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Effects of Shock Loads of Salt on Protozoan Communities of Activated Sludge

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Summary. The effects of wastewater salinity variations on communities of microorganisms taken from activated sludge were studied. Batch cultures were grown for 96 h at final salt concentrations of 3, 5, 10, 20 and 40 NaCl g/l. Protozoa and small metazoa was counted and ciliated protozoan species in these cultures were identified. An increase in salt concentration from 3 to 10 g/l gradually affected the microbial community and few protozoa and metazoa survived at 96 h. Ciliate abundance was species dependent: *Vorticella* spp. and *Opercularia articulata* resisted the high dosages of NaCl better than other ciliates. Total ciliate abundance and diversity fell drastically at 20 g/l, which would compromise reliability in activated sludge processes. At 40 g/l neither protozoa nor metazoa survived after 24 h. This study evaluates the effects of a short-term NaCl shock on the dynamics of activated sludge microorganisms and their community composition. The study also contributes to the understanding of the wastewater treatment process.

Key words: activated sludge, ciliates, microfauna, salinity tolerance, wastewater treatment.

Abbreviations: BOD_5 - five-day biochemical oxygen demand, MLSS - mixed liquor suspended solids, MLVSS - mixed liquor volatile suspended solids.

INTRODUCTION

Wastewater may have high salt contents due to industrial shock loads or occasional sea intrusions. Several studies concluded that shock loads of 0.5-5% reduce the effectiveness of the biological processes in wastewater treatment plants (Lawton and Eggert 1957, Ludzack and Noran 1965, Hall and Smallwood 1967, Kincannon and Gaudy 1968, Burnett 1974). These studies also conclude that rapid shifts in salt concentration typically cause more problems than gradual shifts and also that shifts of 0.5-2% salt usually cause significant disruptions in system performance. Upsets may be temporary and in certain cases organisms acclimatise and give satisfactory reactor performance. Saline loads affect the metabolic functions of microorganisms and reduce the kinetic degradation in activated sludge (Mahmoud and Davis 1970, Woolard and Irvine 1995).

It seems that some acclimatisation of microorganisms to habitats with relatively high saline concentrations is to be expected (Lawton and Eggert 1957, Kincannon and Gaudy 1968, Smurov and Fokin 1999). However, this

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acclimatisation is limited, and even in acclimatised cultures, an adequate process performance depends upon a relatively constant ionic strength. In addition, rapid reductions in salt content of wastewater cause further upsets (Lawton and Eggert 1957).

Pillai and Rajagopalan (1948) reported that an activated sludge plant operated on seawater in India worked as efficiently as fresh-water plants. This plant was analysed for a period over of eight years. The constant characteristics of seawater allowed the development of high numbers of species of marine ciliates (specially those belonging to the genera *Vorticella* or *Zoothamnium*) closely related to those found in fresh-water plants. According Pillai and Rajagopalan effluent quality was closely dependent on the number of active peritrich ciliates.

There have been many studies on the treatment of saline and hypersaline waste waters (>3.5% salt) in constant concentrations (Hockernbury *et al*. 1977, Tokuz and Eckernfelder 1978) or high-polluted groundwaters (Wong 1992). These authors conclude that conventional biological processes cannot be used to treat wastewater containing more than 3-5% salt. However, Woolard and Irvine (1994, 1995) showed that biofilms of halophilic bacteria acclimatised in constant concentrations of up to 15% salt have a great capacity for treating hypersaline wastewater. As Ludzack and Noran (1965) and Kincannon and Gaudy (1968) described, the absence of protozoa in this medium causes the effluent to deteriorate and the suspended solids to increase.

Zobell *et al*. (1937) and Zobell (1946) showed that most wastewater and soil microorganisms die in hypersaline media, and that their survival increased when the salt concentration went down to 3.5 g/l. With regard to protozoa, only experimental studies on natural communities or on single-species cultures have been conducted. Finley (1930) studied the effect of salinity on communities of protozoa from continental waters by diluting seawater. He showed that there was a high mortality when different species were subjected directly to the different saline concentrations studied. In contrast, there was very much better survival when salt content was increased gradually. Smurov and Fokin (1999) studied resistance and tolerance to salinity in 10 species of *Paramecium*, recording the adaptation after prior acclimatisation from 2.1 g/l salt for *P. putrinum* to 18.7 g/l salt for *P. woodruffi*, with a period of acclimatisation not less than 48 h. Studies from Bick (1964), Ax and Ax (1960), Foissner *et al.* (1995) and Albrecht (1984) provided us with a synthetic description of the ranges of salinity found in natural communities for various species of ciliated, flagellated and rhizopod freshwater protozoa.

The objective of this paper is to investigate the effects of shock loads of sodium chloride (between 3 and 40 g/l) on the populations of eukaryotic microorganisms (protozoa and small metazoa) that constitute the activated sludge communities.

MATERIALS AND METHODS

Using activated sludge from a pilot plant for urban wastewater treatment, the effects of several concentrations of NaCl were studied through batch cultures at 20-22°C, pH 7-7.3, 2-5 mg O_2/l and about 900 mg MLSS/l. For each concentration two replicas were placed in 250 ml Erlenmeyers with 60 ml of sludge, 30 ml of synthetic waste water (see composition on Table 1) and 10 ml of saline solution, in order to reach concentration of 3, 5, 10, 20, 40 g NaCl/l, and a control containing synthetic wastewater only (0.4g NaCl/l). After settling, and for the next five days, 40 ml of the supernatant was removed every 24 h and replaced by 30 ml of synthetic wastewater plus 10 ml of saline solution in order to maintain the respective concentrations. The Erlenmeyers thus worked as mini sequencing batch reactors (SBR).

The characteristics of the plant from which the activated sludge came were: an average of 0.7 g/l of salt in the inflow, an hydraulic retention time of about 7 h, and a mass load of 0.25 g BOD_5/g MLVSS d. During the bioassay, the hydraulic retention time was about 16 h and the mass load was 0.1 g of BOD_5/g MLVSS d.

Through optical microscopy, the microorganisms were counted every 24 h for 5 days after the first salt application. These counts were performed in three 25 µl subsamples. The results are given in individuals/ml. The biomass (g/l) of each ciliated species was also computed, using the biomass figures for each 10^6 individuals, following Foissner *et al.* (1995). Thecamoebae, gymnamoebae and rotifers were determined according to Odgen and Hedley (1980), Page (1988), and Koste (1978a, b). Ciliates were identified following Foissner *et al.* (1991, 1992, 1994 and 1995), and using the special silver technique described by Fernández-Galiano (1994). The specific diversity of ciliates was calculated through the number of ciliate species and by means of the Shannon-Weaver diversity index (expressed in bits/individual).

RESULTS

The microfauna composition in the control suffered over time a drop in abundance and number of species (Table 2). This should be considered normal, given the differences in the figures of the operating parameters between the plant of origin and the cultures, and given the nutritional change brought about by the synthetic wastewater. Some loss of species and abundance is to be expected in all such trials. On the basis of the evolution of the control, in general the protozoa sup-

Table 1. Synthetic wastewater composition

Composition	mg/l
Peptone	256.55
Tryptone	354.24
NaCl	407.4
Na_2SO_4	44.6
K_2HPO_4	44.6
$MgCl_2 \cdot 6H_2O$	3.7
$FeCl_2 \cdot 2H_2O$	3.7
$CaCl_2 \cdot 2H_2O$	3.7
$MnSO_4$	0.057
H_2MoO_4	0.031
NaOH	0.008
$ZnSO_4$	0.046
$CoSO_4$	0.049
$CuSO_4$	0.076

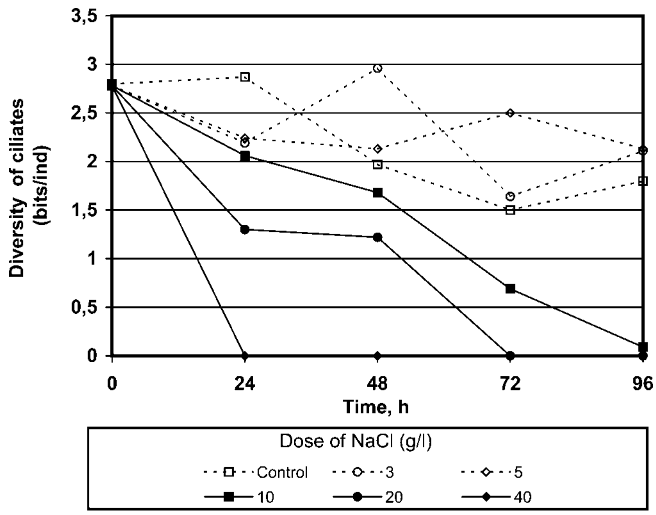

Fig. 1. Evolution of ciliate diversity of each NaCl dose at different exposure times. For all values n = 6

ported NaCl concentrations of up to 5 g/l, but survival began to fall off at concentrations of 10 g/l. Their number dropped markedly at 20 g/l, and they disappeared completely at 40 g/l.

Ciliate survival and diversity index clearly decreased at saline concentrations above 10g/l (Fig.1). However, the limits of salinity tolerance varied greatly between the organisms studied. Hence, the analysis of species provides extremely relevant information. Given the fluctuations for the different species, it was decided to express the maximum tolerances as the maximum concentration of salt in which active individuals were observed for the different times of exposure (Table 3). Within the ciliate protozoa, peritrichs were the ciliates that were most resistant to high concentrations of salt (*Opercularia articulata*, *Vorticella infusionum* and *Vorticella microstoma* supported up to 20 g/l of NaCl) (Fig. 2). However, other peritrichs such as *Carchesium polypinum* and *Vorticella convallaria* were more sensitive. The scuticociliate *Uronema nigricans*, with low density in the control (40 ind/l), increased its abundance in the doses of 5 and 10 g/l of NaCl at 24 h, and even resisted up to the 20 g/l. Thecamoebae did not adapt well to high concentrations and disappeared in doses of over 5 g/l. Large gymnamoebae (belonging to the genus *Mayorella*) behaved in a special way, diminishing after 24 h in all doses, but recovering in doses below 20 g/l (Fig. 3). Unlike the protozoa, rotifers (represented by the genera *Lecane* and *Philodina*), only survived at low concentrations (up to 3 g/l). *Lecane* was significantly more sensitive and disappeared almost entirely at 10 g/l. To the contrary, a few *Philodina* were still present up to 72 h

Table 2. Microbial community composition of the control at time 0

	Initial sludge	
	ind/ml	%
Ciliata		
Acineria uncinata	40	<1
Aspidisca cicada	20	<1
Carchesium polypinum	1020	4.54
Chilodonella uncinata	280	5.35
Chilodonellidae indet.	120	12.47
Epistylis chrysemydis	260	<1
Euplotes affinis	<10	<1
Litonotus lamella	<10	<1
Opercularia articulata	920	4.31
Oxytrichidae	20	<1
Parastrongylidium oswaldi	<10	<1
Spathidium sp.	40	<1
Tokophrya sp.	20	<1
Trochilia minuta	<10	1.58
Uronema nigricans	40	<1
Vorticella aquadulcis	80	1.05
Vorticella convallaria	1020	6.64
Vorticella infusionum	100	<1
Vorticella microstoma	140	<1
Gymnamoebae		
Mayorella sp.	4600	50.27
Thecamoebae		
Arcella sp.	40	<1
Rotifera		
Lecanidae	720	7.99
Philodinidae	100	<1
Total microorganisms	4140	100

Table 3. Maximum NaCl doses (g/l) tolerated at different culture times for different species

	24 h	48 h	72 h	96 h
Ciliates				
Acineria uncinata	10	0.4		
Aspidisca cicada	5	5	0.4	
Carchesium polypinum	20	20		5
Chilodonellidae	20	5	5	0.4
Epistylis chrysemydis	10	10	5	5
Euplotes affinis	10	10	10	10
Litonotus lamella	3	3	3	0.4
Opercularia articulata	20	20	5	5
Oxytrichidae	10	10	10	5
Parastrogylidium oswaldi	5	5	0.4	0.4
Spathidium sp.	3	0.4		
Trochilia minuta	3	3	0.4	
Uronema nigricans	20	10	10	
Vorticella aquadulcis	20	10	10	5
Vorticella convallaria	5	5	5	5
Vorticella infusionum	20	20	20	20
Vorticella microstoma	20	20	10	
Gymnamoebae				
Mayorella sp.	20	20	20	10
Thecamoebae				
Arcella sp.	10	5	3	3
Rotifera				
Lecanidae	10	10	10	5
Philodinidae	10	5	5	1

at 10 g/l, and they only completely disappeared from 20 g/l (Fig. 4). The small flagellates, though few in number, maintained their numbers up to the 20 g/l dose.

Figure 5 shows the total biomass profiles of ciliates per dose (biomass-dose) and total abundance of ciliates per dose for each period (abundance-dose). Within each dose, the behaviour of total biomass between 72 and 96 h was similar. The higher values of total biomass were concentrated in the 3 g/l and especially in the 5 g/l salt dose. Although the tendency in biomass and abundance between 72 and 96 h were similar, while biomass clearly decreased at doses above 5g/l, abundance continued to increase up to 10 g/l. From 10 g/l, both parameters dropped together until extinction at 40 g/l, although as in the 10 g/l doses, at 20 g/l there was also a relative increase in abundance and biomass over time.

The low total biomass figure in the dose of 3 g/l of NaCl at 48 h is due to the marked fall in abundance of the ciliates identified as Chilodonellidae (mainly *Chilodonella uncinata*). At this dose, at 48, 72 and 96 h, total abundance was less than at 5, 10 and 20 g/l of salt. In the 5 g/l dose, the abundance trend was set by the high figure for *Carchesium polypinum* at 48 h and by an explosion in the abundance of *Euplotes affinis*, *Vorticella infusionum* and Oxytrichidae at 72 and 96 h. At 72 h there was an especially significant drop in the abundance of *Opercularia articulata*. At the 10 g/l dose, the explosion of *Vorticella infusionum* and *Vorticella microstoma* was confirmed. The particularly high value of these two species at 96 h should be noted. At this dose both total abundance and biomass declined at 24 h. Finally, at 20 g/l, a minimum was found at 24 h in total biomass and total abundance. Both of these values recovered up to 72 h.

DISCUSSION

The results of this study show that the tolerance limits to salinity variations vary greatly for the organisms found in sludge. In general, a considerable number of species displayed a high range of tolerance to salinity and supported well concentrations of salts (up to 5-10 g/l) considerable above the normal figures for domestic wastewaters (0.2 to 0.5 g/l; Henze *et al*. 1995). Freshwater ciliates, along with some metazoan species, regulate their internal ionic composition and their salt limit is probably 5 g/l (Smurov and Fokin 1999). This limit is consistent with the results of the present study, in which some species tolerated even higher concentrations: for example, the peritrichs *Vorticella microstoma* and *Vorticella infusionum* survived salt concentrations higher than 10 g/l for over 48 h. In contrast, only a few species were intolerant to salinity conditions of 5 g/l. Within the same taxonomic group, differences were also found in response to different concentrations of NaCl. For example, the various species of the genus *Vorticella* responded differently to increasing salt concentrations: in particular, smaller species (*Vorticella microstoma*, *Vorticella infusionum* and *Vorticella aquadulcis*) were more tolerant than bigger ones (*Vorticella convallaria*). This may be due to differences in their ecological strategies.

Our results show that, at salt levels up to 20 g/l, a succession of species can be found in the sludge, with more tolerant species can developing due to the reduction or disappearance of more sensitive species. This change implies a loss of species, which is clearly reflected on the index of diversity. The usefulness of ciliate

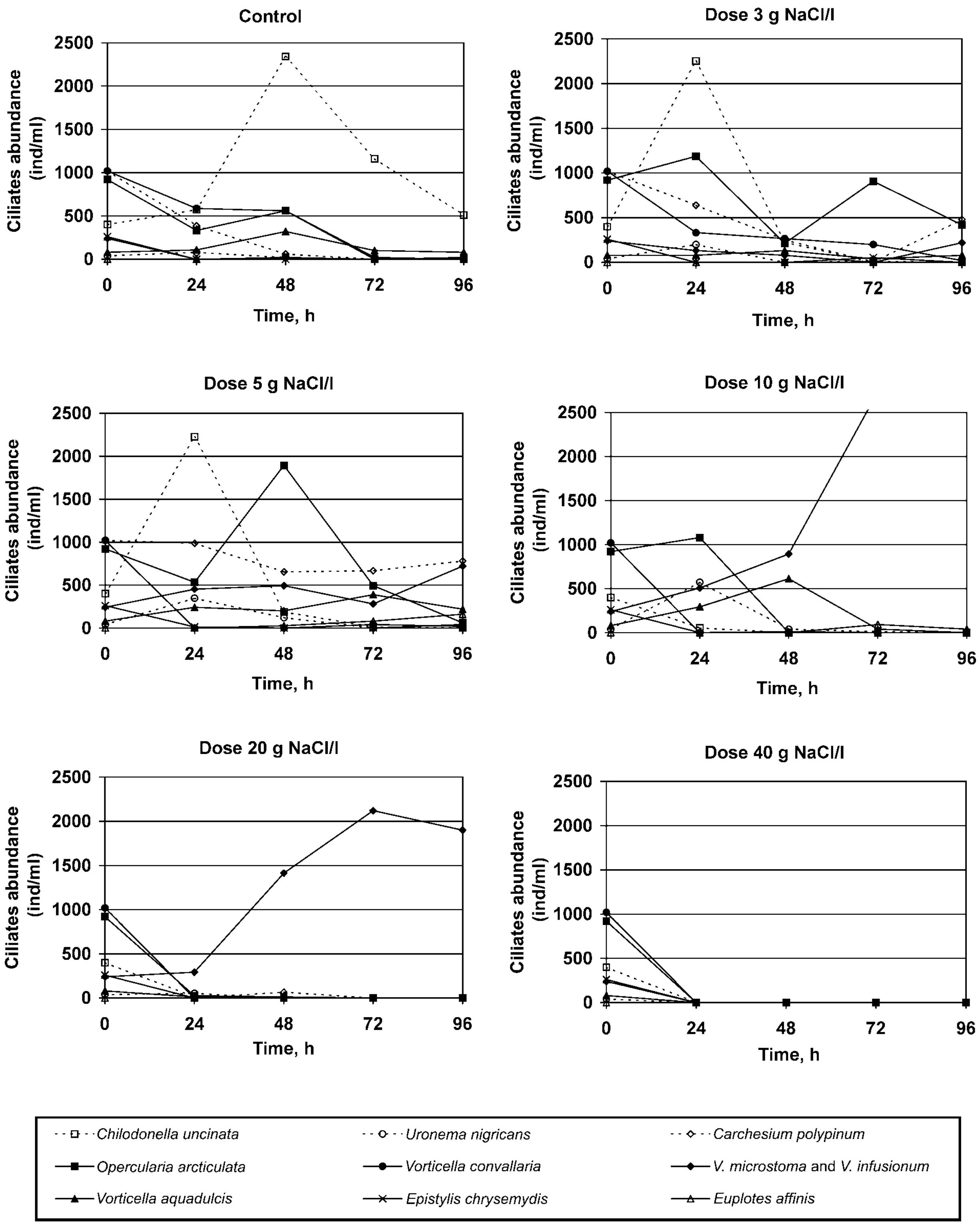

Fig. 2. Evolution of abundance of some ciliate species at different NaCl doses

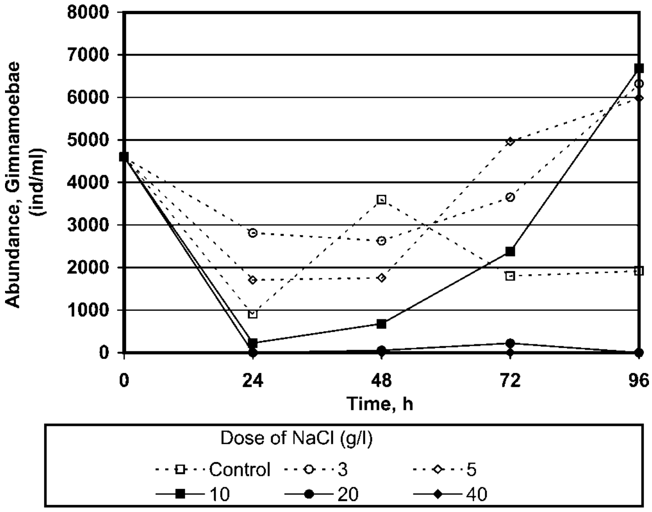

Fig. 3. Evolution gymnamoebae abundance at different NaCl doses

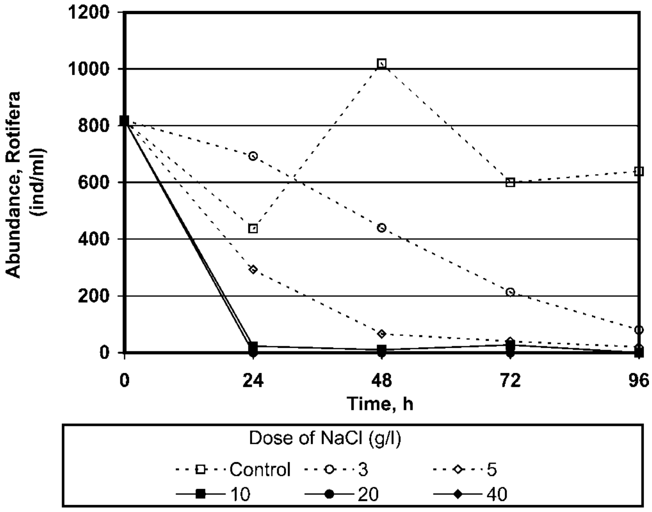

Fig. 4. Evolution of rotifer abundance at different NaCl doses

has been reported for assessing the stress effects caused by toxic substances (chloride and heavy metals) in studies of protozoan communities in activated sludge and other freshwater communities (Fernández-Leborans *et al.* 1988; Gracia *et al.* 1994; Madoni *et al.* 1994, 1996; Salvadó *et al.* 1997, 2000). Our results indicated that diversity decreases significantly above 10 g/l salt and with increased time of exposure. This could be a good indicative parameter for the effects of saline shocks, but only in determining high salt loads.

Nevertheless, the development at higher salt doses of the less sensitive species can be taken as a process of acclimatisation to the sludge, thus enabling ciliates to maintain high biomass figures at up to 10 g/l salt. Several authors (Lawton and Eggert 1957, Kincannon and Gaudy 1968, Smurov and Fokin 1999) already showed that some acclimatisation to habitats with relatively high saline concentrations in ciliate protozoa and other micro-organisms from activated sludge could be expected. We found many examples supporting this such as *Euplotes affinis* and *Mayorella* sp., which increased their populations at doses between 5 and 10 g/l; two kinds of behaviour, however, should be distinguished. At 24 h the gymnamoeba *Mayorella* sp. suffered high and progressive mortality at concentrations between 3 and 20 g/l, but its population then gradually recovered up to doses of 10 g/l, which can be seen as a genuine acclimatisation. The ciliates *Euplotes affinis* and *Vorticella infusionum*, in contrast, did not apparently suffer great losses at 24 h, and over time they increased their populations (between 5-10 g/l in *Euplotes affinis* and up to 20 g/l in *Vorticella infusionum*), which is better considered as a gradual succession of species.

The effect of sodium chloride depends on the ecological characteristics of each species, which are summarised in Table 4. This shows that peritrichs, generally β-mesosaprobic to polysaprobic, are the ciliates that best tolerate high concentrations of sodium chloride (up to 20 g/l in the case of *Opercularia articulata*, *Vorticella infusionum* and *Vorticella microstoma*). These ciliated peritrichs are also indicators of high mass loads (0.4-0.7 $gBOD_5/gMLVSSd$), and display even greater tolerance under stress and in the presence of toxic substances such as heavy metals (Cr, Zn, Cu) and chlorine (Sudo and Aiba 1973, Salvadó *et al.* 1993, Gracia *et al.* 1994, Madoni *et al.* 1996, Salvadó *et al.* 2000). They are very abundant in plants with contributions of wastewater tipped by industry and loaded with toxic substances (Esteban *et al.* 1990, Becares 1991). *Opercularia articulata*, a β-α-mesosaprobic and oligostenohaline species (Foissner *et al.* 1995), increases more than the control at doses of 3 g/l and 5 g/l, with some specimens found even at 20 g/l after 48 h exposure. We thus found that *Opercularia articulata* adapted to salinity concentrations higher than those previously reported (0.4 g/l according to Foissner *et al.* 1995: in our study it survived up to 20 g/l). However, other peritrichs such as *Carchesium polypinum* and *Vorticella convallaria*,

Table 4. Biological and ecological characteristics of the microorganisms of this study, α - alphamesosaprobic, ß - betamesosaprobic, Al - algae, Ba - bacteria, Fl - heterotrofic flagellates, he - holo-euryhaline, os - oligo-stenohaline, oms - oligo- to meso-stenohaline, p - polysabrobic, R - predator. (1) Sládecek (1973), (2) Foissner *et al.*(1995), (3) Madoni (1994), (4) Salvadó *et al.* (2000), (5) Albrecht (1984), (6) Bick (1964), (7) Ax and Ax (1960), (8) Salvadó and Fernández-Galiano (1997)

Ciliates	Saprobity (1, 2, 6, 8)	Main food (2, 8)	Motility (3, 8)	Tolerance of chlorine g/kg·d (4)	Salinity tolerance (5, 6, 7)	Salinity tolerance range g/l (5, 6, 7)	Salinity in this study NaCl g/l
Acineria uncinata	α-p	R	crawling	0 to 15	os	0 to 1	0.5 to 10
Aspidisca cicada	β-α	Ba	crawling	0 to 10	he ?	0 to >30	0.5 to 5
Chilodonella uncinata	α	Ba, Al	crawling	0 to 5	he ?	0 to >30	0.5 a 20
Carchesium polypinum	β-α-p	Ba	attached	-	he ?-oe	0 to >30 - 0 to 10	0.5 to 20
Epistylis chrysemydis	α	Ba	attached	0 to 15	oe	0 to >10	0.5 to 20
Euplotes affinis	β-α	Ba, Al, Fl	crawling	-	he ?	0 to >30	0.5 to 10
Litonotus lamella	α	R	free-swimming	0 to 10	he ?	0 to >30	0.5 to 3
Opercularia articulata	β-α	Ba	attached	0 to 20	os	0 to 1	0.5 to 20
Parastrongylidium oswaldi	α-p	Ba, Fl, Al	crawling	-	-	-	0.5 to 5
Spathidium sp.	-	R	free-swimming	-	-	-	0.5 to 3
Tokophrya sp.	β-α	R	attached	-	os-oms ?	0 to 1 - 0 to 4	0.5
Trochilia minuta	β-α	Ba	crawling	0	os	0 to 1	0.5 to 3
Uronema nigricans	α-p	Ba, Fl	free-swimming	0 to 15	he	0 to >30	0.5 a 20
Vorticella microstoma	α-p	Ba, Al	attached	0 to 20	oms ?	0 to 4	0.5 to 20
Vorticella infusionum	α-p	Ba	attached	0 to 20	he ?	0 to >30	0.5 to 20
Vorticella aquadulcis	α-β	-	attached	-	he ?	0 to >30	0.5 to 20
Vorticella convallaria	α	Ba	attached	0 to 15	he	0 to >30	0.5 to 5

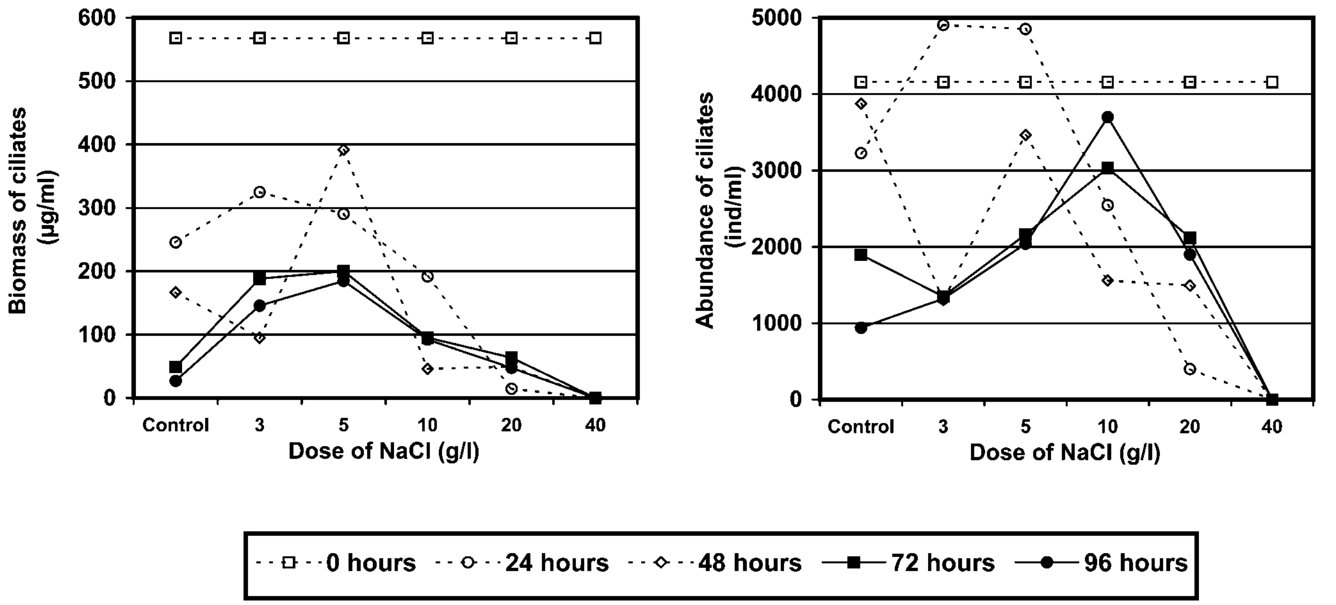

Fig. 5. Biomass and abundance of ciliates for different NaCl doses at different exposure times. Control (0.4 g NaCl/l) and D3, D5, D10, D20 and D40 are respectively doses of 3, 5, 10, 20 and 40 g NaCl/l

those are low-load indicators and give better-quality effluents (Curds 1982, Salvadó and Gracia 1993, Salvadó *et al.* 1995), were more sensitive to increased salinity.

The most sensitive species were those that can considered oligostenohaline (Finley 1930, Foissner *et al.* 1995) and that are also α-β-mesosaprobic, *Litonotus lamella*, *Aspidisca cicada* and *Trochilia minuta*, except for *Acineria uncinata*, which is poly-α-mesosaprobic. These are crawling species that consume different kinds of food: predators or bacteriophages. The first three species are also very sensitive to the toxicity of chlorine (Salvadó *et al.* 2000), and *Trochilia minuta* is especially sensitive to the toxicity of heavy metals Cd, Cr, Cu, Pb and Zn (Madoni 1996). However, *Aspisdica cicada* is not very sensitive to the heavy metals (Salvadó 1993; Gracia *et al.* 1994; Madoni *et al.* 1994, 1996). Moreover, *Acineria uncinata*, which is considered oligostenohaline, lives in a more polluted medium than the three previous species and has greater tolerance to toxic substances, such as chlorine and the heavy metals Zn and Cu, than *Aspidisca cicada* (Salvadó *et al.* 1993, 2000; Gracia *et al.* 1994). In general, there is some correlation between the "System of Saprobic Organisms" and the resistance of certain organisms to a particular toxic substance (Sládecek 1973). However, the results reported here, and those of Foissner *et al.* (1995) do not support a correlation between these two parameters in ciliate protozoa from activated sludge. As such, we should consider them as independent.

The absence of protozoa in general, and ciliates in particular has been described as a limiting factor on the performance of activated sludge, as their absence means that scattered bacteria are not eliminated properly, and so the effluent deteriorates (Curds 1982, 1993). There is no significant loss of protozoa and metazoa up to 10 g/l doses of salt. At 10 g/l, a loss of species, and also a loss of biomass, occurs over time, and at 20 g/l, the loss of individuals and biomass at 24 h endangers the effectiveness of the process. However, the populations of microorganisms have the capacity to recover over time (Curds 1982, 1993; Madoni 1994; Salvadó *et al.* 2000). Together with this fact, the low diversity of microorganisms at this salinity dose reduces the stability of the process even more. Although there are unpredictable fluctuations in the composition of wastewater (Woolard and Irvine 1995), the wide diversity of microorganisms in activated sludge permits a greater capacity of response to shock loads. To sum up, the fact that ciliate protozoa and other microorganisms are capable of surviving in the presence of salt concentrations higher than those normally found in activated sludge increases the stability of the process when shock loads of salt are received.

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