Efficiency of nitrogen removal and protist communities: the potential for introduction of novel biological index

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ABSTRACT

The study of three WWTPs with different nitrogen removal systems during a year has provided valuable information on the characteristic protists communities present in these systems. Population density and diversity are quite different from other wastewater systems and flagellates and amoeba besides ciliates are important members of these N-removal systems. In fact, efficient processes on N-removal, unless what it has been usually found in conventional systems, show large flagellate and amoeba populations, and these populations tend to diminish as nitrogen removal performance is moderate to low. Certain groups and genera of protist are proposed as indicative of high or low performance N-removal besides organic matter removal efficiencies. This information is a valuable step to develop predictive tools easy to use in the day to day laboratory protocols in wastewater treatment sites.

1. INTRODUCTION

The use of protists as indicators of the state of wastewaters treatment plants has been a recurrent idea in the evaluation of biological systems (Curds & Cockburn, 1970b; Kinner et al., 1989; Madoni, 1991; Madoni, 1994a; Luna-Pabello et al., 1990; Esteban et al. 1990; Al-Shahwani & Horan, 1991; Salvadó et al., 1995; Martín-Cerceda et al., 2002; Lee et al., 2004). Environmental conditions within the biological reactors are determinant on which protists species are able to survive. This is why in conventional systems protists and secondarily filamentous dominant bacteria are good bioindicators both of the quality of the purified water and of the biological state of the aeration tanks (Poole, 1984; Luna-Pabello et al., 1996; Chen et al., 2004; Zhou et al. 2006). Therefore a microscopic study of the activated sludge is advisable since a fast observation of the biological treatment conditions would allow an immediate management reaction (Salvadó, 1994)

Nowadays Wastewater Treatment Plants (WWTP) with advanced nutrient removal systems are the alternative being used, seeking to implement the EU Water Framework Directive (2000/60/EC) to prevent further deterioration of aquatic ecosystems. Although it has been assumed that microbial populations in different biological systems must be similar, the fact is that these change clearly with the type of biological treatment to be assessed. Conventional activated sludge microbial populations (Curds & Cockburn, 1970a; Esteban et al., 1991; Madoni 1994b; Martín-Cereceda et al., 1996) have different biological characteristics to those reported in biofilms (Kinner et al., 1988; Pérez-Uz et al., 1998; Martín-Cerceda et al., 2001) or in advanced treatments for nutrient removal (Pérez-Uz et al., 2007; Serrano et al., 2008; unpublished results). Ecological differences in the advanced nutrient removal systems combine aerobic, anoxic and anaerobic stages with distinct strategies of sludge recirculation. This means that previous studies of conventional plants cannot be directly extrapolated to these types of treatment.

Advanced treatments for the elimination of N forms with activated sludge from Spain were assessed during a year in three plants with different designs through collaboration between universities and water companies. This study included the characterization of the biological communities with identification and enumeration of all type of protist (ciliates, flagellates and amoeba) and the recordings of physical-chemical parameters generally used to assess performance of the treatments and especially those related to N forms. These results have been explored to characterize the communities in this systems and to assess possible correlations or associations between biological variables or biological and physical-chemical variables that would allow suggesting bioindicators in these biological systems.

2. MATERIALS AND METHODS

2.1. Wastewater treatment plants

Three WWTPs with different nitrogen removal systems and geographical locations were sampled in Spain during a year. Plants selected were all activated sludge plants with advanced systems for nutrient elimination from different regions in Spain (Sevilla, Valencia and Barcelona). These plants were all designed so that biomass is cycled continuously through alternating oxic, anoxic and anaerobic zones helping the development of nitrifying/denitrifying bacterial populations (Figure 1). All of them receive mainly domestic inputs.

2.2. Activated sludge WWTP samples

Sampling was carried out once a month during a year (May 2007 to July 08). For biological assessment, samples were collected in the final track from the aerobic zone of the biological reactor (Fig. 1) in a container leaving at least half of the container volume empty. Physical-chemical parameters were analyzed before and after the biological treatment.

2.3. Protists counting and microscopic characterization

Samples, maintained under aeration conditions, were analysed.within three hours of sampling. Two replicates of 25 µl were set on a microscope slide below a coverslip and counting was done under x100 (exceptionally x400 for temptative identifications) phase contrast microscopy. Photo and video recordings of alive and silver or flutax stained specimens were used to confirm species identification.

2.4. Physical-chemical analysis

Physical-chemical parameters (BOD_5, COD, $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and Total-N/Kjeldahl N) were analyzed following the standard methods (APHA, 1998).

2.5. Statistical analysis

Univariate and multivariate analysis were carried out with the statistical software packages Statgraphic Plus v.5.1 and, SPAD v.7.0. Since absolute abundances showed large magnitude differences in some of the counts, relative abundances (as a percentage of total protists present) were used (Parker & Arnold, 1999). Only those biological variables (protist counts) that showed a frequency of appearance in samplings higher than 20% were selected for multivariate analysis. Principal Component Analysis (PCA) was used for the ordination of samplings based on biological variables and then factors accounting for at least an explained 80-90% cumulative percentage were selected for Hierarchical Cluster Analysis (Nearest Neighbors, RECIP module in SPAD v.7.0) using then physical-chemical variables as supplementary continuous variables. Cluster partitions were set and description and characterization of sampling clusters were assessed with a t-test to define biological and/or physical-chemical variables significantly associated to them.

The total number of taxons, individuals and the Shannon–Weaver diversity index (Shannon and Weaver, 1963) were calculated for each sample site and for each group of protist considered (flagellate, amoeba and ciliates) with the software package PAST v.1.89. Relationships between these variables were stablished through simple regression analysis.

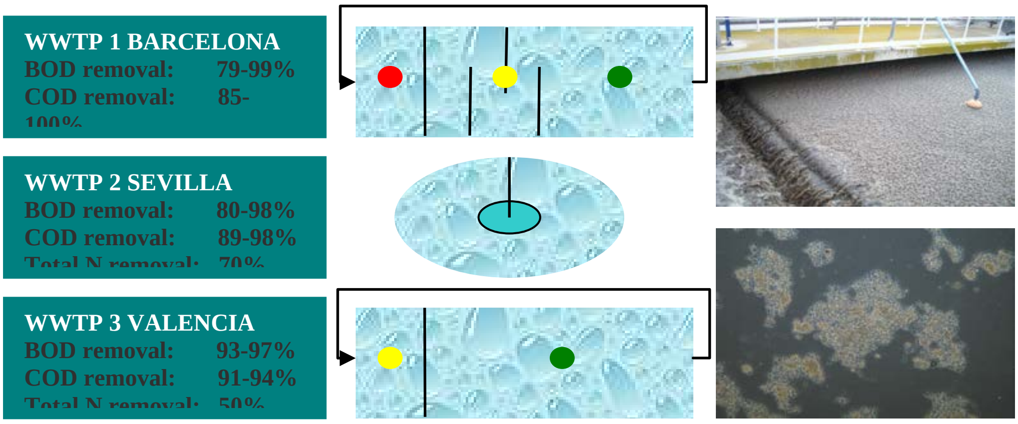

Figure 1. Mean efficiencies (BOD_5, COD, nitrogen removal) of the three WWTP. Biological reactors designs, aeration tank and phase contrast microscopic appearance of flocs.

3. RESULTS

Data collected showed that the protist community associated to the oxic zone of the biological reactor where de nitrification process occurs, had a different composition compared to that found in a conventional WWTP.

3.1. Flagellates

These advanced systems showed that flagellates represent a stable population within the biological reactor (87% of relative cell density), and their density decrease drastically as nitrogen removal performance is moderate or low. Percentage of occurrence of the flagellate populations showed that the number of taxa increases when efficiency on N-removal increases (p=0.01). The bodonids were the most representative flagellate group (Figure 2a), being present in more than the 50% of the samples when nitrification performance was higher than 85% (absolute frequency data confirmed the significant presence of bodonids in the total protists population). In fact, the bodonids were significantly associated (p<0.0001) to high performance on N elimination (both N-NH_4^+ and Total-N/Kjeldahl N) and BOD_5 removal typical in most Barcelona plant samples and in some Sevilla samples. A reduction in N-removal efficiency also diminished significantly both occurrence and density of the flagellate populations (p=0.0159).

3.2. Amoeba

Free living amoeba –both naked and testate amoeba- were also a representative group and their percentage of occurrence (11%) decreased as N-removal efficiency decreased, specially the occurrence of *Arcella* species (Figures 2b-d). Percentages of occurrence within the samples of both types of naked amoeba (larger and smaller than 50 µm) were similar, however, small amoeba were the most abundant populations (ind/ml). These small amoeba and specially those under 20 µm size, showed a significant association (p<0.01) to high performance on N elimination (both N-NH_4^+ and Total-N/Kjeldahl N) and BOD_5 as it happened with bodonids. Occurrence and abundance of amoeba decreased significantly as the nitrogen removal efficiency was reduced (p=0.0101).

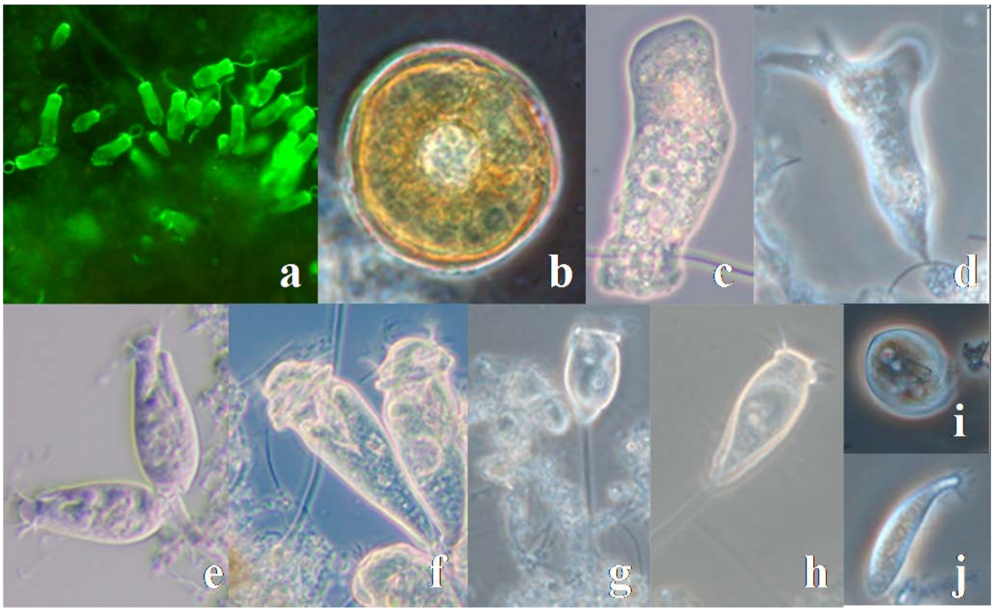

Figure 2. (a) Small flagellates, (b) *Arcella*, (c-d) naked amoeba (>50, <50 µm), (e) *Opercularia articulata*, (f) *Epistylis chrysemidis*, (g) *Vorticella aquadulcis* complex, (h) *Vorticella infusionum* complex, (i) *Trochilia minuta*, (j) *Acineria uncinata*.

3.3. Ciliates

Ciliates were the group of protists with the highest species diversity, although they presented lower total abundance when compared to other waste water treatment processes. Data referred to percentage of occurrence showed that the ciliate community was diverse and that all the typical ciliate groups described in conventional wastewater treatments were present. However, ciliates showed a very low abundance (5 %) in samples with a good nitrification performance compared to other protist populations, the tendency was to decrease with higher N-NH_4^+ elimination (p=0,011). The diversity of this group was found to decrease as well with an increased BOD_5 elimination performance (p=0.005)

The most representative species in terms of percentage of occurrence and abundance were the Peritrichids (Figures 2e-h). From these ciliate populations only *Pseudovorticella elongata* and *Opercularia articulata* (Figure 2e) appeared associated to higher N-removal efficiencies, although these were not statistically significant.

The crawling ciliate community was composed by few species of litostomates and cirtoforids (belonging to phylopharynges), specially *Acineria spp.* and *Trochilia minuta* (figures 2i,j) although these did not appear to show a significan relationship to N-removal. However, samples with low N removal (<50%) showed low number of species, oligohymenophores –mostly scuticociliates- reached percentages of occurrence not observed in good performance situations and phylopharynges cirtoforids were substituted by the "classical crawling " hypotrichs (*Aspidisca* and *Euplotes*).

The free swimming prostomatid ciliate, *Plagiocampa rouxi*, appeared to show as well an association to high N elimination, although no statistical significancy was found either.

3.4. Community analysis

Results obtained from hierarchical cluster analysis on the communities observed in the different samples indicated that two groups were clearly supported. These included a first group with those samples with high performance on N and BOD_5 removal (Figure 3) and another cluster with lower performance results on the same physical-chemical parameters. First cluster grouped all the samples from the WWTP in Barcelona and some samples from the Sevilla plant. The second cluster grouped all samples from Valencia and some from Sevilla. Within those good performance samples the main statistically significant associations found were those of Bodonids and small amoeba (<20 µm) to nitrification. Although, as it has been mentioned, the PCA results showed as well that ciliates *Pseudovorticella elongata* and *Opercularia articulata* appeared to be associated to higher N-removal efficiencies, these were not statistically significant. However, results also indicated that *Uronema nigricans*, *Gastronauta membranaceus*, and the peritrics *Vorticella aquadulcis* and *Epistylis balatonica*, were asociated to high COD removal, that occurred when lower nitrificant performance was going on in those samples (p=0.0039) (Fig. 3).

The second cluster confirmed the results obtained in the first cluster, indicating that those ciliates, flagellate or amoeba populations associated to the second factor heavily associated to COD where more abundant in those samples with worse nitrification performance than those population of bodonids or small amoeba (<20 µm) that were practically absent.

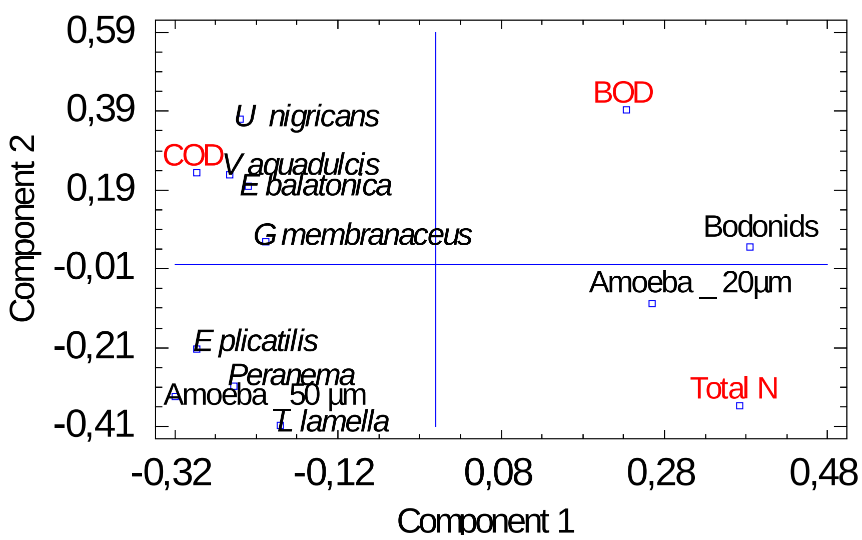

Figure 3. Graphic representations of the first two components from the PCA analysis. Only those significant variables from first cluster analysis results have been selected.

4. DISCUSSION

The results obtained in systems designed for nitrogen removal show that protist communities are quite different from those found in other conventional systems specifically those with activated sludge (Curds & Cockburn, 1970a; Esteban et al., 1991; Madoni, 1994a; Serrano et al., 2008). Data collected have shown that the protist community associated to the oxic zone of the biological reactor, where de nitrification process occurs, had a different composition compared to that in other conventional WWTP.

First of all, these observations indicated that one of the main components of protists populations in advanced treatments are not only ciliates, since amoeba and flagellates become representative members of the stable populations. This observation differs clearly from conventional systems (Curds & Cockburn, 1970a; Esteban et al., 1991; Madoni 1994b; Martín-Cereceda et al., 1996). Flagellate and amoeba in activated sludge conventional systems have been cited as unusual, except in those highly loaded plants during start-up stages of a plant or temporary overloading. In fact these protists have always been associated in these conventional systems to deficient performance and therefore have always been considered as indicators of a functional deficiency (Curds & Hawkes, 1975). However, amoeba and flagellates in advanced systems for nutrient removal seem to play a different role, becoming representative members of the stable populations and are also clearly associated to good performance on N-elimination. Therefore are appropriate candidates to take into account for the development of a performance associated index in these systems. These flagellates, specifically Bodonids and small amoeba (<20 µm) are predators of bacteria and they might keep other bacterial populations at lower levels, allowing a better performance of the nitrificant bacteria within the flocs (Mitchel et al., 1988). This fact has also been observed in other ecosystems (Verhagen et al., 1994), and it seems that the bacterivory might enhance mineralization of immobilized nitrogen in this way. Although nitrifying bacteria might also decrease in presence of flagellates through selective predation (Pernthaler et al., 1996, Verhagen, 1992, Mitchel et al., 1988) our results indirectly indicate their activity must increase.

Ciliate populations were still the main components of protists communities in these advanced treatments, however, they were less abundant and diverse than in other wastewater biological systems (Curds & Cockburn, 1970a; Esteban et al., 1991; Madoni 1994b; Martín-Cereceda et al., 1996; Kinner et al., 1988; Pérez-Uz et al., 1998; Martín-Cerceda et al., 2001). The lower abundance found in samples with a good nitrification and BOD_5 performance compared to other protist populations was initially quite surprising and the result obtained in this case is that some of them could be used as indicators of a lower performance of the system, since until now no significant association to N-removal has been found. These unexpected results are obviously a characteristic that could be used nonetheless as a part of an index design as well. Other studies have found that ciliates do not seem to have an influence on abundance and growth of nitrifying bacteria and nitrification (Neubacher et al., 2008), and our initial results seem to confirm those observations.

On the other hand, it has also been observed that some free swimming ciliates, that were also considered as indicators of bad performance as flagellate and amoeba, in this study seem to be associated to good nitrification performance. This was the case for the predator *Plagiocampa rouxi*, a prostomid ciliate predating on large particles. It is interesting to point out that other prostomids like species of the genus *Coleps* have been found to indicate good N-removal (Poole, 1984; Madoni et al., 1993).

Finally, the approach to explore the results through multivariate statistical techniques has allow to find associations between biological variables or biological-physical-chemical variables which indicate that in this case Bodonids and small amoeba could be appropriate indicators of good performance in these systems, while it has also shown that certain ciliates might be also representative of lowered N-removal performance.

These promising results should be explored further to design a biological index considering flagellate and amoeba population plus those negatively associated ciliates. Some ciliates could also be possible candidates as indicators of good performance as well as flagellates and small amoeba, however further sampling effort will be needed to really confirm statistically significant relationships of these ciliates to N-performance.

These results are a valuable step to develop predictive tools easy to use in the day to day laboratory protocols in wastewater treatment sites. The final goal will be to establish and test an experimental protocol through an inter-laboratory reproducibility assessment involving specialists from water companies and university.

5. CONCLUSIONS

Bodonids and small amoeba (<20 µm) are important members of the biological populations within nitrogen removal systems, and they indicate good rates of nitrification in these systems. Although some ciliates also appear to be related to N-removal efficiencies, no significant results were obtained with these data so far, therefore futher sampling efforts, might allow reaching better understanding of these relationships.

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