3. ACUTE AND CHRONIC ECOTOXICITY TESTS TO EVALUATE THE RISK OF KNOWN CHEMICAL: INTERNATIONAL VIEW

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Cr(III) BEHAVIOR IN FRESHWATER ENVIRONMENTS: HOW RELIABLE ARE ECOTOXICOLOGICAL DATA FOR *Daphnia magna*?

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Abstract: Chromium occurs in the environment in two thermodynamically stable oxidation states, Cr(VI) and Cr(III). Always trivalent chromium is considered relatively harmless. Upon addition to laboratory test media, Cr(III) rapidly undergo hydrolysis and form sparingly soluble Cr oxyhydroxydes that may lower the actual concentration of Cr(III) that potentially leading to a underestimation of its toxicity. On this basis, we investigated the ecotoxicity of Cr(III) to *Daphnia magna* in two exposure media of similar hardness, but of different alkalinity. Chromium concentrations were repeatedly measured over test duration and its chemical speciation was estimated using the software Visual MINTEQ-3.0. In the high alkalinity medium, no Cr remained in the exposure solution after 24h and, no acute toxic effects were observed. Modelling of Cr speciation indicated insoluble $Cr(OH)_3$ as the dominant species. Conversely, in the low alkalinity test medium, charged, soluble Cr(III) species were predicted to be predominant and toxicity was observed. Acute and chronic EC50s were 14.1 and 1.9 mg/L, respectively, when based on the time weighed mean for total Cr concentration. The same values expressed in terms of soluble Cr(III) species, assumed to be responsible for the observed toxicity, were around 8 and 0.3 mg/L, respectively. The ecotoxicity of Cr(III) needs a re-assessment based on the use of protocols for sparingly soluble substances to account for the rapid decrease in exposure concentrations.

Keywords: Trivalent chromium; Chromium speciation; Chromium toxicity; *Daphnia magna*; Risk Assessment

INTRODUCTION

The world production of Cr is a about $26.3 \cdot 10^3$ tons per year; 60 to 70% is used in alloys, including stainless steel, and 15% is used in chemical industrial processes, mainly leather tanning, pigments and electroplating [1]. Due to the widespread use, large quantities of Cr compounds are discharged as liquid, solid and gaseous wastes into the environment and can ultimately have significant adverse biological and ecological effects [2]. Cr concentrations in non-polluted waters vary from 0 to 114 (typical 0.52) µg/Lin rivers and from 0.1 to 0.7 µg/L in lakes [3]; while levels as high as 80 mg/L have been observed in paper mill effluents [4]. In the environment, chromium occurs in two thermodynamically stable oxidation states, Cr(VI) and Cr(III). Hexavalent chromium is highly toxic to humans and aquatic organisms due to its ability to easily cross cellular membranes and its strong oxidizing power [5]. Once inside the cell, Cr(VI) reacts with cellular constituents and is reduced to Cr(III), resulting in the formation of hazardous free radicals and leaving behind freshly formed Cr(III) in the inner environment of the cell. Chromium(III) has been reported to show adverse effects on e.g., some metallo-enzyme systems [6], but the scientific community agrees in considering Cr(VI) as the redox form of actual environmental concern. As a consequence reduction of Cr(VI) to Cr(III) is a typical approach in remedial actions dealing with Cr(VI)-contaminated water and wastewater [7,8] and the study of the toxicity of Cr(III) attracts little interest from scientists and regulators.

However, several studies published after 2000 have shown that Cr(III) can be as toxic as, or more toxic than, Cr(VI) to freshwater algae, aquatic mosses, yeasts and pollen of terrestrial plants [9,10,11]. Furthermore, some aspects of Cr(III) chemistry well-known by (environmental) chemists have so far been poorly appreciated for their relevance and implications in ecotoxicology. Cr(III) has more complicated reactions in the environment compared to Cr(VI). Specifically, at circumneutral pH values typical of many natural waters and laboratory culture media, Cr(III) undergoes hydrolysis reactions rapidly leading to the formation of Cr oxyhydroxides. This chemical forms of Cr(III) have an extremely low solubility (about 5 µg/L according to Rai et al. [12]); well below most, if not all, the acute and chronic toxicity values considered in extensive risk assessment reports [13,14].

Assessment of Cr(III) toxicity to *Daphnia magna*, one of the most commonly used invertebrate in regulatory testing of chemical [15,16,17], is no exception to this general situation. Values for acute toxicity of Cr(III) to *D. magna* are in the range 1.2 to 111 mg/L according to Munn et al. [13] and 0.1 to 59 mg/L according to CICAD [14]. Chronic toxicity is expected in the range 0.047 to 3.4 mg/L [13,14]. Because all these values are well above the expected solubility of Cr(III), the concentrations of Cr in test solutions amended with such levels Cr(III) can markedly decrease with time. Neglecting the chemical behaviour of trivalent Cr after addition to the test medium can therefore lead to an underestimation of its toxicity of Cr(III). Furthermore, Cr hydrolysis can cause a decrease in the pH of poorly buffered media with possible repercussions on the formation of insoluble Cr species during an ecotoxicological test. In practice, the alkalinity of the exposure media will be a key factor in controlling Cr(III) speciation during ecotoxicological

tests. While the effect of water hardness in regulating the toxicity of trace elements is well appreciated and included in regulation

Table 1 Composition of each exposure medium for acute (Medium A, B) and chronic (Medium C) test. Values are in mg/L unless otherwise indicated.

Component	Medium A	Medium B	Medium C
Ca^{2+}	48.6	80.5	89.6
Mg^{2+}	28.2	12	12.6
Na+	5.8	17.8	9.6
K+	1	3.2	3.6
HCO_3^-	301	47	51.6
SO_4^{2-}	4.1	48	50.5
Cl-	2.4	143.8	144.4
pH	7.84	7.55	7.69
Conductivity [a]	417	565	583
Hardness [b]	240	250	269
Alkalinity [c]	4.9	0.77	0.85

[a] µS/cm
[b] as mg/L $CaCO_3$
[c] meq/L

[18], we have been unable to find studies on effect of alkalinity on Cr(III) toxicity.
Based on all these considerations, the aims of the present study are1) to show that rapid formation of Cr(III) insoluble species, leading to a fast decrease of Cr(III) exposure concentration, occurs in representative ecotoxicological test media, 2) to highlight that neglecting Cr(III) chemistry during ecotoxicity test can underestimate its toxicity, and 3) to show that, at equal hardness, differences in the alkalinity of test media affect Cr(III) speciation and, hence, toxicity.

MATERIALS AND METHODS

Temporal stability and speciation of trivalent chromium concentrations in culture media

The temporal stability of trivalent chromium concentrations was studied in three laboratory culture media for daphnids (Table 1). Medium A corresponds to the medium used for culturing daphnids in our laboratory, while medium B and medium C were prepared according to guideline OECD 202 [15] by adding analytical grade salts to ultrapure water and a commercial mineral water, respectively. This choice has been made to avoid problems with the use of ultrapure water for the long term test. The compositions of each exposure medium are show in Table 1.
Stock solution of Cr(III) (2 g/L) was prepared in ultrapure water using analytical grade chromium chloride ($CrCl_3 \cdot 6H_2O$, purity > 96%). Depending on the objective of the corresponding ecotoxicological test, each medium was spiked with suitable concentrations of Cr(III) (Figure 1).
Aliquots of each working solution were recovered and transferred into polystyrene bottles of 25 ml, at the start of the test (t0) and after 6, 24, 48 hrs,
filtered through polycarbonate filters of 0.45 µm pore size and acidified with concentrated nitric acid (VWR, Suprapur).
All material was washed with 10% nitric acid and thoroughly rinsed whit ultrapure water.
Total chromium concentrations were determined by FAAS (Perkin Elmer 3300, detection limit of 8 µg/L) for medium A and GF-AAS(Perkin Elmer AA600 detection limit of 0.2 µg/L) for media B and C.
Chromium speciation in each medium was estimated with the software package Visual MINTEQ version 3.0; a freeware chemical equilibrium model for the calculation of metal speciation. Analytical concentration

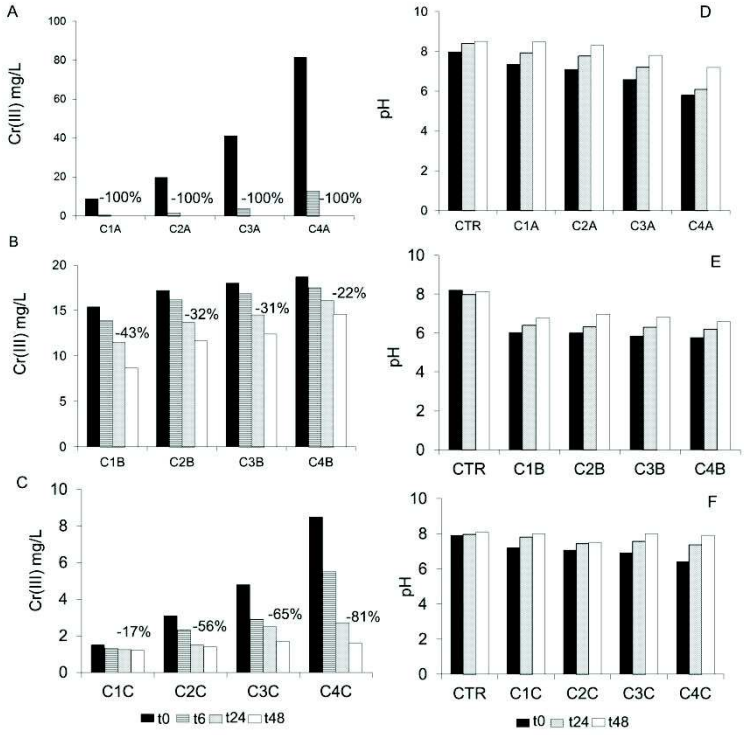

Figure 1. Temporal changes in Cr concentrations under static conditions in medium A, B and C (homonymous panels) and corresponding variations of pH values (panels D, E, and F, respectively) at t=0, 6, 24 and 48 hrs. For each exposure medium the percentages of concentrations decrease at t=48 are indicated. Chromium concentration in control solutions were always below detection limit and are not reported. Note the different scales on y-axes for panels A, B, and C. Acronyms C1A etc. indicate the different exposure concentrations for each test medium. CTR: control.

of Cr(III) at t=0; t=24 and t=48 were used as input for the component Cr(III) and the tabulated composition of each medium (Table1) as input for the other model components. For the chronic test, only the first 48-h were monitored assuming that Cr solutions would show a similar behaviour at each medium renewal. For each simulation at different analytical Cr(III) concentration, measured pH values at t=0; 24 and 48 hours were specified in the input menu. No changes were made to default complexation constants contained in the model database.

Daphnia magna culture

Parthenogenetic *Daphnia magna* were cultured in a mineral water (medium A, see Table 1) with hardness of 240 mg/L ($CaCO_3$) and pH of 7.8, as recommended by OECD [15]. The culture was maintained at 20 ± 1 °C under 16 hrs light: 8 hrs dark photoperiod with a light intensity of 1000 lux. Culture medium was renewed and offspring discarded three times a week. On each renewal, daphnids were fed with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (0 to 8-days-old daphnids: $8 \cdot 10^6$ cells·ind/day and from 8-days-old daphnids: $16 \cdot 10^6$ cells ind/day) and yeast *Saccharomyces cerevisiae* ($15 \cdot 10^6$ cells ind/day). Brood daphnids were renewed every 6 weeks and replaced with neonatal organisms.

Daphnids from the third generation were used for culture renewal and for exposure experiments to reduce variability [15].

Chromium toxicity test

The concentrations for acute and chronic tests were prepared from stock solution following a geometric progression with a ratio not higher than 2. Acute and chronic toxicity assays were performed following OECD Protocol 202 and 211 [15,16] and all tests were conducted in a 50-ml glass beakers. In acute toxicity test, four replicates containing 20 mL of solution with five newborns (< 24 hours) for each beaker were exposed to four concentrations and a control for each exposure medium. Beakers were covered with glass sheet and incubated for 48-h at 20 ± 1 °C without feeding. After 48 hrs, the number of immobilized organisms in
each vessel was counted and ecotoxicity data were reported as EC50 with the 95% confidence interval estimated by EPA Probit analysis programme.

For the chronic toxicity test, ten replicates containing 20 mL of solution and one newborn for each vessel were exposed to four concentrations and a control in medium C. The incubation was carried out for 21 days in controlled conditions with feeding (same conditions and feeding as the culture). Test solutions were renewed every other day, when number of neonates produced by each female was counted. EC50 with 95% confidence intervals, estimated from reduction of fecundity compared to control, were obtained from log-logit analysis. The ECs50 were estimated based on both total dissolved nominal concentrations and Time-Weighted Mean (TWM) concentrations [16]. The latter procedure estimates the mean concentrations to which organisms are exposed during a toxicity test when the dissolved concentrations of a substance decrease over time (as it is the case in our acute and chronic test). During each test pH values were measured at 0, 24 and 48 hrs.

RESULTS

Results chemical analysis

For all experiments, measured Cr concentrations at t=0 were within 20% of the expected nominal values, but decreased during the exposure time for both acute and chronic tests (Figure 1). In medium A, a Cr levels dropped by 84 to 90% of their initial values over the first 6 h and were below detection limit at the end of test (Figure 1). In this medium, the addition of Cr(III) caused marked decreases in pH values with respect to the control; especially for the highest exposure concentration where the pH at t=0 dropped to 5.8 units (Figure 1). Measured pH reverted to circumneutral or slightly alkaline values over the 48 hours, but remained lower than in the control for C3A and C4A (Figure1). In medium B, the decrease of Cr concentration between t=0 and t=48 was 20 and 40 % respectively for C4B and C1B (Figure 1). Addition of Cr(III) caused a substantial reduction of pH values also in this exposure medium. Furthermore, pH values at 48 hours remained between 1 to 1.5 units lower than in the control for all treatments.

In medium C, chromium concentration remained within 20% of the initial value in solutions amended with the lowest level of Cr (C1C) where no substantial changes in pH at t=24 and t=48 were observed either (Figure 1). On the other hand, a temporal decrease of Cr concentrations were observed in solutions C2C, C3C and C4C; with losses up to 80% of the initial value at t=48. Addition of Cr(III) in medium C lowered the pH by as much as 20% (in C4C), but pH values after 48 hrs were similar to those of the controls. (Figure 1).

	% charged species [a]			% insoluble species [b]			TWM mg/L	
	t0	t24	t48	t0	t24	t48	charged species	insoluble species
C1A	11.1	-	-	88.9	-	-	0.1	0.5
C2A	19.5	-	-	80.5	-	-	0.3	1
C3A	47.5	-	-	52.5	-	-	1.2	1.3
C4A	44	-	-	56	-	-	2	2.4
C1B	78.1	64.6	39.7	21.9	35.7	60.3	7.5	4.2
C2B	76.6	69.3	26.7	23.4	30.7	73.3	8.7	5.3
C3B	78.8	69.7	36.8	21.2	30.2	63.2	9.5	5.3
C4B	79	72	52.7	21	27.8	47.3	11.3	4.9
C1C	15.7	4	2.5	84.3	96.1	97.5	0.1	1.2
C2C	22	9.3	7.9	78	90.7	92.1	0.2	1.6
C3C	30.2	6.7	2.5	69.8	93.3	97.5	0.4	2.4
C4C	66.8	10.9	3.1	33.2	89.1	96.9	1	2.6

[a] $Cr(OH)^{2+}$; $Cr(OH)_2^{+}$; Cr^{3+}

[b] $Cr_3(OH)_4^{5+}$; $Cr_2(OH)_2^{4+}$; $Cr(OH)_3$; $CrOHSO_4$ (aq)

Table 2. Speciation results of Cr(III) in in medium A, B, and C (Visual MINTEQ freeware, version 3.0) and time weighted mean concentrations for total Cr (calculated from analytical measurements), charged Cr(III) species and insoluble Cr(III) species. All speciat results are given as percentage abundance of the total. For each simulation, measured pH values and analytical Cr concentrations at t=0, 24 and 48 hrs were used as input values. Time Weighted Mean (TWM) concentrations of Cr(III) species were estimated from percentages of Cr species abundance.

In the Table 2 it is possible to see the results obtained from the thermodynamic equilibrium speciation model.

According to thermodynamic speciation calculations, insoluble species such as such as the oxyhydroxides $Cr(OH)_3$ and the polynuclear forms $Cr_3(OH)_4^{5+}$ and $Cr_2(OH)_2^{4+}$ would dominate Cr speciation in medium A at t=0; their contribution being comprised between approx. 50 to 90% (Table 2). More specifically, $Cr(OH)_3$ would account for the totality of insoluble species at in C1A and C2A, while the contribution of polynuclear species would become more important (about 10%) in C3A and dominant in C4A. No Cr speciation was estimated at t=24 and t=48, because no Cr was actually remained in the solution. In medium B, speciation model predicted a low proportion of insoluble species and a predominance of charged species such as $Cr(OH)^{2+}$, $Cr(OH)_2^{+}$, and Cr^{3+} at t=0 and t=24 in each concentration. At t=48 when pH values increased above 6.8 except for C4B hydroxide insoluble species became dominant. It was possible to calculate the Time Weighted Mean(TWM) concentration based on concentrations of Cr in charged species estimated from Visual- MINTEQ simulation at time of 0, 24, 48 hrs (Table 2). The TWM concentration of the charged species was between 64% (C1B) and 70% (C4B) of the TWM total Cr concentration.

In medium C, oxyhydroxide insoluble species were predicted to be predominant in all treatments with the exception of C4C at t=0 (Table 2).

TWM concentrations obtained from percentage of charged species estimated from model are between 0.1 and 1 mg/L, corresponding to 8 and 28% of the total

MEDIUM A				MEDIUM B				MEDIUM C			
Cr(III) [a]		TWM[b]	% effect	Cr(III) [a]		TWM[b]	% effect	Cr(III) [a]		TWM[b]	% effect
Control	0	Bdl	0	Control	0	bdl	0	Control	0	bdl	0
C1A	10	0.5	0	C1B	15	11.7	0	C1C	1.5	1.3	39
C2A	20	1.1	0	C2B	17	14	50	C2C	3	1.8	49
C3A	40	2.2	0	C3B	18	14.8	80	C3C	5	2.8	64
C4A	80	5.3	0	C4B	20	16.2	90	C4C	8	3.6	67
No acute toxic effect				Nominal EC50 18.3 (17.7-18.9)		Analytical EC50* 14.1 (13.1-14.7)		Nominal EC50 3.2 (3.01-3.33)		Analytical EC50* 1.9 (1.81-1.99)	

[a] Nominal concentrations.
[b] Time Weighted Mean (TMW) concentrations based on analytical concentrations measured at time of 0, 6, 24, 48 hrs.
* Analytical EC50 was estimated from TWM concentrations of total Cr.

Table 3. Nominal and Time Weighted Mean (TMW) concentrations of Cr (III) in exposure media A, B, and C (see table 1 for the respective compositions). % effect: percentage of immobile organisms after 48 hrs of exposure (acute tests, media A and B) or percentage inhibition of reproduction compared to control (chronic test, medium C). Bdl: below detection limit. All concentrations values are in mg/L.

concentrations.

Results chromium toxicity tests

Acute toxicity tests showed clear differences in the responses in relation to exposure medium. In medium A, characterized by high hardness and alkalinity (240 mg/L $CaCO_3$; 4.9 meq/L), no toxic effects were observed.

A preliminary toxicity test set in the medium B with the same concentrations used in medium A, showed 100% lethality at 20 mg/L Cr(III) (nominal concentrations, data not shown). After this range finding test, the final one was performed using the exposure concentration range reported in Table 3. Results obtained from ecotoxicity test showed no observed effect at nominal concentrations of 15 mg/L. This concentration can be considered as the NOEC (No Observed Effect Concentration). 48-hrs LC50 value (95% Confidence Intervals) obtained by EPA Probit analysis with nominal concentration was of 18.3 (17.7-18.9) mg/L Cr(III). Due to decrease of exposure concentration during time observed thanks to analytical measure (Figure 1) same statistical analysis based on Time Weighted Mean was carry out. A 48-hrs EC50 with 95% Confidence Intervals of 14.1 (13.1-14.7) mg/L Cr(III) was obtained.

In chronic toxicity test, significant differences (ANOVA, $p < 0.001$) between reproductive output of the exposed organisms and the control were found starting from a nominal exposure concentration of 3 mg/L total Cr. After 21 days, a reduction of 39% in the fecundity of organism exposed concentration C1C (1.5 mg/L nominal concentration) was alsoobserved with respect to the control (Table 3), but it was not statistically significant (Bonferroni t-test, $p > 0.05$). Log-logit analysis established a nominal EC50 value with 95% Confidence Intervals of 3.2 (3.01-3.33) mg/L Cr(III) at 21 days. Considering the temporal decrease of Cr concentration, the corresponding EC50 value would be 1.9 (1.81-1.99) mg/L Cr(III).

DISCUSSION

Chromium chemistry in exposure media

The Cr(III) oxidation state is the most thermodynamically stable and it is known that Cr(III) presence, concentration and

forms in the environment depend on different chemical and physical processes, such as hydrolysis, complexation/redox reactions and adsorption [12]. If we consider the solubility product of chromium(III) hydroxide $[Cr^{3+}][OH-]^3 = 6.3 \cdot 10^{-31}$ so at neutral pH, when $[OH-]^3 = 10^{-7}$ mol/L, free chromium(III) concentration would be 6.3 10^{-10} mol/L or less than 1 μg/L [19] and thus there would be practically no chromium dissolved. The formation of complexes other than oxyhydroxides would depend on the presence of other ligands and on the relative stability constant of the different complexes. In media A, B or C, alternate ligands to OH- are limited to sulphate, chloride and carbonate ions. Stability constants of sulphate complexes are orders of magnitude lower than those with OH- ligands [20], those with chloride are even smaller and the formation of Cr chloride complexes is kinetically slow [21].

As a consequence, Cr speciation in our test media is controlled by the availability of OH- ligands (i.e., pH), with little of no importance of other inorganic complexes.

The predicted behaviour of Cr(III) agrees well with the experimental observations, because insoluble Cr species become predominant in our media as soon as the pH value approaches neutrality (Table 2). Thus, during the acute assay in exposure medium A, more than 80% of the added Cr(III) was already 'lost' after only 6 hrs and no Cr was detected after 24 hrs. Only when we added high Cr quantities (C3A; C4A) causing a decrease in pH to 5.8, speciation calculation estimated that more than 40% of the added Cr existed as free charged species at t=0 (Table 3). However, high hardness and alkalinity of medium A progressively buffered the moderately strong acid hexa-aquacrhromium(3+) so that, after 24 hrs, pH values in each treatment again reached values around 7 and all Cr was present as unfilterable species. In contrast in medium B characterized by low alkalinity $Cr(H2O)_6^{3+}$ exhibits its amphoteric behaviour and a decrease in pH values respect to the control was observed in all concentrations tested. At t=0 pH values for C3B and C4B were 5.84 and 5.77, respectively, and remained below 7 for the entire duration of the assays.

In this situation, hexa-aquachromium (3+) deprotonated forms such as $Cr(OH)^{2+}$ and $Cr(OH)_2^{+}$ are expected to dominate [12] as confirmed by speciation calculation (Table 2). Analysis of the temporal evolution of filterable Cr concentration highlighted a slight decrease over time, so that toxicity could be expressed also in term of TWM concentration for each treatment. A TWM concentration in terms of free ionic species could also be estimated as a better proxy of the real quantity of Cr(III) available to organisms in medium B.

Similarly, in medium C, a high proportion of ionic Cr species (up to 66%, Table 3) was also predicted, as expected because of the equal hardness and alkalinity of media B and C. However, due to comparatively low Cr(III) concentrations in medium C, a good buffer capacity was observed and pH values at 24 and 48 hours were similar to control pH values; resulting in a predominance of insoluble hydroxo complexes (Table 3).

Chromium toxicity in relation to chemical behaviour in exposure media

Regarding aquatic ecosystems the possible toxic effects of Cr(III) on *Daphnia* were already investigated but the data present in literature are dissimilar and sometime the methods used to obtain ecotoxicity parameters are not clear. The ecotoxicity results obtained in present work seem to

indicate a sensibility to chromium(III) in specific conditions of exposure.

In medium A Cr(III) concentrations expected to elicit a response from organisms were tested, but no acute toxic effect was observed. Chemical analysis showed that Cr concentrations dropped abruptly after 6 hours and that no Cr remained in the exposure medium after 24 hrs. It is therefore more appropriate to conclude that no inferences can be made as to the toxicity of Cr(III) in medium A, rather than to speak of an absence of toxicity of Cr(III). Exposure media having high hardness associated with high alkalinity buffer capacity can mitigate the pH variation caused by introduction of Cr(III) salts. As a consequence, the pH of the test solution will remain around (or rapidly return to) circumneutral values so that all Cr(III) in solution will occur as insoluble hydroxo complexes with little or no bioavailability for organisms. Furthermore, Cr losses in medium A were extremely rapid even at high concentrations and slightly acidic pH (Figure 1) suggesting that formation of insoluble Cr species occurs even more rapidly and frequently than predicted by chemical equilibrium models. Hardness can affect Cr toxicity indirectly through nonspecific ionic interferences which decrease the ionic activity of the metal ion, and directly by interfering with metal uptake and action through competition for binding sites.

Several studies present in literature consider pH and hardness as significant parameters for predicting metal speciation and bioavailability, and current water-criteria for the protection of the aquatic life have been developed taking into account hardness as possible modifier of toxicity (USEPA). Park et al. [22] have evaluated the combined effect of pH and hardness on acute metal toxicity to *D. magna* and hardness was the most significant parameter to Cr(VI) toxicity; Yim et al. [23] have observed approximately four or five times higher toxicity in soft (45 mg/L $CaCO_3$) rather than hard water (150 mg/L $CaCO_3$) for different metals including Cd, Cu, Zn and Pb.

Since the chemical of natural freshwater is characterized by a general interrelationship among pH, hardness and alkalinity, even the latter needs attention when developing predictive water-quality criteria for the protection of aquatic life. Alkalinity in fact can directly affect metal toxicity through the formation of poorly soluble metal carbonates of low toxicity, exactly as observed in the exposure medium A.

In medium B, the low quantity of carbonates (and the low alkalinity) resulted in a decrease of pH values with respect to the control (Figure 2) and a toxic effect on survival of daphnids was observed (Table 3).

Cr(III) oxidation in circumneutral solutions and in the absence of suitable oxidants such as Mn oxides and hydroxyl radicals is expected to be rather slow [24] and for these reasons denoted during our experiments, we could attribute toxic response to effect of trivalent chromium.

The difference of these two exposure medium is evident from a comparison between the same exposure concentrations: C2A, where no toxic response was observed, analytical analysis show a total disappearance of Cr after 6 hrs and estimation of chemical speciation showed 80% of insoluble Cr species at t=0 and C4B where 90% of toxic effect was observed and only 20% of Cr loss was observed respect to the control. In medium B the EC50 calculated from TWM concentrations was of 14 mg/L. Due to results obtained from chemical speciation estimation that predict a high percentage of free ionic species and

due to the fact that free metal ions are usually considered to be more toxic than other metal species.

Comparison with previously published results is difficult because available data are from studies using very different matrices and mostly performed between 1950s and 1980s. Anderson [25] found a threshold of toxicity of of 1.2 mg/L for a 64 hrs exposure in Lake Eire water. This author reports the formation of a precipitate for added Cr concentration between 0.14 and 15 mg/L and a drop in pH from about 8 to 5.6 in solution amended with the higher concentration. The threshold for toxicity of 1.2 mg/L fell in the concentration range favouring the formation of precipitates so that no conclusion can be drawn as to the actual Cr level responsible for the observed effect. Chapman et al. [26] studied the effect of water hardness on the toxicity of metals to *Daphnia magna* and for Cr(III) found a range of EC50 values calculated from geometric mean concentrations between 9.3 mg/L for hardness of 52 mg/L $CaCO_3$ and 34.63 mg/L for hardness of 215 mg/L $CaCO_3$. These authors also report a drop in pH (from 7.9 to 8.4 in controls to 6.2 to 6.4) and total alkalinity (from 2.8 meq/L in controls to 0.41 meq/L) for solutions amended with Cr concentrations corresponding to EC50 values. Note that we assumed that total alkalinity values reported by Chapman et al. [26] are given as mg/L HCO_3^- to calculate the corresponding value in meq/L. All considered, Cr behaviour in the medium used by Chapman et al. [26] should have been between what was observed in our media A and B. It is therefore possible that the EC50 of 34.6 mg/L reported by Chapman et al. [26] actually corresponds to a much lower value. Indeed, geometric means for C3A and C4A would be approximately 13 and 32 mg/L, four to five time higher than the corresponding TWM. Once again, the interplay of hardness and alkalinity determines combinations of Cr behaviour that must be considered to obtained reliable values of acute EC50 for Cr(III).

Kuhn et al. [27] reported a 24 h EC50 of 22 mg/L Cr(III), added as $CrCl_3$ and also 0.93 mg/L for Cr(VI) added as $K_2Cr_2O_7$. Because details of medium composition are given, it is possible to estimate to calculate Cr speciation in the exposure medium. We refer the reader to Kuhn et al. [27] for details, but we note that, according to our calculation, the medium used by those authors had the same alkalinity as medium B (0.77 meq/L based on a HCO_3^- content of 47 mg/L) and a hardness of 363 mg/L as $CaCO_3$. Kuhn et al. [27] also report some shifts in the pH of test solutions: from 8 units (control value) to as low as 7 units after 48 and 72 hours after the addition of test substances. This observations are similar to our findings for medium B and, for a pH value of 7, about 75% of Cr(III) is expected to have occurred as insoluble species during the experiments of Kuhn et al. [27]. From these model calculations, we obtain an EC50 of 5.5 mg/L as soluble Cr(III) species; in reasonable agreement with our results for medium B (Table 3). Similarly to acute assay in medium B, chronic assay carried out in medium C showed a statistically significant effect on reproduction of *Daphnia* respect to the control.

The variation in Cr concentration during the time observed in the chronic assay could be modified by the presence of algae used to feed *Daphnia*. Roy et al. [28] conducted an algal biomass adsorption studies with different metal as As, Cd, Co, Cr, Pb, Ni and Zn, the green alga, *Chlorella minutissima*, adsorbed greater than 90% of the initial Pb,

and greater than 98% of the initial Co concentrations. Recent study were made on the sorption of Cr(VI) from solutions by biomass of filamentous algae *Spirogyra* species and the results indicated that the biomass of *Spirogyra* species is suitable for the development of efficient biosorbent for the removal of Cr(VI) from water [29].

Literature data for chronic toxicity of Cr(III) are also scarce. Brisinger et al. [30] assessed chronic effect of different metals including Cr(III) on *Daphnia magna* in Lake Superior water characterized by low hardness (45 mg/L $CaCO_3$) and low alkalinity (0.7 meq/L) and estimated a chronic EC50 of 0.6 mg/L for *D. magna*. Considering the differences in total hardness and medium composition, this result agrees quite well with the EC50 of 1.9 mg/L obtained in medium C (Table 3). Furthermore, the EC50 for medium C expressed as concentration of charged Cr(III) species (Table 2) falls between 0.23 and 0.34 mg/L; again in good agreement with Brisinger et al. [30]. Furthermore, Chapman et al. [26] calculated a NOEC of 0.047 mg/L Cr(III) in a low hardness exposure medium (52 mg/L $CaCO_3$); a value very similar to the NOEC of 0.03 mg/L estimated in present work in terms of free ion charged species (Table 2). Finally, Kuhn et al. [27] give a chronic EC50 of 0.7 mg/L for Cr(III) and 0.018 mg/L for Cr(VI). Performing speciation calculation gives chronic EC50 for soluble Cr(III) species of 0.168, 0.056 and 0.018 mg/L for pH values in the test medium of 7, 7.5 and 8 units, respectively. Considering the similar alkalinity of the medium of Kuhn et al. [27] and medium C along with the temporal changes in pH values in medium C (Figure 2), chronic EC50 in terms of soluble Cr(III) for Kuhn et al. [27] would likely have been closer to the lowest value.

Implications for ecotoxicology and risk assessment of Cr(III)

The removal or reduction of Cr(VI) to Cr(III) serve as a key process for removal of Cr(VI) contaminated water and wastewater and several methods such as adsorption, biosorption, reduction, and filtration reverse osmosis, ion-exchange, foam flotation, electrolysis, and surface adsorption are used for chromium removal. Furthermore Cr(III) is used in texile (printing, dying), tannery and industrial processes (chrome plating) [31]. Therefore the possible effects of chromium (III) cannot be ignored in the environmental assessment; recent studies have documented a greater availability/toxicity of Cr(III) respect to Cr(VI) to aquatic and terrestrial organisms [9,32].

High dissimilarity between ecotoxicity data of Cr(III) available in literature is attributable to different characteristic of different exposure medium used for the tests and to the fact that in many work aimed to establish Cr(III) Environmental Quality Standard no analytical concentration measures were performed or were made only at the start of the test.

Chromium (III) is usually removed from the water column and supersaturation with respect to chromium solid phases is expected only in zone directly impacted by e.g. tannery wastewater [33]. Furthermore, the influence of ligands such as sulfidieds and organic matter that can occur in natural environments should be considered.

Current water quality standards and risk assessment procedures of metals are predominantly based on total or dissolved metal concentrations. However, there is extensive evidence that neither total nor dissolved aqueous metal concentration is good predictors of metal bioavailability and

toxicity [34]. Because the bioavailability of metals depends on chemical form or speciation it is necessary make the evaluation of free metal ions, inorganic and organic complexes, and organometallic compounds. Chemical equilibrium computer programs, as Visual MINTEQ, are useful for computing the distribution of species in samples containing defined total concentrations of metal and ligands, if appropriate stability constants are available. Probably more a module such as Biotic Ligand Model (BLM) that linked metal speciation in solution, competition of cations for binding to and accumulation on physiologically active sites and ensuing toxicity responses would be more appropriate [35]. BML has been developed as a computational alternative to completion of bioassay tests for use in assessing the effects of water chemistry on metal bioavailability and toxicity. Comparison of BLM predictions with toxicity test results shows excellent agreement for copper and other metals (e.g. Ag; Zn) [35]. Nonetheless, the level of development of the BLM currently remains uneven among metals, especially regarding trivalent trace metals. This as for most trivalent metals, uptake mechanisms are still speculative and the links between their speciation and accumulation remain elusive.

Crémazy et al. [36] studied scandium (Sc) uptake in *Chlamydomonas reinhardtii* in order to explore the applicability of the BLM to trivalent metals and they found that BLM cannot be expected to predict Sc uptake as it greatly underestimates fluxes at pHs where the free metal proportion is very low.

In spite of this use of the BLM in conjunction with water quality monitoring data provides a cost-effective means of determining regional, seasonal, and long-term trends in metal bioavailability and toxicity. More recently, short- and long-term BLMs have been developed with crustaceans and algae, and the ability of these models to predict metal toxicity in different types of surface waters (e.g. geographic variability in Europe) has been assessed [37] and these confirm the importance to develop BLM for chromium too.

CONCLUSIONS

The outcome of laboratory tests to evaluate the toxicity of Cr(III) to *Daphnia magna* is largely dependent on the test medium employed during the experiments. Upon addition to media with circumneutral pH, the hydrolysis of Cr(III) causes i) a decrease in pH values of up to 2 units and ii) the formation of insoluble Cr oxyhydroxides. The initial decrease in pH also occurs in well-buffered media and, depending on medium alkalinity, can be partially on entirely reversible over 48 hours; i.e., the typical duration of acute tests or the usual interval between medium renewals in chronic toxicity tests.

In laboratory media of high hardness and alkalinity, Cr(III) is predicted to exist mainly as hydroxo insoluble complexes and its concentration decrease vary fast with time; in agreement with results of the speciation model. In such situations no toxicity to *Daphnia magna* is observed. In low alkalinity media, and at equal hardness, Cr(III) soluble ionic species can represent up to 80% of the total Cr concentration, resulting in acute and chronic negative effects on *Daphnia magna*. In any case, Cr speciation will vary markedly over test duration (acute toxicity) or between the beginning and the end of renewal periods (chronic toxicity). While the information

necessary to develop predictive models is collected, an accurate estimation of Cr(III) toxicity in standard conditions requires the adoption of protocols for sparingly soluble substances exemplified in this work.

In terms of risk assessment, our results show that the hazard component of the process is probably underestimated and that Cr(III) water quality criteria must be adjusted for both water hardness (as already endorsed by US EPA) and alkalinity (which is currently not considered in the specific case of Cr(III)). However, the exposure component will be controlled by the parameters examined in this study (hardness and alkalinity) and also by the presence of a variety of organic ligands in natural waters. In general, we can expect water bodies characterized by medium-low hardness and very low alkalinity values, such as subalpine lakes, to be particularly sensitive to Cr(III) contamination.

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4. ACUTE ECOTOXICITY TESTS TO EVALUATE THE RISK ASSOCIATED TO NEW POLLUTANTS

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The Potential of Zosteric Acid as New Strategy for Mitigating Biofouling in Membrane Bioreactor Systems

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Abstract

This study provides data to define an efficient biocide-free strategy based on zosteric acid to counteract biofouling on the membranes of submerged bioreactor system plants. 16S rRNA gene phylogenetic analysis showed that gammaproteobacteria were the prevalent taxa on fouled membranes of an Italian wastewater plant. *Pseudomonas* was the prevalent genus among the cultivable membrane-fouler bacteria and *Pseudomonas putida* was selected as the target microorganism to test the efficacy of the antifoulant. Zosteric acid was not a source of carbon and energy for *P. putida* cells and, at 200 mg/L, it caused a reduction of bacterial coverage by 80%. Biofilm experiments confirmed the compound caused a significant decrease in biomass (-97%) and thickness (-50%), and it induced a migration activity of the peritrichous flagellated *P. putida* over the polycarbonate surface not amenable to a biofilm phenotype. The low octanol-water partitioning coefficient and the high water solubility suggested a low bioaccumulation potential and the water compartment as its main environmental recipient and capacitor. Preliminary ecotoxicological tests did not highlight direct toxicity effects toward *Daphnia magna*. For green algae *Pseudokirchneriella subcapitata* a significant effect was observed from 100 mg/L together with a protozoan growth that may be connected to algal growth inhibition.

Keywords: antifouling system, biofilm, *Pseudomonas putida*, wastewater treatment plant, membrane bioreactor, ecotoxicological tests.

Abbreviations: MBR, membrane bioreactor systems; WWTP, wastewater treatment plants; COD, chemical oxygen demand; BOD, biological oxygen demand; TSS, total suspended solids; P_{tot}, total phosphorus; N_{tot}, total nitrogen; DGGE, denaturing gradient gel electrophoresis; PCA-analysis, principal component analysis; PCA, plate count agar medium; SWA, agarized synthetic wastewater medium; SWB, Synthetic wastewater broth; PBS, phosphate buffer solution; DAPI, 4',6-diamidino-2-phenylindole dye; BCF, arnot–gobas bioconcentration factor; BAF, bioaccumulation factor; Kow, octanol-water partitioning coefficient; pK_a, acid dissociation constant.

1 Introduction

Membrane-based separation processes have gained increasing interest over the last decade and are becoming the promising technology in wastewater treatment as well as in drinking water and high purity water production and purification in biorefining and bioenergy processes [1, 2, 3, 4].

Membrane technology is attractive to complement or supplant conventional filtration and sedimentation processes and it is also now being integrated into membrane bioreactor systems (MBR) as a reliable and advanced option to improve the performances of the conventional activated sludge processes in wastewater treatment plants (WWTPs) [5]. Membrane based separation processes often offer the following significant advantages over conventional unit operations: easy scale-up and flexibility due to modular design, production of a very high quality effluent, simplification of operation and great reduction in space requirements [6]. In contrast, a major disadvantage is the high energy consumption due to the significant aeration intensity applied in MBR for biofouling control. Indeed, the rapid decline of the permeate flux over time due to biofouling is recognized as the major obstacle in the application of membrane technologies [7].

Biofouling is an operation definition, referring to the undesirable accumulation of microorganisms in form of biofilm on membrane surface [8]. Biofouling increases feed flow pressure, extending system downtime, operation costs and decreasing the lifespan of the membrane modules and process productivity [9].

Antifouling strategies for membrane-based separation processes have focused mainly on the optimization of operating parameters [10, 11], membrane surface modification [12], and chemico-physical cleanings [13]. However, the influence of variations in the operation parameters on microbial communities is still scarce and in full scale WWTP it is not possible to control oscillations in the composition of the wastewater or environmental variables [11]. The membrane surface modifications have also several disadvantages: mainly the flux declines rapidly and thus frequent backwashing is necessary, the effluent quality is unstable especially after regeneration, the main module configuration applied is flat sheets for which the packing density is low and non-renewable materials are often used [12]. Physical cleaning and antimicrobial agents are not effective to control biofouling since biomass is not effectively removed from surfaces, resulting in rapid bacterial regrowth and might provide nutrients for subsequent colonization [14, 15, 16]. Indeed, some biocides increase the nutrient content by oxidizing recalcitrant organics, making them more bioavailable and improving

microbial growth [8]. In addition, it is well established sessile microorganisms express phenotypic traits that are distinct from those that are expressed during planktonic growth, displaying a much higher tolerance to antimicrobial agents [17]. Some mechanisms that may protect biofilm are limited agent penetration, presence of stationary phase dormant zones and existence of a subpopulation of resistant phenotype in the biofilm [18]. After exposure to the killing effects of biocides, a small surviving population of persistent bacteria can repopulate the surface immediately, and become more resistant to further biocide treatment [19]. Finally, the growth in public concern over the use of biocides may pose a regulatory risk for applications in water treatment.

To guarantee minimal biofilm coverage on membrane surface for extended time, a novel biocide-free approach aimed at interfering with the key steps of biofilm genesis is here proposed. P-(sulphooxy) cinnamic acid sodium salt (zosteric acid) represents a potential powerful antifouling agent able to significantly reduce, at sublethal concentrations, both bacterial and fungal adhesion and to successfully counteract the effects of colonization-promoting factors like temperature and time [20, 21]. However, to date no data exist about its environmental fate and ecotoxicological effect.

In this study the ability of zosteric acid in mitigating, at sublethal concentrations, the surface biofouling phenomena by the membrane colonizer *P. putita* isolated on the membranes of a submerged MBR plant was studied. In addition, for the first time, preliminary screenings of the potential environmental partitioning behavior and ecotoxicological tests of this promising molecule toward the target aquatic organisms *Daphnia magna* and *Pseudokirchneriella subcapitata* were performed. The work represents a preparatory study to transfer the compound performance to the membrane-based separation process technology in WWTPs.

2 Methods

2.1 Samples collection and preparation

To characterize the fouling process, fouled filtration membranes were collected from the MBR WWTP of Aldeno (Trento, Italy), a plant fed with real municipal wastewater after sieving and degritting. Samples of about 1 cm^2 of filtration membrane were taken from both fouled and apparently unfouled areas by sterile scalpel and transferred in sterile plates in laboratory. The samples of filtration membranes were suspended in tube with 900 µl of saline solution and vortexed for 5 min to remove sessile cells. Tubes were stored at -20°C. Some

filtration membrane samples both covered and uncovered of fouling were placed on glass slides, confined by in-situ frames (1 cm^2 area; Eppendorf) for microscopy analysis.

In addition, four samples of sludge (2 L each) were collected from the same MBR plant near to the panels with the filtration membranes and conserved in sterile bottles. Fifty mL of sludge samples were filtered by sterile 0.22 μm filters (Millipore). Filters were placed on sterile tubes with 1.8 mL of lysis buffer (EDTA 40 Mm, TrisHCl 50 Mm pH 8, sucrose 0.75 M), vortexed for 5 min and stored at -20°C for the DNA extraction.

The chemical-physical features of the influent wastewater during the sampling were as follows: temperature 13.1°C, pH 7.7, chemical oxygen demand (COD) 251 mg/L, biological oxygen demand (BOD) 55 mg/L, total suspended solids (TSS) 52 mg/L, total phosphorus (P_{tot}) 10.1 mg/L, total nitrogen (N_{tot}) 63.5 mg/L, ammonia nitrogen 35.6 mg/L, organic nitrogen 27.1 mg/L, nitric nitrogen 0.8 mg/L.

2.2 Analysis of bacterial community by culture independent methods

An accurate understanding of the microbial community composition in membrane systems is crucial to identify target microorganisms.

Total DNA was extracted directly from microbial cells collected from sludge and from filtration membrane samples as described by Murray et al. [22] to characterize the fouling community.

Bacterial communities were analyzed amplifying 16S rRNA gene fragments with primers GC-357 F and 907 R [23] with the following chemical conditions: 1× of PCR Rxn buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.3 μM of each primer and 1 U of Taq DNA polymerase (GoTaq, Promega). The thermal cycling program included an initial denaturation at 94°C for 5 min followed by thirty-five cycles consisting of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

All PCR reactions were performed in a total volume of 50 μl containing 2 μl of template DNA. The amplicons obtained were separated by denaturing gradient gel electrophoresis (DGGE), as previously described by Polo et al. [23]. The electrophoresis run was performed for 15 h at 90 V by D-Code Universal Mutation Detection system (Bio-Rad). Amplicons from sludge and membrane samples were loaded in the same gel to make the DGGE profiles comparable. The DGGE was performed with the denaturant gradients 40-60 %. The gels were stained by SYBR-Green (Armersham Pharmacia Biotech) and the results observed by GelDoc

(Bio-Rad) apparatus. The excised bands were eluted in 50µl milli-Q water by incubation at 37°C for 3 h and successively overnight at 4°C. All the excised bands were identified by sequencing (Primm, Milan). The sequences were analyzed in June 2011 using BLASTN software (www.ncbi.nlm.nih.gov/BLAST) and deposited in the European Nucleotide Archive (ENA; www.ebi.ac.uk/ena/) under the accession number HF546518 to HF546526 and HF546538 to HF546548.

Using 16S DGGE profiles, the line plots were generated by ImageJ software [24], and then imported into an Excel® file as x/y values. The matrix were analyzed using principal component analysis (PCA-analysis) in order to study the relationship between bacterial communities in sludge and on filtration membranes. The multivariate investigations were conducted by XLSTAT (vers. 7.5.2 Addinsoft, France) on autoscaled data normalized using the Pearson correlation as similarity index. Line profile data were scaled in order to consider even the small peaks, downweighting the relative importance of the predominant bands [25]. The significance of the PCA-analysis model was tested by a cross-validation procedure.

2.3 Isolation and identification of bacterial strains from fouled filtration membrane

To isolate fouler strains, one hundred µL of cells suspensions collected from filtration membrane samples were inoculated in two cultural media: plate count agar medium (PCA; Merck) for aerobic heterotrophic bacteria and on the agarized synthetic wastewater medium (SWA) reported by Khan et al. [26] modified in order to have the same COD, N_{tot} and P_{tot} values measured in the wastewater of the Aldeno MBR plant and added with agar (258.8 mg/L glucose, 242.6 mg/L NH_4Cl, 43.9 mg/L KH_2PO_4, 10 mg/L $CaCl_2$, 10 mg/L $MgSO_4 \cdot 7H_2O$, 3 mg/L $FeCl_3$, 2 mg/L $MnCl_2$, 200 mg/L $NaHCO_3$, agar 17 g/L, pH 7-8), with incubation at 30°C for 3-5 days. All different colonies observed were isolated by subsequent plating. The genomic DNA of isolated strains was extracted as described by Murray et al. [22] and analyzed amplifying 16S rRNA gene fragments with primers 357 F and 907 R with the following chemical conditions: 1× of PCR Rxn buffer, 1.5 mM of $MgCl_2$, 0.12 mM of dNTP mix, 0.3 µM of each primer and 1 U of Taq DNA polymerase (Invitrogen). The thermal cycling programme included an initial denaturation at 94°C for 5 min followed by thirty-five cycles consisting of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

All PCR reactions were performed in a total volume of 50 µl containing 3 µl of template DNA. The sequences were analyzed in June 2011 using BLASTN software

(www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences were deposited in the ENA (www.ebi.ac.uk/ena/) under the accession number HF546527 to HF546537.

2.4 Synthesis of zosteric acid

Zosteric acid was prepared by treating a N,N-dimethylformamide solution of trans-4-hydroxycinnamic acid with the sulfur trioxide pyridine complex, as previously described by Villa et al. [20]. The obtained sodium zosterate contains < 5% of sodium sulfate. Proton nuclear magnetic resonance spectroscopy of sodium zosterate showed a downfield shift of aromatic protons adjacent to the strong electron withdrawing sulphooxy group with respect to the same protons of trans-4-hydroxycinnamic acid [20].

2.5 Cellular growth with zosteric acid

Pseudomonas putida (accession number HF546530) was used as target microorganism for all the experiments with zosteric acid. The ability of bacteria to grow on zosteric acid as their sole carbon and energy source was tested using synthetic wastewater broth (SWB) medium without glucose and supplemented with zosteric acid at sublethal concentrations 200 and 600 mg/L (0.73 mM and 2.2 mM respectively), the same quantities used in later experiments [20]. The positive control was represented by the SWB supplemented with both 395.7 mg/L (2.2 mM) and 4000 mg/L (22.2 mM) of glucose [20]. Microbial growth was followed by determination of absorbance (OD_{600}). All the experiments were repeated three times.

2.6 Microplate-based biofilm assay

P. putida cells adhesion was assessed quantitatively using fluorochrome-labeled cells in hydrophobic 96-well blacksided plates as previously reported by Villa et al. [20]. Briefly, 200 µl of SWB containing 10^6 cells and different sublethal concentrations (from 200 to 600 mg/L) of zosteric acid were placed in microtiter plate wells. After 72 h incubation at 30°C, the microtiter plate wells were washed one times with 200 µl of phosphate buffer solution (PBS; Sigma Aldrich) and adhered cells were firstly permeabilized with a 70% ethanol solution for 2 min and then stained using 10 µg/mL of 4',6-diamidino-2-phenylindole dye (DAPI; Sigma-Aldrich) in PBS for 30 min in the dark at room temperature. After three wash with 200 µl of PBS, fluorescence intensity was measured using the fluorometer VICTOR™ X Multilabel

Plate Readers (Perkin Elmer) at excitation wavelength 433 nm and emission wavelength 335 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the performance of the anti-biofilm compound. For all the combinations a negative control without the potential anti-biofilm agent was present.
Four measurements were performed for each combination and their average was computed.

2.7 Biofilm preparation by colony-biofilm method

P. putida biofilms were cultivated on sterile polycarbonate membrane filters (diameter, 2.5 cm; pore size, 0.2 μm; Whatman) placed onto PCA plates using the colony biofilm protocol by Anderl et al. [27]. A tissue paper moistened with PBS or with PBS plus 200 mg/L zosteric acid was placed between the agar culture medium and the membrane filters for the control and the treated samples respectively and replaced every 24 h. After 48 h of incubation at 30°C, the membrane-supported *P. putida* biofilms were visually inspected, photographed, removed, suspended in PBS and serially diluted in microtiter plates. Biofilm cells were enumerated using the drop-plate method in which 20 μL drops of each dilution were placed onto PCA plates. All the experiments were conducted in duplicate.

2.8 Biofilm sectioning

The biofilms grown on polycarbonate membranes by colony-biofilm method were carefully covered with a layer of optimum cutting temperature (OCT formulation, Tissue-Tek) and placed in dry ice until completely frozen. The frozen samples were dipped vertically into the center of a cryosectioning mold filled with fresh OCT. The frozen samples were sectioned at −19°C using a Leica CM1850 cryostat. The 5-μm thick cryosections were mounted on glass slides treated by Vectabond (Vector laboratories).

2.9 Biofilm imaging by confocal laser scanning microscopy (CLSM)

Both biofilm on membrane filtration samples from the MBR WWTP and biofilm sections were stained with green-fluorescent nucleic acid stain SYTO9 (5 μM) (Invitrogen) and Texas red-labeled Concanavalin A (ConA, 200 μg/mL) (Invitrogen) for 10 min in the dark at room temperature. After incubation, samples from filtration membranes were rinsed three times with PBS. Samples were visualized using a Leica TCSNT CLSM with a 10x dry objective,

excitation filters at 488 and 568 nm, and emission filters at 530 and 590 nm. The images were analyzed with the software Imaris (Bitplane Scientific Software, Switzerland). All the experiments were conducted in duplicate. For the biofilm sections, the biofilm thickness of the control and treated samples was measured for each image at three different locations randomly selected along the profile. More than five images per sample were taken for microscope analysis. These measurements were used to calculate the average thickness and the associated standard deviation.

2.10 Physicochemical profiling of zosteric acid

Different chemical and physical properties of zosteric acid were obtained with the goal of determining the general features of its behavior in the environment.

The chemical and physical properties of zosteric acid are reported in table 1.

The octanol-water partition coefficient (Kow) of zosteric acid was determined by potentiometric titration with the Scirus GLpKa instrument coupled with a computer-aided system for the evaluation of acid dissociation constant (pK_a) values (Scirus RefinementPro software Ver. 1.0) as reported by Vistoli et al. [28]. To obtain the Kow, separate titrations of the compound were carried out using various volumes of n-octanol. In the presence of n-octanol, the pK value shifts as a consequence of the partitioning of the substance into the organic phase, allowing a new pK constant to be determined. These shifts in the pK values were used to determine log Kow, the logarithm of the partition coefficient of the neutral form [28].

Table 1. Physico-chemical properties of zosteric acid measured and/or predicted by EPIsuite (http://www.epa.gov/opptintr/ exposure/pubs/episuite.htm).

Physico-chemical properties	Value	Source
Molecular weight (g/mol)	244	-
Melting point (°C)	260	Nakazawa and Ohsawa 2000
Water solubility (mg/L at 20°C)	2.5E+005	Villa et al. 2011
Vapor pressure (Pa, 25°C)	3.81E-009	Predicted
log Kow (octanol water coefficient, 20°C)	-0.25	Measured in this work
log BCF (bioconcentration factor)	0.94	Predicted

2.11 D. magna acute ecotoxicity test

Parthenogenetic *D. magna* were cultured (40 ind/L) in 1L glass beaker filled with 500 mL of culture medium (hardness 240 mg/L, pH 7.8) [29], maintained at 20±1°C under 16-h light:8-h dark photoperiod. Culture medium was renewed and offspring discarded three times a week. In these occasions, daphnids were fed with a suspension of the unicellular green alga *P. subcapitata* (0- to 8-days-old daphnids: $8 \cdot 10^6$ cells ind^{-1} day^{-1} and from 8-days-old daphnids: $16 \cdot 10^6$ cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* ($15 \cdot 10^6$ cells ind^{-1} day^{-1}). Brood daphnids were renewed every 6 weeks and replaced with neonatal organisms. Daphnids from the third generation were used for culture renewal and for exposure experiments to reduce variability [29].

Stock solutions were prepared by dissolving the appropriate amount of zosteric acid in culture medium. Two acute toxicity tests were carried out. In a preliminary test, organisms were exposed to solutions within the range 10 mg/L-700 mg/L, in the final test, zosteric acid concentrations were between 700 mg/L and 7000 mg/L in geometric progressions no higher than 2. Acute toxicity assays were performed following the OECD Guideline n. 202 [29]. Five newborn daphnids, less than 24 hours of age, were exposed to 20 mL of test solution. Each exposure was run in four replicates for 48 h at 20±1°C without feeding. Daphnids immobilization was used as acute toxicity end-point after 24 h and 48 h, when pH was also measured.

2.12 P. subcapitata growth inhibition ecotoxicity test

In order to adapt the test alga *P. subcapitata* to the test conditions, an inoculum culture in the test medium [30] was prepared 3 days before the start of the test. The algal density was adjusted in order to allow exponential growth to prevail in the inoculum culture until test start. Test was carried out following the OECD guideline n. 201 [30]. Exponentially growing algal cultures were exposed to five concentrations ranging from 10 mg/L to 2000 mg/L of zosteric acid and control under standard conditions. Algal density of each exposure concentration was measured at 72 h by cell counting with a Burker counting chamber; the assay was run in three replicates starting from an algal density of 50000 cells/mL. Exposure flasks containing 80 mL of test solution were maintained at 20±1°C under 16-h light:8-h dark photoperiod (7000 lux) with stirring to facilitate transfer of CO_2 for 72 h. To be valid, test cells density in the control culture should have increased at least 16 times during the 72 h exposure period. Data are reported as the average specific growth rate [30] for each concentration, included control

calculated as:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}\ day^{-1}$$

where: μ_{i-j} is the average specific growth rate from time *i* to *j*, X_i is the density at time *I*, and X_j is the density at time *j*.

Protozoa density of each exposure concentration was measured at 72 h by organisms counting with a Burker counting chamber. A subsample of each flanks were fixed with methanol to immobilize organisms in order to facilitate the counting.

2.13 Statistical analysis

For the microplate-based biofilm assay and toxicity data, the mean values, standard errors of the means and variance analysis with ANOVA of all replicates were calculated using GraphPad Prism 4 to assess the significance of differences in the results collected at several antifoulant concentration. Differences were considered significant with P-values <0.05. Individual comparisons were made post hoc with the Tukey–Kramer test.

For the biofilm assay, statistical significance of CFU/cm^2 and biofilm thickness was determined by Student's *t*-test analysis ($P < 0.05$).

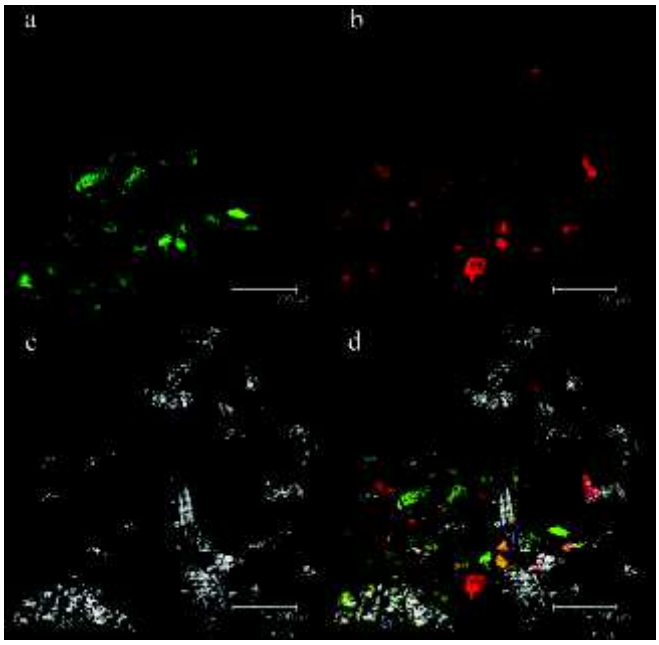
3 Results

3.1 CLSM analyses of fouled filtration membranes

Figure 1 illustrates the fouled filtration membrane sample visualized by CLSM. Microscopic observations showed the presence of sessile cells (green fluorescence) embedded in exopolysaccharides (red fluorescence), thus demonstrating the presence of microbial biofilm.

Figure 1. Confocal analysis of the biofouling on the filtration membrane of the Aldeno MBR WWTP. Panel (a) shows bacterial cells (green fluorescence), panel (b) the biofilm matrix

exopolysaccharides (red fluorescence), panel (c) the membrane fibers in reflection mode and panel (d) the superimposition of panels (a), (b) and (c).



3.2 DGGE, sequencing and community profiles analysis

Table 2 reports the identified strains obtained from DGGE analysis. The sequences from sludge and fouled membranes were phylogenetically most closely related to alphaproteobacteria (64%), bacteroidetes (27%) and betaproteobacteria (9%), and to gammaproteobacteria (45%), betaproteobacteria (22%), acidobacteria (11%), bacteroidetes (11%) and other unclassified bacteria (11%), respectively.

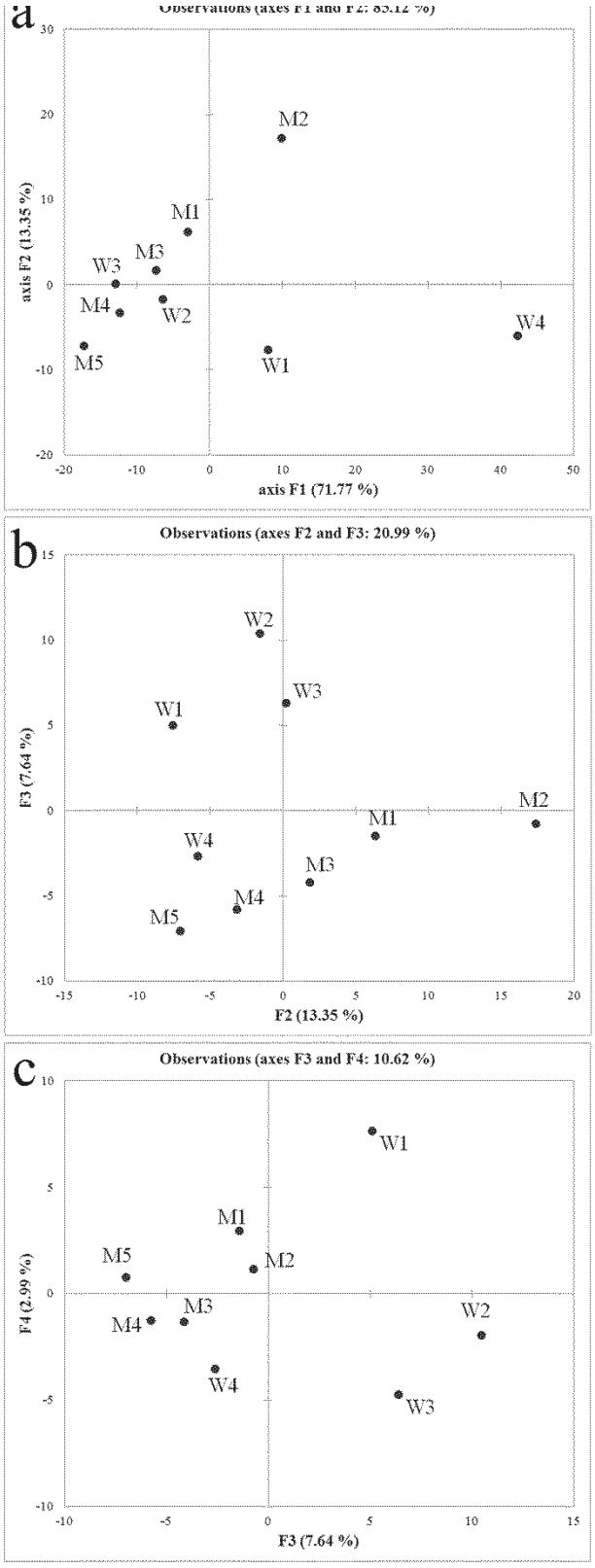
PCA-analysis was applied to study possible structural similarities in the bacterial communities evidenced by the 16S rDNA DGGE profiles. The plot of the two-dimensional scores accounted for 85.12% of the input data variability for bacteria and was therefore adequate to describe the system under investigation (figure 2a). The samples W4 and M2 dominated the first principal component (F1, explaining the greatest percentage of the system variability), masking any other interesting grouping among samples. The study of the following principal components (F2 vs. F3 and F3 vs. F4) was used to gain useful information concerning the differences between sludge samples and samples collected on the membrane (figure 2b and 2c). The PCA-analysis excluded a close relationship among bacterial

communities as several samples from both fouled membranes and sludge are in separate clusters.

Table 2. Identification of partial 16S gene sequences from excised bands observed in DGGE profiles.

Source	BlastN reference strains			RDP tassonomic classifier	
	Closest relative strain	**Similarity (%)**	**GenBank accession number**	**Most probabile taxon**	**Similarity (%)**
Sludge	*Paracoccus* sp.	92%	AY646160	Alphaproteobacteria	99
	Novosphingobium sp.	99%	EU430056	Alphaproteobacteria	100
	Novosphingobium aromaticivorans	93%	JF459981	Alphaproteobacteria	100
	Novosphingobium sp.	96%	FM886872	Alphaproteobacteria	100
	Alpha proteobacterium	91%	AB578881	Alphaproteobacteria	100
	Sphingomonas sp.	99%	AJ617690	Alphaproteobacteria	100
	Sphingomonas sp.	89%	GQ181132	Alphaproteobacteria	97
	Sphingobacteriaceae bacterium	97%	FJ386545	Bacteroidetes	100
	uncultured *Niastella* sp.	98%	GU326307	Bacteroidetes	100
	Flavobacteriales bacterium	95%	EF636194	Bacteroidetes	100
	uncultured Oxalobacteraceae bacterium	91%	HM141157	Betaproteobacteria	100
Fouled membranes	uncultured Xanthomonadales bacterium	89	EF073609	Gammaproteobacteria	99
	uncultured Gammaproteobacteria bacterium	99	CU926678	Gammaproteobacteria	100
	Rhodanobacter sp.	99	EU876661	Gammaproteobacteria	100
	Rhodanobacter sp.	97	EU876661	Gammaproteobacteria	100
	uncultured Betaproteobacterium	97	JF703550	Betaproteobacteria	100
	uncultured *Comamonas* sp.	89	JF808874	Betaproteobacteria	99
	uncultured bacterium	98	AB241559	Bacteroidetes	100
	Uncultured Acidobacteria bacterium	98	HM062044	Acidobacteria	100
	uncultured Betaproteobacterium	89	FJ184029	unclassified bacteria	98

Figure 2. PCA analysis results for bacterial DGGE band patterns: (a) first principal component (F1) vs. second principal component (F2); (b) F2 vs. third principal component (F3); (c) F3 vs. fourth principal component (F4). W = sludge samples; M = fouled filtratio membranes.



3.3 Bacterial strains isolated from fouled filtration membrane

Table 3 reports the identified cultivable bacterial strains obtained from fouled membranes. The sequences of isolated strains were phylogenetically most closely related to gammaproteobacteria (55%), firmicutes (27%), actinobacteria (9%) and betaproteobacteria (9%).

Table 3. Identification of partial 16S gene sequences from bacterial strains isolated from fouled filtration membrane.

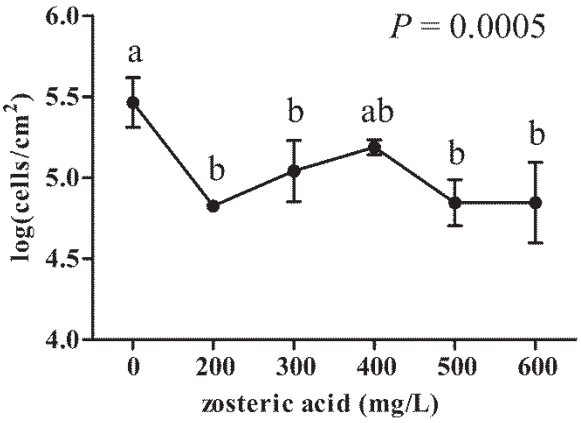
Closest relative strain	Cultural media	Taxa	Similarity (%)	GenBank accession number
Pseudomonas putida	PCA	Gammaproteobacteria	99%	JF703662
Pseudomonas sp.	PCA	Gammaproteobacteria	99%	HQ718413
Pseudomonas sp.	SWA	Gammaproteobacteria	97%	AM689975
Pseudomonas sp.	SWA	Gammaproteobacteria	99%	HQ588845
Shewanella xiamenensis	PCA	Gammaproteobacteria	99%	HQ418493
Enterobacter cloacae	PCA	Gammaproteobacteria	99%	HQ231214
Bacillus arvi	PCA	Firmicutes	99%	AM260979.1
Lysinibacillus fusiformis	PCA	Firmicutes	99%	GQ844965
Bacillus cereus	PCA	Firmicutes	100%	HQ333012
Rhodococcus sp.	PCA	Actinobacteria	96%	EU293153
Chitinimonas sp.	SWA	Betaproteobacteria	97%	GQ354569

3.4 Zosteric acid was not a carbon and energy source for P. putida but affected cell adhesion

Although *P. putida* grew in SWB supplemented with glucose, it did not exhibit growth on zosteric acid as the sole carbon and energy source.

Bacterial cells stained with DAPI appeared uniformly labeled and revealed a linear relationship between cell number and fluorescent intensity in a range from 10^2 to 10^7 cells. Figure 3 shows *P. putida* cells adhered to the microplate walls for cm^2 of surface. Zosteric acid at sublethal concentrations induced a significant inhibition of cell adhesion compared to the control without the antifoulant. Adhered cells were reduced of ca. one order of magnitude by using 200 mg/L of the compound. The increase of the antifoulant concentration did not improve its performance.

Figure 3. *P. putida* cells adhesion in presence of different concentrations of zosteric acid. In the graph, y-axis shows log(CFU/cm^2) values and x-axis represents different tested concentrations. Each value corresponds to the mean of four replicates. The graphs provides the P-value obtained by ANOVA analysis. According to post hoc analysis (Tukey–Kramer, $P < 0.05$), means sharing the same letter are not significantly different from each other. Bar errors show standard deviations.



3.5 Zosteric acid affected the morphology, thickness and biomass of P. putida biofilm

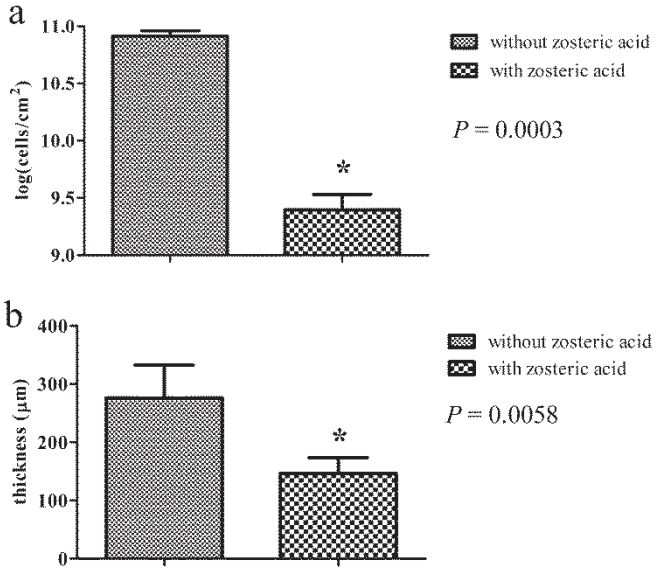
Visual inspection of *P. putida* biofilm grown in presence of zosteric acid highlighted different morphological development in comparison to the same cells without the antifoulant. *P. putida* biofilm without zosteric acid shows a flat development.

After 48 h of growth as a colony biofilm, a considerable decrease in biofilm formation was observed in the presence of the compound. Treatment with zosteric acid produced biofilms containing $2.6 \cdot 10^9 \pm 7.2 \cdot 10^8$ CFU/cm^2 (control $8.3 \cdot 10^{10} \pm 8.7 \cdot 10^9$ CFU/cm^2) (figure 4a), leading to a cell reduction of 97%.

Measured biofilm thickness obtained by cryosectioning combined with microscopy analysis revealed that in the presence of zosteric acid, *P. putida* biofilm had a thickness of 146±27 μm, significantly thinner than the film formed without it where the biofilm thickness was 276±56 μm (figure 4b). The yield reduction in biofilm thickness was by 50%.

Zosteric acid-treated biofilms retained similar morphological patterns to those observed in the control. The fluorescent staining procedure demonstrated the presence of EPS and thus the formation of a biofilm. In both the control and the treated samples, ConA staining resulted in an intense red fluorescent signal, revealing that the exopolysaccharide component of EPS matrix did not show quantitative changes in presence of zosteric acid.

Figure 4. Effect of zosteric acid on the amount of P. putida sessile cells on the polycarbonate membranes (a) and on the thickness of cryosectioned P. putida biofilm (b). Data represent the mean ± standard deviation of replicates. The star (*) indicates statistically significant difference at the 95% confidence level between control and treated samples.



3.6 *General features of the zosteric acid behavior in the environment*

Zosteric acid is a practically odorless white crystalline powder that is readily soluble in water and has a high melting temperature (table 1). Zosteric acid has low vapor pressure and Kow close to zero that enhance its stability and presence in water.

3.7 Ecotoxicity responses

The *D. magna* preliminary test showed no immobilization effects within the concentrations between 10 to 700 mg/L of zosteric acid. Taking into account this information, higher exposure concentrations were tested and in the final test no toxicity was observed even at 7000 mg/L zosteric acid. No significant variations of pH were observed during both exposures.

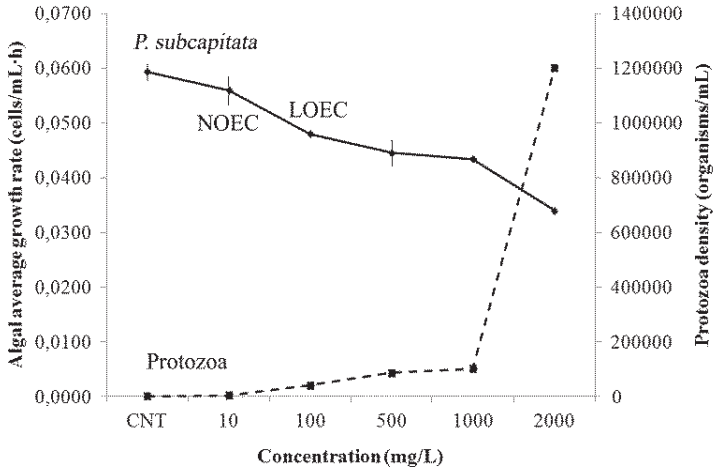
A significant effect on *P. subcapitata* algal growth was observed starting from 100 mg/L (ANOVA, $p<0.05$) (figure 5). A decrease of growth of algae was gradually observed with a simultaneous unexpected growing development of ciliates protozoa (with a density from

40000 organisms/mL at 100 mg/L of zosteric acid to $1.2 \cdot 10^6$ organisms/mL at the highest concentration).

4 Discussion

The present work was designed to explore for the first time the potential of the zosteric acid as antifouling strategy to counteract the membrane fouling in MBR of WWTPs equipped with membrane-based separation technology.

Figure 5. Algal average growth rate of P. Subcapitata (cells/mL·h) and protozoa density (organisms/mL) at the different concentrations of zosteric acid and control. NOEC (No Observed Effect Concentration) and LOEC (Low Effect Observed Concentration) on algal growth rate are also reported.



Villa et al. [20] have already demonstrated its efficiency in hindering biofilm formation in vitro using different model microorganisms. However it was widely reported that zosteric acid exhibits a species-specific behaviour and the reduction in microbial adhesion depends on the environmental conditions [20, 21]. In addition, bacterial variance in WWTPs leads to an extremely complex biofilm system that is poorly known and the dominant species would be quite different with the changes of feed water, process configurations, membrane materials, and operating conditions [31]. Therefore, it was necessary to characterize the microbial communities in MBS, to isolate the microorganisms involved in the membrane biofouling process, and to investigate the performance towards a target fouler strains in order to evaluate the potential of an antifouling strategy based on zosteric acid. This work represents the first

step to transfer the antifouling performance of zosteric acid to the membrane based separation process technology in WWTPs.

Initial inspection by CLSM of fouled membranes from the Aldeno MBR plant highlighted the presence of sessile cells included in a EPS matrix demonstrating that the biofouling was a considerable constituent of fouling process on such membranes as reported in several other works [32]. Observations demonstrated that microbial biofilms were constituted by separate agglomerates of cells with a good production of exopolysaccharides.

Bacterial communities from both sludge and fouled membranes were studied by culture independent methods in order to characterize bacterial taxa involved in the biofouling process. The sequences from sludge and fouled membranes were phylogenetically most closely related to alphaproteobacteria (64%), bacteroidetes (27%) and betaproteobacteria (9%), and to gammaproteobacteria (45%), betaproteobacteria (22%), acidobacteria (11%) and bacteroidetes (11%), respectively. In both cases, proteobacteria were found to be the most prevalent classes in agreement with several previous researches [33, 34, 35, 36]. These results agree with several works reporting that proteobacteria play a major role in the development of membrane biofouling in WWTPs [31, 37]. Therefore, the knowledge of these dominant species in membrane biofilms of the specific MBR represents a crucial step to test potential antifouling strategies designed to hindering biofouling. As PCA-analysis did not highlight significant similarities among bacterial communities (both in sludge and those that grew in sessile form on fouled membrane), it demonstrated that only some taxa present in sludge caused biofouling on the membranes and that the bacterial community causing biofouling can be different on the several point of membrane surface. Therefore, a bacterial collection of cultivable strains was obtained only from fouled membranes to investigate the antifouling activity of zosteric acid against fouler microorganisms. Many identified strains were previously reported in WWTPs. *Shewanella xiamenensis* was commonly isolated from fresh water and seawater [38, 39]. *Bacillus arvi*, *B. cereus, Lysinibacillus fusiformis*, and strains belongs to *Chitinimonas* and *Pseudomonas* genera were generally isolated from soil and rhizosphaera [40, 41] and in the latter case they could have come from agricultural waste as crops are widespread in Trentino region. Members of *Pseudomonas* genus were also frequently isolated from WWTPs [33, 42], growing on several of type of membranes [43]. *L. fusiformis* was also isolated from chromium contaminated industrial wastewater [44]. Strains belonging to *Chitinimonas* genus were isolated from soil and freshwater pond [32, 45]. *Enterobacter cloacae* was previously identified from a range of natural environments including contaminated soil [46]. Members of the genus *Rhodococcus* have been isolated

from a large range of habitats such as soil, rocks, ground water, marine sediments, animal dung, the gut of insects and from infected animals, plants and humans, and they have great significance in environmental bioremediation and a number of industrial processes [47].

The observed bacterial variety highlighted an extremely complex biofilm on fouled membranes. Therefore, for this work, a single representative strain was used as a target bacterium. These comprehensive phylogenetic analyses revealed that in this research Gammaproteobacteria resulted to be the prevalent taxon on fouled membrane both by culture dependent and independent methods (they represent 55% and 45% of identified bacterial community, respectively). Gammaproteobacteria were considered to have a distinctive role in initial membrane colonization since they were more predominant than betaproteobacteria and alphaproteobacteria in primary biofilms as compared with mature ones [48]. In this case study, *Pseudomonas* was the prevalent genus belonging to gammaproteobacteria isolated from fouled membranes. *Pseudomonas* spp. are known for their relevant abundance in municipal wastewater and in activated sludge compared to other bacterial taxa, and for their ability to foul surfaces rapidly [49, 50, 51]. In addition, they were frequently found in biofilms and were reported to play a major role in the development of the mature biofilms, which led to the severe irreversible membrane biofouling [52]. Therefore, *P. putida* (accession number HF546530) was chosen among the isolated *Pseudomonas* strains as target organism to study the antifouling properties of zosteric acid. In fact, *P. putida* is commonly present throughout the investigated environment and readily forms biofilms [53, 54]. In addition, it was diffusely isolate from activated sludge in WWTPs [55, 56], and it has been already adopted as model biofoulant to test antifouling technology on membranes [43, 57, 58]. Although Xu and coworkers [59] and Newby and coworkers [60] have already tested the effectiveness at preventing the attachment of *P. putida* planktonic cells, on the best of our knowledge this is the first time that the effects on the *P. putida* biofilm were investigated. In addition, for the first time the attachment experiments were performed simulating the chemical condition (COD, N_{tot} and P_{tot}) measured in the wastewater of the MBR plant where the fouling strain was isolated.

Zosteric acid did not represent a carbon and energy source for *P. putida* at the sublethal concentrations tested and at 200 mg/L reduced cell adhesion by 97% on hydrophobic surfaces of 96-well plates without raising the selective pressure. Pitts et al. [61] claimed that a compound which could reduce biofilm by at least 40% can be considered as a satisfactory antibiofilm agent. Xu and colleagues [59] observed a reduction in bacterial biofilm coverage by 98.2% at 500 mg/L zosteric acid. The mathematical model developed by Villa and

colleagues [20] predicted that 200 mg/L zosteric acid reduced cell adhesion on the hydrophobic surface by about 55% and 78%, for *E. coli* and *B. cereus* respectively, whereas 500 mg/L zosteric acid ensured a percentage reduction of bacterial adhesion of more than 90%. Taken together these results showed that, although zosteric acid exhibited a species-specific behaviour, it always decreased bacterial adhesion, thus displaying potential as a broad range antifoulant.

For the first time, the membrane-supporting biofilm reactor was used to further investigate the effect of zosteric acid on *P. putida* biofilm formation. This technique permitted to force cells surface attachment, a feature that allowed to i) mimic the membrane scenario in which foulers are retained by the membrane surface during gravity-driven filtration and ii) directly investigate the effect of zosteric acid biofilm structural development and organization bypassing the effect on the adhesion phase. Although a biofilm-like structure was observed in presence of zosteric acid, *P. putida* biofilm development was impaired with significant decrease in biomass and mean thickness. These findings corroborated previous results that revealed the ability of zosteric acid to slow down surface colonization and reduce biofilm thickness [20, 21].

Noteworthy, zosteric acid treatment induced a migration activity of the peritrichous flagellated bacterium *P. putida* over the polycarbonate surface not amenable to a biofilm phenotype. Recently, Villa et al. [20] reported the ability of zosteric acid treatment to induce a hyper-motile phenotype in *E. coli* by controlling the degree of flagellation in the swim cell-state. These results suggested that sub-lethal zosteric acid concentrations act as environmental cue driving the transition between motile and sessile lifestyle and making *E. coli* cells too motile for proper biofilm formation [20, 62].

By identifying the surface colonizers and testing the efficacy of zosteric acid against the membrane-fouler *P. putida*, this study proposes a potentially interesting biocide-free approach for the prevention and reduction of biofouling in membrane-based separation processes. However, despite these promising properties to date no studies have addressed the fate and ecotoxicological effects of zosteric acid in the environment. To evaluate the suitability of zosteric acid for future application as antifouling technology in membrane-based separation processes in submerged MBR plant, in this study, for the first time, preliminary screening investigations and speculations about its potential environmental partitioning behavior and its ecotoxicity effects toward target aquatic organisms were performed. The first group of properties that must be estimated in an assessment of environmental risk are the basic physical and chemical properties that describe a chemical's partitioning between solid, liquid

and gas phases. These include melting point, vapor pressure, water solubility and the Kow. Considering the application of zosteric acid as antifouling agent in wastewater treatment plant, the water ecosystem would be the principal recipient of primary emissions and fate processes. High water solubility and low Kow suggest that water likely retains most of the compound introduced in the system. This means that when emitted only to water, zosteric acid remains in that phase and it is expected that this compound does not partition appreciably into soils, settling particles in water and sediments. Exposure of the terrestrial environment to the compound is to be expected being limited to areas subjected to flooding or intensive runoff and direct deposits of sewage or wastewater sludge. It follows that any exposure via the sediments, while unlikely, would be due to zosteric acid dissolved in the pore water. High water solubility and low vapor pressure suggest that volatilization of zosteric acid from surface water to the atmosphere is negligible.

Because the Kow characterizes partitioning between aqueous and organic, lipid-like phases, it is used to estimate a variety of toxicological, and environmental fate parameters. One specific use of the Kow is as a gauge for the potential for bioaccumulation [63]. If a chemical tends to partition into the organic phase (is lipophilic), then the chemical can be stored in fatty tissue of fish and will bioaccumulate in animals that consume the fish [63]. The low Kow and the high water solubility of zosteric acid, suggests a low bioaccumulation potential. Predictions using the Arnot–Gobas bioconcentration factor (BCF) and bioaccumulation factor (BAF) model from the EPIsuite prediction software [64] clearly suggest that zosteric acid should not be expected to bioaccumulate either at the lower or upper trophic levels (BCF of 0.94) and it possesses the biotransformation half-life of 2 hours. To date, only few toxicological data exist about the zosteric acid. Xu et al. [59] reported that zosteric acid EC50 values were 167 ± 3.9 and 375 ±10 mg/L for *P. putida* and Lake Erie bacteria, respectively, and the EC50 value from the Microtox assay was 442 ± 100 mg/L. Using in vitro experiments with suitable primary cell based models, Villa et al. [21] demonstrated that 10 mg/L of zosteric acid did not compromise the cellular activity, adhesion, proliferation or morphology of either the murine fibroblast line L929 or the human osteosarcoma line MG-63. Flemming [65] reported no detectable LD_{50} toward larval fish, and an acute toxicity profile similar to table sugar: it was fully biodegradable and had a half-life in seawater of a few days. To our knowledge, until now no data on the toxic effects of zosteric acid on freshwater invertebrates are available. Therefore, considering possible future uses and environmental fate of zosteric acid in freshwater environments, the importance of knowing the potential toxic effects on target aquatic organisms seems crucial. The freshwater invertebrate *D. magna* and the algal species

P. subcapitata were selected as target aquatic organisms as widely used for ecotoxicity studies [29, 30, 66, 67, 68]. No toxicity effect toward *D. magna* at any tested concentration was observed. A significant inhibition of growth of algae was observed starting from 100 mg/L which represents the 72 h LOEC (Lowest Observed Effect Concentration); in our bioassay NOEC (No Observed Effect Concentration) was 10 mg/L. It has to be underlined that an increasing density of grazers protozoa was observed, probably contributing to the progressive decrease of algal density with increasing concentrations of zosteric acid. As no protozoa were observed in control flasks their presence may be connected to the exposure medium. Protozoa are well known to feed on algae [69, 70, 71] and their grazing effects on algal density cannot be excluded even in this case. As a consequence caution is requested in the evaluation of the toxic effects of zosteric acid on algal communities and indepth investigations should be performed to have more extended ecotoxicological data on algae and to evaluate if the growth inhibition is directly related to zosteric acid or to protozoa grazing activity. Taking into account that LOEC of algae is referred to a chronic exposure, these results can support the development of a biocide-free strategy based on zosteric acid to counteract unwanted biofilm in membrane-based separation processes.

5 Conclusions

In this study we characterized the biofilm microbial communities on the membranes of a submerged MBR plant, located in Aldeno, Trento (Italy) and the membrane colonizer *P. putita* was selected as a biofilm target system to investigate the ability of zosteric acid in mitigating surface biofouling phenomena and the antifouling concentration to be used. On the best of our knowledge, this is the first time that the antibiofilm properties of zosteric acid were tested against an isolated bacterium from biologically fouled membranes of a submerged MBR plant at sublethal concentrations. These investigations exhibited that zosteric acid is effective in reducing biofilm formation by *P. putida* without raising the selective pressure. In addition, in view of an application, a preliminary screening of the potential environmental partitioning behavior of this promising molecule suggested that the water compartment represents the main environmental recipient and capacitor of this molecule while the fraction associated to any other compartment, including atmosphere, soils, settling particles in water and sediments would be virtually negligible. In addition, the low Kow and the high water solubility of zosteric acid suggested a low bioaccumulation potential. Finally, preliminary

ecotoxicity tests suggested that zosteric acid does not represent a threat for the target aquatic organisms *D. magna* while for the impact on algal communities more ecotoxicological data are recommended.

This study encourages the use of zosteric acid-based methods as an innovative, effective, biocide-free and green strategy for the prevention and reduction of biofouling in membrane-based separation processes. However, the biodegradability of zosteric acid in freshwater, its potential toxicity toward a wider range of aquatic organisms, human and animals, and its consistent performance toward a bacterial consortium of sludge in a pilot scale MBR will have to be further investigated for optimization of its use on a field scale.

Conflict of Interests

The authors of the manuscript do not have any direct financial relation with the commercial identities mentioned in the paper.

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