD, the N–NH_4 load was removed for the largest part in reactor R1 with the exceptions, however, of the aeration regimes corresponding to 30.2 and 40.2 kg O_2 d^{-1} (Fig. 2B). Finally, with the only exception of the regime 15/45, the N–NH_4 concentrations in the final effluent out of R3 were always lower than 1.0 mg l^{-1}.

The plant and the activated sludge showed good flexibility and adaptability to the varying aeration regimes: in fact, passing from 30.2 kg O_2 d^{-1} (aeration regime 15/45) to 60.4 kg O_2 d^{-1} the operative system recovered efficiency and full functionality after only two weeks (Fig. 2A and B).

As for the depletion of the phosphate ion (P–PO_4), a marked negative effect was observed possibly due to the release of this ion by the activated sludge under prolonged anoxic conditions (Majed et al., 2009; Jeon et al., 2001) (Fig. 3). However, negative removal efficiencies were also recorded at the highest aeration regimes (90.6 e 60.4 kg O_2 d^{-1}). This observation is somehow consistent with what reported by Danesh and Oleszkiewicz (1997) who hypothesized that in shortage of volatile fatty acids the phosphates released during the anaerobic phase would not be re-absorbed by the phosphorous accumulating organisms in the subsequent aerobic phase.

3.3. *Effect of the aeration regime on the protozoan community*

The variation of the aeration regimes markedly influenced the populations of protozoa present in the activated sludge in terms of both density and structure (Figs. 4 and 5). By confronting the frequencies of the various taxa present in the three reactors (Fig. 4A, B and C refer to the reactors R1, R2 and R3, respectively), a marked similarity becomes apparent likely due to the fact that the three reactors worked in series. Regardless of the aeration regimes, in fact, the taxa most present in R1 were also majority in R2 and, afterward, in R3, observation fully confirmed by the richness (*S*) and the Shannon-Weaver index (*H*) (Fig. 5A and B, respectively).

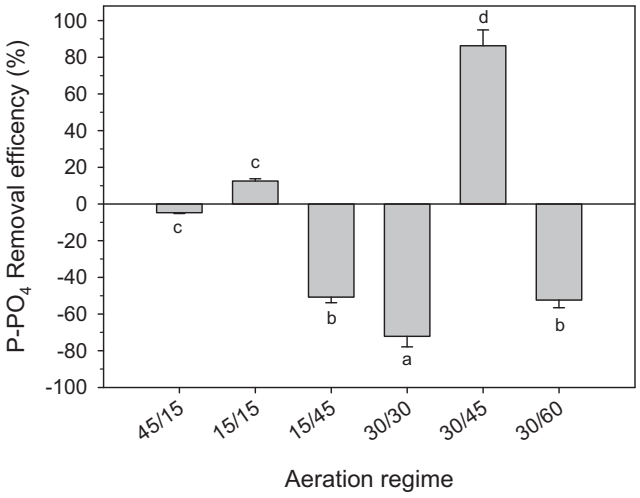

Fig. 3 – Overall phosphate removal efficiency at different aeration regimes (On/Off cycles). Values are the means of two replicates and error bars indicate standard deviations. Pairwise comparisons were performed by the Tukey test ($P \leq 0.05$): same lower case letters denote absence of statistical significance between different aeration regimes.

Under the aeration regimes 45/15 and 15/15, the crawling ciliates were prevalent (Fig. 4); other forms of ciliates and thecamoebians were also present, while absent were the flagellates. The sludge biotic index (SBI), calculated in correspondence of these regimes of aeration, was very high (between 9 and 10) in all three reactors, thus confirming the good COD and N–NH_4 degradation performances.

Lower aeration levels significantly influenced the protozoa populations present in the activated sludge. Under the aeration regime 15/45, in fact, there was a decrease in biodiversity with reduction of the number of taxa (*S*) and of *H* (Fig. 5A and B, respectively). The disappearance of various taxa was likely due to their difficulty in adapting to prolonged anoxic conditions (Dubber and Gray, 2011). Under that regime of aeration, a modification of the protozoa groups frequency was observed with clear prevalence of flagellates (Fig. 4A–C) belonging, in particular, to the genus *Trepomonas*, an obliged anaerobe (Priya et al., 2008), able, therefore, to live under the anoxic conditions present in the three reactors (Table 2). In this respect, it is worth remembering that, in his pioneristic and foundation work, Lackey (1932) reported that *Trepomonas* sp. needed anaerobic conditions to proliferate, while after only 6 h of aeration it disappeared in favor of the ciliates. Surprisingly, under the anoxic conditions caused by the aeration regime 15/45 no sessile ciliates, such as *Vorticella microstoma* (Madoni,

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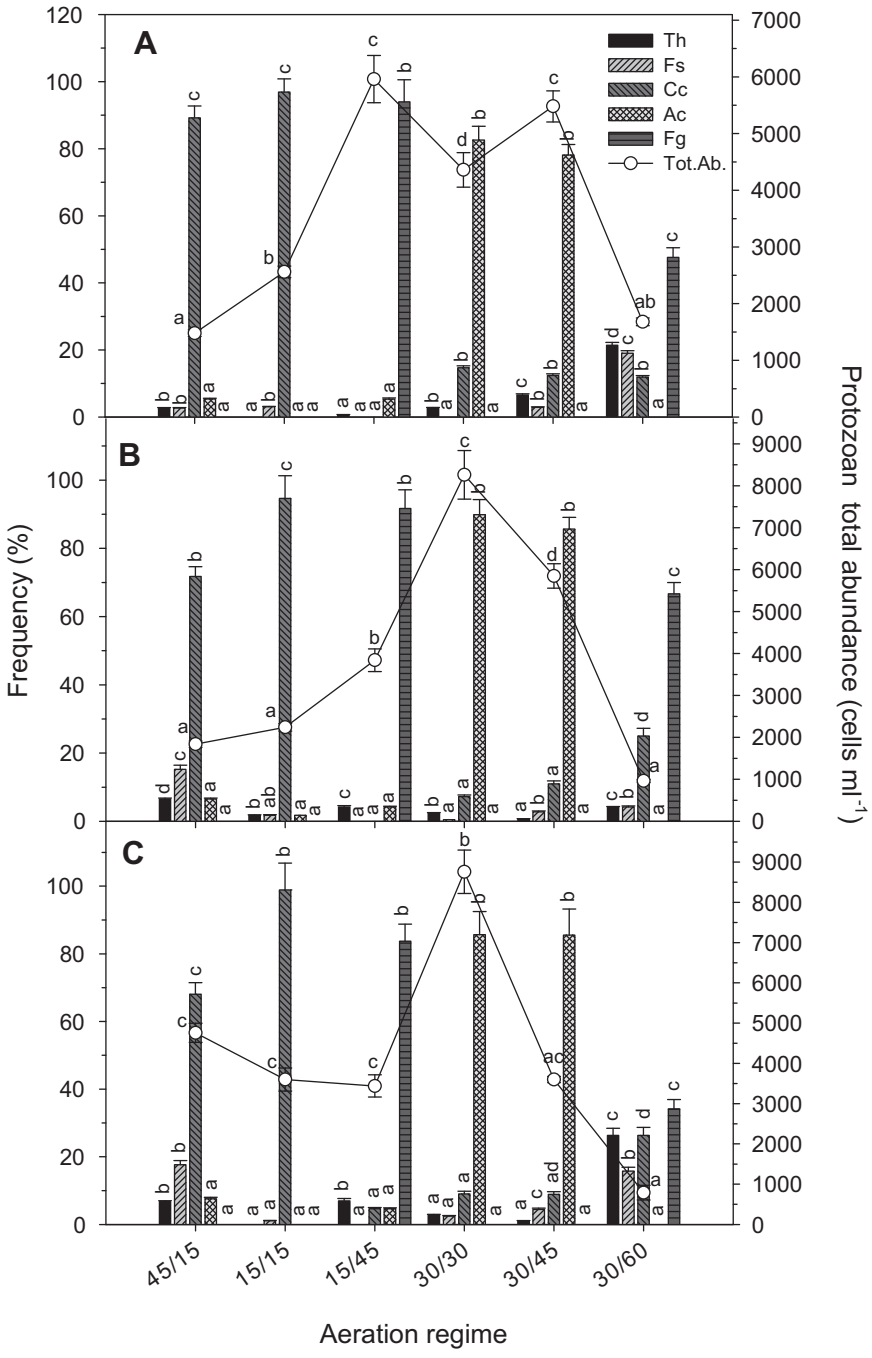

Fig. 4 – A–C - Frequency of various protozoan classes (Th, Thecoamoebians; Fs, Free-swimming Ciliates; Cc, Crawling Ciliates; Ac, Attached Ciliates; Fg, Flagellates) and the total abundance of protozoa in the three aerated reactors in series R1 (A), R2 (B) and R3 (C) at different aeration regimes (On/Off cycles). Values are the means of two replicates and error bars indicate standard deviations. Pairwise comparisons were performed by the Tukey test ($P \leq 0.05$): same lower case letters denote absence of statistical significance between different aeration regimes within the same group (i.e., Th, Fs, Cc, Ac, Fg or Tot.Ab.).

2003; Arevalo et al., 2009) and *Opercularia* sp. (Madoni, 2003; Lee et al., 2004; Arevalo et al., 2009), could be found. In the case of the latter, in particular, it can be hypothesized that the DO concentrations were too low to allow survival: in fact, again Lackey (1932) compared the reactions of *Trepomonas* and *Opercularia*, concluding that increasing aerations had a negative effect on the former group before than on the latter. The low treatment efficiencies under the 15/45 aeration regime (see above) were also dependent upon the effluent's high turbidity probably associated to the low efficiency of bacterial predation by the flagellates, the prevalent group (Madoni, 2003). In all the three oxidation reactors, the SBI

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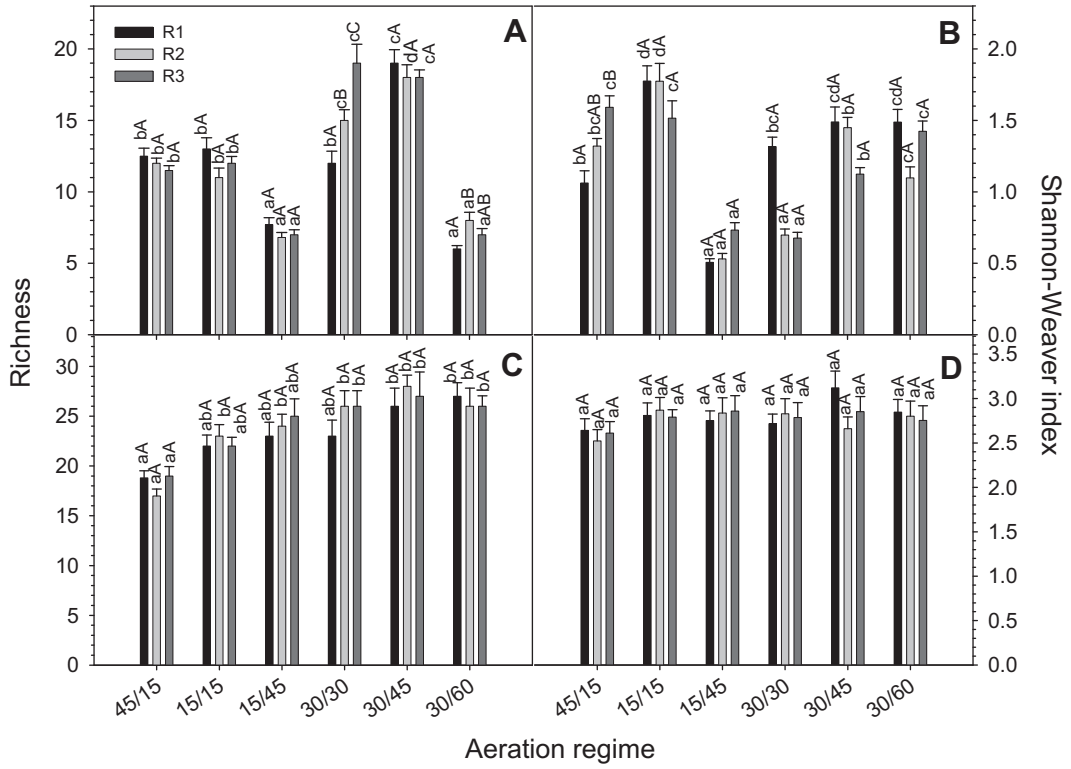

Fig. 5 – A–D -Richness and Shannon–Weaver index of protozoan (A and B, respectively) and bacterial (C and D, respectively) populations in the three aerated reactors in series (R1, R2, R3) at different aeration regimes (On/Off cycles). Values are the means of two replicates (error bars indicate standard deviations) and are calculated on the data reported in Fig. 4 and on DGGE analysis showed in Fig. 6, for protozoa and bacteria, respectively. Pairwise comparisons were performed by the Tukey test ($P \leq 0.05$): same lower case and upper case letters denote absence of statistical significance between different aeration regimes within the same reactor (i.e., R1, R2 or R3) and between the three reactors within the same aeration regime.

index was only 3 thus confirming the anomalous functioning of the oxidation sector of the plant at this aeration condition.

Under the aeration regimes 30/30 and 30/45, the attached ciliates were prevalent with a marked increase of *S* as compared to the above aeration regime (Fig. 5A); also the *H* index increased but only in R1 (Fig. 5B). The SBI index reached values of 9–10, thus indicating full recovery of the microfauna functionality (Madoni, 1994) even if the community presented a different structure. In fact, the crawling ciliates, abundant before the onset of anoxia, were replaced by the attached ciliates after restoration of optimal aerobic conditions.

Under the aeration regime 30/60, though the condition was not as strictly anoxic as under the 15/45 regime, the ciliates population varied in a similar way showing again species belonging to the genus *Trepomonas* with contemporaneous decrease of *S* and *H* index. The SBI index value was between 4 and 5, thus confirming an anomalous functioning of the biological compartment. However, the predominance of flagellates, generally associated to low treatment performances (Madoni, 1994, 2003; Seviour and Nielsen, 2010) can not be considered a rule: Perez-Uz et al. (2010), in fact, found that the N-removal performance was highest when flagellates were prevalent in full-scale wastewater plants.

Varying the aeration regimes, the protozoa cell density of the activated sludge was always over 1×10^6 cells l^{-1} independently of the aerobic (45/15 e 15/15) or anoxic (15/45) conditions. The 30/30 and 30/45 regimes showed high cell densities, with the only exception of R3 at the latter regime (Fig. 4A–C), due to the presence of sessile ciliates, particularly belonging to the genera *Carchesium* and *Zoothamnium*, protozoa characterized by colony growth (Miao et al., 2004). Under the aeration regime 30/60 the sessile ciliates disappeared leading to a lower total cell number.

It is interesting to note that the relationship between the *S* values of the protozoa populations and the COD removal efficiencies, as already observed by Madoni (1994), showed a positive correlation ($R = 0.766$, $P < 0.001$) (Fig. 6A); also the *H* index showed similar behavior but with a lower correlation coefficient ($R = 0.619$, $P = 0.001$) (Fig. 6B).

3.4. *Effect of the aeration regime on the bacterial community*

The dynamics of the bacterial communities in the three reactors (R1, R2 e R3) following the variation of the aeration regime were studied by PCR-DGGE analyses of the 16S rRNA genes (Fig. 7A); *S* and *H* index are shown in Fig. 5C and D, respectively; the cluster analysis is reported in Fig. 7B.

As also observed for protozoa, the three reactors showed, independently of the aeration regime, similar bacterial

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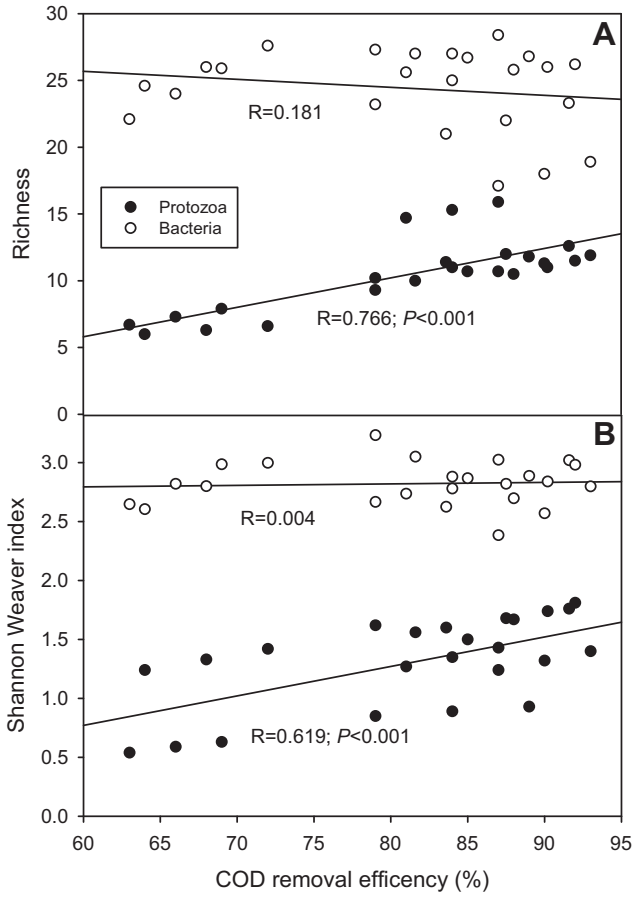

Fig. 6 – Relationship between COD removal efficiency and Richness (A) and Shannon–Weaver index (B) of protozoan and bacterial populations in the reactor R1 for all samplings. Correlation coefficients (Pearson) and their levels of significance are reported on the graph.

communities (similarity >76%) (Fig. 7B), likely due to the fact that they worked in series; in fact, common bacterial populations settled in the three reactors responding in the same manner to different inputs of organic loads and to different operational conditions.

On the contrary, the variations of aeration regime had great impact on the bacterial communities as clearly indicated by the low similarity (ranging from 38 to 74%) among the DGGE profiles. Our results are in good agreement with the findings by McGarvey et al. (2007) who, using 16S rRNA gene sequence libraries, studied the bacterial population dynamics during treatment of dairy waste. Interestingly, the condition changes did not appear to affect the overall amount of different bacterial populations, as indicated by the biodiversity indexes S and H, which remained stable (Fig. 5C and D). In fact, and differently from the protozoa population, the bacterial community biodiversity indexes showed low correlation with the removal efficiency ($R = 0.181$ and $R = 0.0065$, respectively) (Fig. 6A and B). Recently, Denecke et al. (2012) have reported that two activated sludge reactors, one intermittently and one continuously aerated, showed similar efficiencies but the former was characterized by a five-fold higher species richness, thus suggesting the limited relevance of the overall number of bacterial species.

Decreasing the oxygenation from 90.6 to 60.4 kg O_2 d^{-1} (45/15 and 15/15 regimes, respectively) caused noticeable changes in the bacterial community structure (similarity, 55%) even though did not lead to loss of removal efficiency of the organic and ammonium loads (Fig. 2A and B).

Comparing the 15/15, 15/45 and 30/30 aeration regimes, characterized by predominantly aerobic, anoxic and aerobic conditions, respectively (Table 2), marked effects on the bacterial community composition were observed as also confirmed by the variations of the plant performance (Fig. 2). DGGE profiles and related dendrogram (Fig. 7A and B, respectively) showed that the similarity between the two regimes 15/15 and 15/45 was low (61%) probably due to the unfavorable change in the aeration regime that caused an important decrease in the plant performance. It is particularly interesting to note how the return to favorable

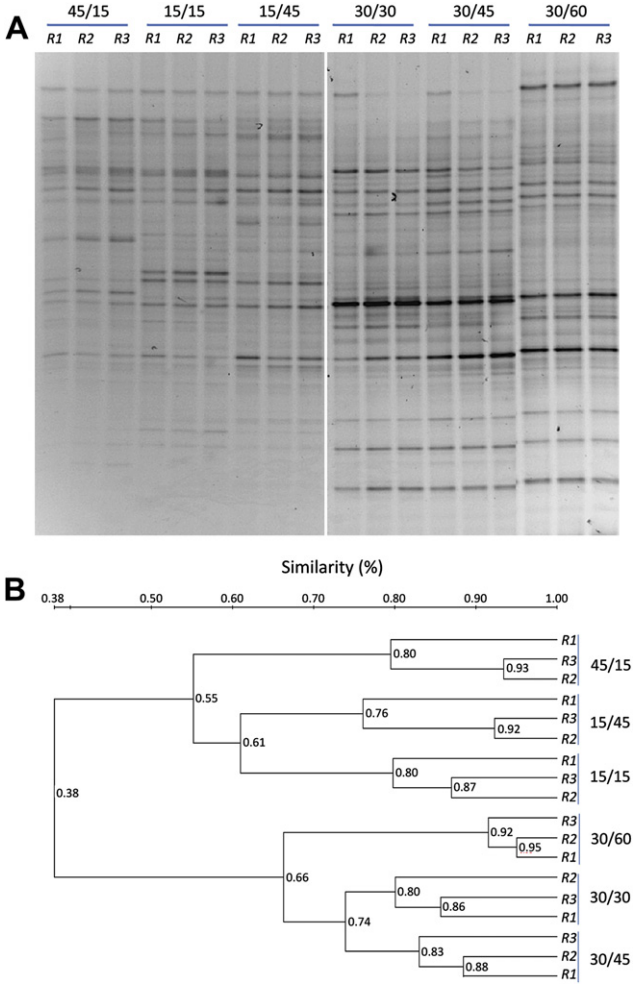

Fig. 7 – A, B - DGGE analysis of the bacterial populations in the three aerated reactors in series (R1, R2, R3) at different aeration regimes (On/Off cycles) (A). Cluster analysis obtained from the DGGE profiles based on the averaged similarity matrix (B). Scale indicates the degrees of similarity along of the nodes.

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conditions (regime 30/30 with 60.4 kg O_2 d^{-1}) was able to restore ideal performances but not to make the bacterial community recuperate the initial structure. In fact, the bacterial community changed its own structure in an even more marked way so that the similarity between the profiles obtained in this condition and the first two (15/15 and 15/45) was only 38%. This behavior of the bacterial community is similar to what observed for the protozoa. In both cases, in fact, the restoration of optimal oxygenation after a period of anoxia led to the recovery of optimal plant performance but not to the re-settling of the same microbial populations. This is somehow different from the ecological interpretation of the bacterial populations dynamics of Marzorati et al. (2008) who suggested that broad changes in bacterial community structure might cause loss of overall coherence.

In the passage from 30/30 to 30/45 and then to 30/60 regimes, the aeration conditions changed from favorable to unfavorable. The first two conditions, however, showed similar DGGE profiles (74%) and, in fact, the plant performances remained optimal despite the reduction in oxygen supply (Fig. 2). On the contrary, at the 30/60 regime the DO levels were sufficiently low to cause a clear decrease in the COD removal performance to which corresponded a marked variation of the bacterial community (similarity, 66%).

As already found by other authors (Lee and Oleszkiewicz, 2003), changes in aeration conditions did not cause relevant variations of the total number of heterotrophic bacteria, assessed by viable counts (data not shown).

4. Conclusions

To the best of our knowledge, this is the first investigation carried out in a real scale dairy WTP aimed at studying the effects of aeration regime variations on the degradation performances and the microbial communities of the activated sludge, including both bacterial and protozoan populations. The following main evidences can be highlighted: i) of the six aeration regimes tested, best performances were obtained at 30/45 (45.4 kg O_2 d^{-1}), while higher amounts of oxygen did not lead to significant performance increases; ii) with aeration regimes 30/60 (40.2 kg O_2 d^{-1}) and 15/45 (30.2 kg O_2 d^{-1}) serious losses of performance were recorded; iii) these anoxic conditions caused reduction in protozoan diversity and modification in the community structure (prevalence of flagellates of the genus *Trepomonas* at the expense of the mobile and sessile forms) which resulted in a less efficient activated sludge but, as the oxygen was brought back to adequate levels, the ciliate population quickly recovered a more performing configuration; iv) varying aeration regimes did have marked effect on the bacterial community structure although the overall amount of bacterial diversity (based on the *S* and the *H* indices) remained stable.

It can be concluded that, although aeration regime variations strongly influence the structure of both protozoan and bacterial communities in the activated sludge, a high biodiversity among the protozoan population is fundamental for reaching high plant performance.

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