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Zinc-induced antibiotic resistance in activated sludge bioreactors

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ABSTRACT

Increased levels of bacterial resistance to antibiotics noted in recent decades poses a significant obstacle to the effective treatment and prevention of disease. Although overuse of antibiotics in agriculture and medicine is partially responsible, environmental exposure to heavy metals may also contribute to antibiotic resistance, even in the absence of antibiotics themselves. In this study, a series of eight lab-scale activated-sludge reactors were amended with Zn and/or a suite of three antibiotics (oxytetracycline, ciprofloxacin, and tylosin), in parallel with unamended controls. Classical spread-plating methods were used to assess resistance to these compounds in culturable bacteria over 21 weeks. After seven weeks of general acclimation and development of baseline resistance levels (phase 1), 5.0 mg/L Zn was added to half of the reactors, which were then operated for an additional 7 weeks (phase 2). For the final seven weeks (phase 3), two of the Zn-amended reactors and two of the control reactors were amended with all three antibiotics, each at 0.2 mg/L. Zn amendment alone did not significantly change resistance levels at the 95% confidence level in phase 2. However, tylosin resistance increased significantly during phase 3 in the Zn-only reactors and resistance to all three antibiotics significantly increased as a consequence of combined Zn+antibiotic amendments. Ambient dissolved Zn levels in the reactors were only 12% of added levels, indicating substantial Zn removal by adsorption and/or precipitation. These results show that sub-toxic levels of Zn can cause increased antibiotic resistance in waste treatment microbial communities at comparatively low antibiotic levels, probably due to developed cross-resistance resulting from pre-exposure to Zn.

1. Introduction

Bacterial resistance to currently available antibiotics poses a significant obstacle to the effective treatment and prevention of disease (Davies, 2007; Mølbak, 2004; Neu, 1992; Witte, 1998). Increases in the occurrence of antibiotic resistant strains of multiple infectious pathogens, including *Salmonella typhimurium*, Streptococcus *pneumoniae* and *Staphylococcus aureus*, have been tracked both in the United States and around the globe (Lee et al., 1994; Simor et al., 1997). In addition to the obvious health risks associated with the spread of treatment resistant pathogens, managing antibiotic resistance issues has a substantial economic cost, estimated at up to $10 billion per year in U.S. hospitals alone (Levy, 2002).

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There is substantial overlap between known mechanisms for metals and antibiotic resistance, such as those for copper and tetracyclines, copper and ciprofloxacin, and arsenic and β-lactams (Baker-Austin et al., 2006). Various studies have also pointed to strong patterns of co-occurrence between metal and antibiotic resistance in environmental settings (e.g. Baker-Austin et al., 2006; De Souza et al., 2006; McArthur and Tuckfield, 2000; Soltan, 2001), including soils amended with Cu (Berg et al., 2005), freshwater microcosms amended with Cd and Ni (Stepanauskas et al., 2006), and liquid pure cultures containing Cu and Zn (Caille et al., 2007). Such exposures have been shown to increase the incidence of bacterial antibiotic co-resistance through the transfer of genetic elements containing both metal and antibiotic resistance genes (Aviles et al., 1993; Ugur and Ceylan, 2003), and also through the selection of organisms that contain elements, such as non-specific efflux pumps, which can convey cross-resistance to both metals and antibiotics (Summers, 2002).

Antibiotics in aquatic systems are frequently degraded quickly via photolytic and other pathways (Cardoza et al., 2005; Engemann et al., 2006). Therefore, there is debate about whether antibiotics themselves actually select for resistance in exposed bacteria in the environment (Knapp et al., 2008) or whether other factors are more important in maintaining or spreading antibiotic resistance (Knapp et al., 2010). Metals, which are relatively common and persist indefinitely in the environment, may be one such factor. Wastewater treatment plants are ideal locations to examine this question, as metal and antibiotic contamination often co-exist in these systems due to varied domestic and industrial waste inputs. Further, such settings contain diverse bacterial communities that might facilitate the conservation and/or promotion of metal and/or antibiotic resistance. In this study, we investigated bacterial resistance in wastewater treatment units with different metal and antibiotic inputs. Bacterial resistance to three antibiotics (ciprofloxacin, oxytetracycline, and tylosin) was assessed in lab-scale treatment reactors in conjunction with differing co-exposure levels of these antibiotics and sub-toxic levels of Zn. We hypothesized that moderately low levels of Zn might increase the observed levels of resistance to some or all of these antibiotics in the treatment reactors, providing an alternate explanation for varied levels of antibiotic resistance observed in different wastewater treatment plants.

2. Experimental methods

2.1. Reactors

Eight bench-scale activated sludge reactors were constructed from 4-L amber glass bottles. The bottom of each bottle was cut off and the neck filled with a rubber stopper. Each bottle was then inverted and placed in a wood stand. Aquarium air pumps (Aquatic Gardens 800) were used to provide aeration and mixing at a constant rate to each reactor. During the experiments, a foil cap was placed over each reactor to minimize evaporative losses.

Prior to the start of the experiment, two of the reactors were seeded with a mixture of return-activated sludge and primary clarifier effluent from the Lawrence, Kansas wastewater treatment plant. Once the mixed liquor suspended solids concentrations (MLSS) in the "seed" reactors were >1000 mg/L, the solids were re-distributed among the other six reactors and all eight reactors were grown through supplemental feeding with fresh wastewater until each unit achieved MLSS levels >1000 mg/L for a period of at least two weeks. On Day 1, the mixed liquor from all eight reactors was homogenized again in a sterile container, and 2 L of the homogenized solution was reintroduced to each reactor to start the experiment.

2.2. Microbial growth experiments

The study was performed over a 21-week period divided into three seven-week phases: initial growth, Zn amendment, and Zn+antibiotic amendment. During the initial phase (Phase 1), all reactors were fed primary effluent as described below. At the beginning of week 8, reactors 1–4 were provided an initial amendment of 10 mg of Zn as $ZnSO_4$ (reactors 5–8 were unamended) and were subsequently provided 5 mg/L Zn in their daily primary effluent feed throughout Phases 2 and 3. This Zn feed rate was chosen because Cabrero et al. (1998) found toxic effects at 10 mg/L Zn in bench-scale activated sludge reactors and it was desired to assess the influence of Zn levels that were "typical" and not innately toxic. In fact, Petrini and Grechi (1984) found soluble Zn levels in municipal wastewater plants up to 1.2 mg/L, which is about double levels observed here after Zn amendment (see later). Therefore, our Zn levels were typical of levels found in treatment plants.

In Phase 3, two of the Zn-amended units (Reactors 3–4) and two of the non-Zn-amended units (Reactors 5–6) were additionally amended with 0.2 mg/L (each) of ciprofloxacin as ciprofloxacin hydrochloride (Serological Proteins), oxytetracycline as oxytetracycline dihydrate (Acros Organics) and tylosin as tylosin tartrate (MP Biomedicals). The full amendment schedule for each reactor is summarized in Table 1.

The reactors were operated on a fill-and-draw basis using a hydraulic residence time of 5 days via the following procedure. Each day, 450-mL of mixed liquor was removed (out of a total volume of 2 L) and allowed to settle for 10 min. Four hundred-mL of supernatant was then removed for analysis, while the remaining 50-mL (including the settled solids and remaining liquid) was returned to the reactors. To replace the discarded supernatant, each reactor was amended with 10-mL of concentrated feed solution (Table 2) and 390-mL of primary clarifier effluent. This primary effluent was collected bi-monthly and stored at 4 °C in plastic carboys, but allowed to

Table 1 – Reactor amendment schedule.

Reactors	Phase 1	Phase 2	Phase 3
1–2	None	Zn	Zn
3–4	None	Zn	Zn+antibiotics
5–6	None	None	Antibiotics
7–8	None	None	None

Zn = 5 mg Zn/L as $ZnSO_4$, Antibiotics = 0.2 mg/L of ciprofloxacin, oxytetracycline and tylosin.

Table 2 – Synthetic feed solution composition (after Knapp and Graham (2007)).

Component	mg/L
Peptone	12,800
Meat extract	7600
$(NH_4)_2SO_4$	2640
Urea	1200
Yeast extract	1200
K_2HPO_4	560
KH_2PO_4	440
$CaCl_2 * 2H_2O$	160
$MgSO_4 * 7H_2O$	80

warm at room temperature prior to addition to the reactors. The pH of each reactor was monitored daily and adjusted to pH 7.0–7.5 using Na_2CO_3 as needed, in order to offset the effects of microbial CO_2 production in the units. Minimal variations in reactor volume were observed throughout the experiment.

2.3. Resistance measurements

Samples from each reactor were analyzed for metal and antibiotic resistance using classical spread-plating techniques. Classical methods were chosen over genetic methods because they do not rely on individual genes for quantification, which was considered less appropriate given the genetic diversity found in wastewater mixed liquor (Auerbach et al., 2007; Tennstedt et al., 2003). This approach clearly missed unculturable species, but was considered sufficient to observe general trends, especially in a carbon-rich environment like mixed liquor.

For each analysis, separate plates were prepared using Luria Bertani agar (Difco) amended as follows: no amendment (control), 161 mg/L (1 mM) $ZnSO_4$, 16 μg/mL ciprofloxacin, 16 μg/mL oxytetracycline, and 16 μg/mL tylosin. LB media was used to isolate as many organisms as possible from the relatively rich environment of mixed liquor. This media tends not to select for stressed organisms, which could potentially result in the under-reporting of antibiotic- or metal-resistant organisms. Antibiotic concentrations were chosen based on recommended values (Clinical and Laboratory Standard Institute, 2006), whereas Zn levels were chosen based on previous Zn toxicity assessments (Sabry et al., 1997; Ugur and Ceylan, 2003).

Samples for plating were collected at the end of each week and plated immediately. For each individual sample, 10-mL of mixed liquor was removed per reactor, homogenized in a sterile glass test tube, and serially diluted in de-ionized water, with three dilutions per sample being plated in duplicate. The plates were incubated for 7 days at 23–26 °C before colony enumeration. Antibiotic and metal resistances are reported as the ratio of colony forming units (CFU) on the different "amended" plates relative to parallel unamended controls. Only dilutions with 20–300 CFU per plate were used for colony enumeration.

2.4. Reactor chemistry

Daily samples were analyzed for total and volatile suspended solids (MLSS and MLVSS, respectively) using Standard Methods 2540 D and E, respectively (American Public Health Association, 2001). Chemical analyses included chemical oxygen demand (COD, Standard Method 5220 D), NO_3 (4500-NO_3-B), NO_2^- (4500-NO_2-B), NH_3 (4500-NH_3-F) and total dissolved nitrogen (TDN) by the alkaline persulfate digestion method (Solorzano and Sharp, 1980). Dissolved Zn levels were measured after filtration through 0.45 μm nylon filters, using an AAnalyst 300 atomic absorption spectrophotometer (Perkin–Elmer).

2.5. Data interpretation

Experimental outliers in the plate count data were identified using the interquartile range (IQR) method by removing points with >1.5 times the IQR in either direction (Schiff and D'Agostino, 1996), which removed <5% of the data. The Kruskal–Wallis one-way ANOVA test then was used to check for variation in resistance and biomass levels within reactor pairs at a 95% confidence level. No significant differences were observed between any duplicate reactors; therefore, data from pairs were averaged to produce a single value for each pair-set.

The antibiotic and Zn resistance data were statistically analyzed to assess changes in resistance levels between experimental treatments and phases. Differences in mean resistance levels were assessed using two separate tests. For normally distributed data (as measured by the Anderson-Darling test), the two-tailed two-sample Student's *t*-test was used, whereas if the sample groups were non-normally distributed, the non-parametric Mann–Whitney test was used. Significant differences were determined at the 95% confidence level unless otherwise noted. To corroborate apparent differences in resistance levels between treatments, we also compared fractional changes in resistance between experimental phases by calculating the ratio of mean resistances from one phase to the next. For example, oxytetracycline resistance in the Zn+antibiotic-amended reactors averaged 8.5 ± 1% in phase 2 (after Zn amendment, but prior to antibiotic amendment) and 45 ± 8% in phase 3, which gives a fractional change of 5.3 ± 2.1 between phases. Implicitly, a fractional change of one suggests no change in mean resistance in subsequent phases.

3. Results

Overall treatment performance of carbon and nitrogen removal was not substantially affected by either metal or antibiotic amendments during the study. COD levels in the reactor feed averaged 572 ± 75 mg/L (95% of confidence level) over the course of the experiment, with COD removal efficiencies between 84 and 88%. No significant differences were observed between experimental phases. Feed ammonia levels averaged 40 ± 8 mg-N/L, with ammonia removal rates >97% throughout the experiment and no NO_2^- accumulation. Reactor biomasses (as MLVSS) were consistent across reactor pairs during phase 1 (Fig. 1), but in phase 2, MLVSS levels increased by 20–30% in the unamended reactors, compared to no change in the Zn-amended units, a significant difference (95% of confidence level). MLVSS values were further elevated

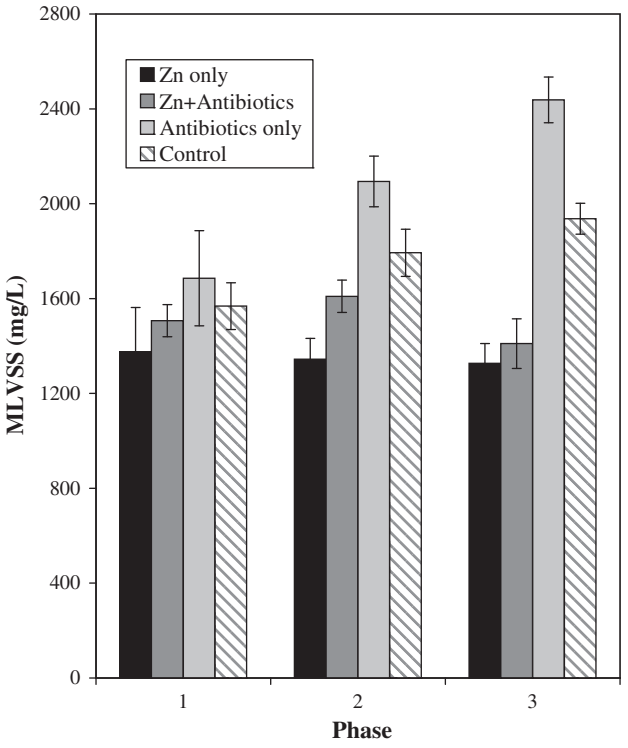

Fig. 1 – Mean mixed liquor volatile suspended solids concentrations in replicate reactors. Bars represent standard errors (n = 14 samples).

in antibiotic-only reactors in phase 3, whereas Zn amendment continued to result in comparatively lower MLVSS levels. Analysis of primary CFU data from control plates for each week (see Supporting Information) show no consistent trend in the total number of culturable organisms as a result of Zn amendment, confirming that the concentrations used here were sub-toxic.

3.1. *Antibiotic and Zn resistance between phase 1 and phase 2*

Ciprofloxacin resistance in phase 1 was the lowest among the three antibiotics tested with mean resistance levels between 5% and 7% for all reactors (Table 3). Other antibiotic resistance levels showed more variation during the initial phase, although no significant trends were apparent among different compounds. Antibiotic resistance levels were practically unchanged in the reactors between phases 1 and 2 (Fig. 2). Table 3 suggests that reactors 3 and 4 had lower resistance levels to both oxytetracycline and tylosin between phases 1 and 2, but the overall differences in antibiotic resistance in the Zn-amended reactors were not significant at a 95% confidence level. Zinc resistance levels increased in all reactors between phases 1 and 2 (Table 3), although increases were only significant at a 95% confidence level in reactors 7–8. However, there was no overall significant difference in Zn resistance between the two phases.

3.2. *Antibiotic and zinc resistance in phase 3*

In contrast to phases 1 and 2, relative patterns of antibiotic resistance changed dramatically between phases 2 and 3. Fig. 3 shows fractional changes in antibiotic and zinc resistance between the latter phases with increases being most pronounced when antibiotics were added to reactors with previous Zn amendment. For example, mean resistance levels to ciprofloxacin more than doubled between phases 2 and 3 in the Zn+antibiotic units, while the Zn-only and antibiotic-only treatments only saw small increases in ciprofloxacin resistance, similar to the control reactors. Even more dramatic changes were observed in oxytetracycline resistance in the Zn+antibiotic reactors, fractionally increasing five-fold. In this case, an increase in resistance also was noted in the antibiotic-only units, but the magnitude was less profound than when Zn was present (Fig. 3).

All four treatments in phase 3, including the control reactors, saw statistically significant increases in tylosin resistance, although the extent of this increase was not equal among treatments. Similar to ciprofloxacin and oxytetracycline, the largest fractional increases in resistance were seen in the Zn+antibiotic units, although this partially results from lower tylosin resistance levels seen in these two reactors in the second phase. Overall tylosin resistance levels in the Zn-only and Zn+antibiotic treatments during phase 3 (52% and 60%, respectively) were significantly higher than the control reactors (32%). Mean resistance in the antibiotic-amended reactors was very similar to the Zn-only treatment

Table 3 – Antibiotic and Zn % resistance data.

Phase	Ciprofloxacin			Oxytetracycline			Tylosin			Zinc		
	1	2	3	1	2	3	1	2	3	1	2	3
Zn only[a]	6 ± 1	6 ± 1	8 ± 1	21 ± 4	17 ± 3	18 ± 2	18 ± 3	21 ± 3	52 ± 8*	34 ± 6	44 ± 6	62 ± 6*
Zn+antibiotics[a]	5 ± 1	5 ± 1	14 ± 2*	15 ± 2	9 ± 2*	45 ± 8*	29 ± 5	9 ± 1*	60 ± 8*	36 ± 6	38 ± 7	54 ± 9
Antibiotics only[a]	6 ± 1	6 ± 1	6 ± 1	15 ± 3	16 ± 4	26 ± 4†	22 ± 3	23 ± 4	50 ± 8*	24 ± 2	34 ± 7	48 ± 8
Control[a]	7 ± 1	7 ± 1	9 ± 2	17 ± 3	23 ± 4	20 ± 3	19 ± 3	18 ± 3	31 ± 6†	31 ± 5	46 ± 7†	37 ± 5

* indicates significant change in mean resistance from the previous phase at the 95% confidence level.
† indicates significant change in mean resistance from the previous phase at the 90% confidence level.
a Amendments are as follows: i) Zn only – 5 mg/L Zn in phases 2 and 3 ii) Zn+antibiotics – 5 mg/L Zn in phases 2 and 3, plus 0.2 mg/L of each antibiotic in phase 3 iii) Antibiotics only – 0.2 mg/L of each antibiotic in phase 3 iv) Control – no amendments. All values include standard error of the measurements, with 12–14 samples per phase.

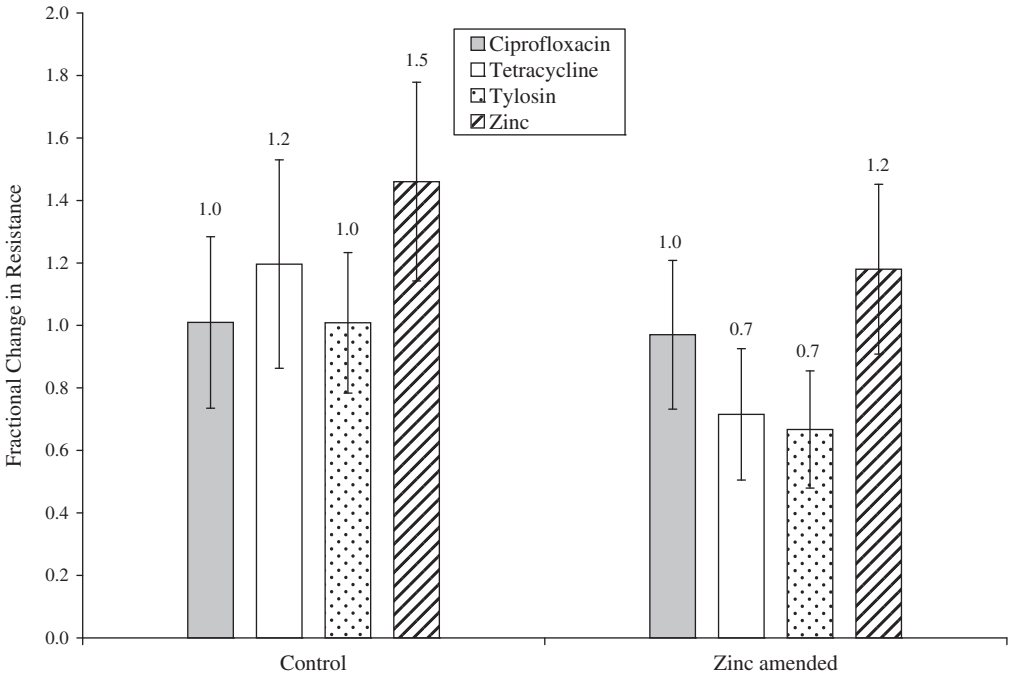

Fig. 2 – Fractional change in mean antibiotic and zinc resistance from phase 1 to phase 2, with standard errors of each ratio.

(50%), and was significantly different from the control reactors at a 90% confidence level.

There were also differences in the weekly pattern of tylosin resistance related to the presence or absence of Zn in the reactors. Fig. 4 shows the moving average of actual percent tylosin resistance during phase 3 (moving averages were used to better demonstrate actual trends). Initial resistance levels were low in all reactors; however, tylosin resistance in the Zn-only and Zn+antibiotics-amended reactors started to increase in week 17 cresting at almost 90% resistance to tylosin among CFU by the end of the experiment. In the reactors without Zn amendment, increases in tylosin resistance were not apparent until week 19, which implies Zn amendment may accelerate the acquisition of tylosin resistance relative to units without Zn addition.

Zinc resistance levels in the control reactors, which had somewhat increased in the second phase, generally decreased during the final phase of the experiment, implying no

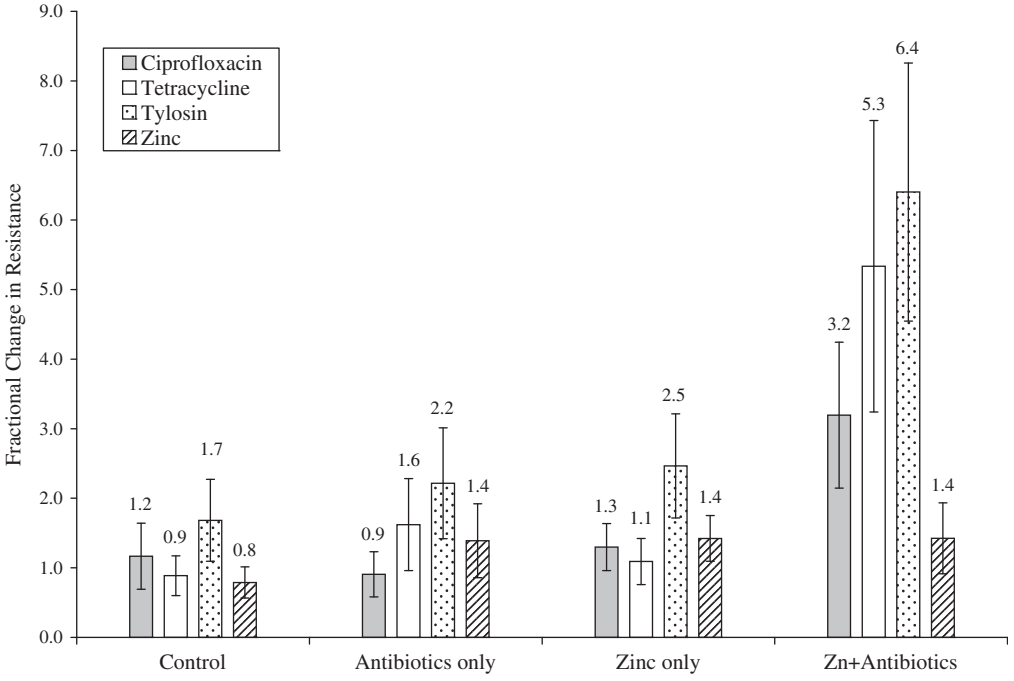

Fig. 3 – Fractional change in mean antibiotic and zinc resistance from phase 2 to phase 3, with standard errors of each ratio.

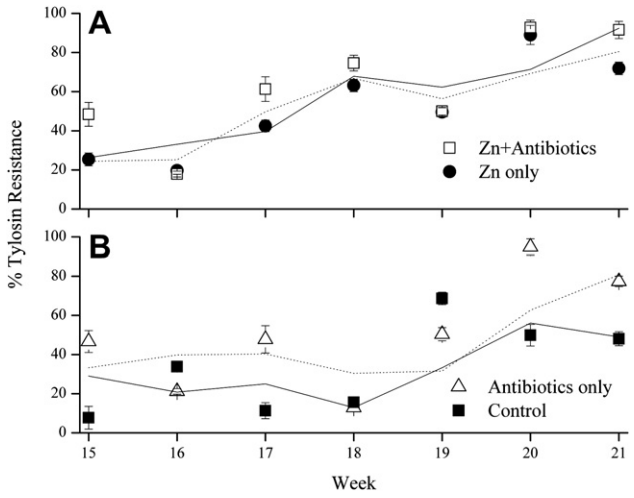

Fig. 4 – Weekly tylosin resistance levels during phase 3, with 95% confidence intervals. Trendlines are 2-week moving averages of the time-series. Trendlines are as follows: (A) (——) Zn+antibiotics, (– – – –) Zn only; (B) (——) Control, (– – – –) Antibiotics only.

consistent or dramatic trend in Zn resistance itself over the whole experiment. Zinc resistance did increase in all three "amended" treatments during the third phase and was comparatively higher relative to the control reactors. However, the only statistically significant change in mean Zn resistance levels was seen in the Zn-only units, which averaged >60% resistance during the final phase.

3.3. *Soluble Zn levels and speciation*

The average soluble Zn level in pre-amended reactor influents over the whole experiment was 119 μg/L. When 5 mg/L Zn was added in phases 2 and 3, soluble Zn levels increased to ~600 μg/l in Zn-amended reactors, which is below typical minimum inhibitory concentrations (Harrison et al., 2005) and similar to soluble Zn levels often seen in wastewater mixed liquors (Goldstone et al., 1990; Madoni et al., 1996). Given that the mixed liquor dissolved Zn levels (including background Zn) were only 12% of the added Zn concentration, the large majority of added Zn was apparently being removed from solution, either by adsorption to the bacterial cells or by chemical precipitation. There were no significant differences in dissolved Zn levels among all Zn-amended reactors in phases 2 and 3 regardless of antibiotic addition.

4. Discussion

We hypothesized that co-exposure to sub-toxic levels of certain metals might influence the extent of bacterial resistance to antibiotics observed in wastewater treatment plants. Zn was assessed as the model co-contaminant because it is found at moderate levels in most wastewaters (Petrini and Grechi, 1984) and it also is known to influence antibiotic resistance in other settings through various mechanisms (Baker-Austin et al., 2006).

Results from phases 1 and 2 suggested that Zn additions had minimal effect on levels of resistance to Zn, ciprofloxacin, oxytetracycline, and tylosin among culturable bacteria. However, phase 3 data showed that Zn addition coupled with moderate levels of antibiotics caused consequential increases in resistance to all three antibiotics. This suggests that co-exposure to Zn appears to fortify the microbial community towards increased antibiotic resistance, although the exact cause of fortification is unclear. Interestingly, increased resistance to tylosin also was seen with Zn alone, but not the other two antibiotics. In the cases of ciprofloxacin and oxytetracycline, the lack of increased resistance in Zn-only units suggests that co-resistance via the transfer of mobile genetic elements is unlikely. Specifically, no changes in resistance to either antibiotic or to Zn were observed in phase 2, and the increased antibiotic resistance in Zn+antibiotic-amended units in phase 3 was associated with the smallest increase in Zn resistance among all three amended reactor pairs. While this result may have been influenced by specific Zn and antibiotic levels employed in the plate media, an increase in antibiotic resistance clearly occurred when additional Zn was present, especially coupled with added antibiotics.

Bacterial Zn resistance mechanisms include reduced uptake, uptake and then efficient efflux, internal or external sequestration, and transformation to less toxic forms (Choudhury and Srivastava, 2001), some of which could coincidently result in resistance to other compounds (i.e., cross-resistance). For example, if even subtle changes in Zn resistance resulted in altered uptake or efflux activity, such resistance also could confer resistance to other compounds, including antibiotics (Baker-Austin et al., 2006). Alternately, although Zn addition might not necessarily result in increased Zn resistance, it might influence the composition of the bacterial community, possibly by selecting for Zn-tolerant species in response to prolonged Zn stress. Regardless of the actual cause, sub-toxic Zn exposure clearly increases resistance to unrelated antibiotics, which implies previously unidentified factors can be consequential to the development of subtle resistance in a system. Generalizations about resistance levels in treatment plants, especially based solely on genetic data, must therefore be made with care.

The results for tylosin resistance development are slightly different than for ciprofloxacin and oxytetracycline resistance. Increased tylosin resistance occurred in all units in phase 3, although the presence of Zn was still a central factor to observed increases. Clearly, prolonged Zn exposure during phase 3 increased tylosin resistance in the Zn-only and Zn+antibiotic reactors, although this does not explain increased tylosin resistance in the control or antibiotic-only reactors during the final phase. Although it is strictly speculation, it is possible that tylosin resistance-associated genes or organisms might have been introduced to all reactors in phase 3 in the primary effluent. This study used wastes from an operating wastewater treatment plant as its reactor feed, and it is possible that a sub-population with elevated tylosin resistance was introduced to the system. Once introduced, this sub-population triggered the proliferation of tylosin resistance, which was amplified under Zn stress (Fig. 4). This scenario highlights the need for experimental controls, such as those used in this study, and for care in interpreting

resistance data from wastewater treatment systems because these systems clearly are not insular. The introduction of a key gene or organism in the waste might alter the gene resistance pool, which is more or less manifested based on selective conditions.

5. Conclusions

The results presented here show that pre- and/or co-exposure to Zn at sub-toxic levels can result in increased incidence of resistance to tylosin, oxytetracycline and ciprofloxacin among culturable bacterial populations in wastewater treatment reactors when those antibiotics are also present in the water. However, Zn addition alone also appeared to amplify the increase in tylosin resistance observed in all reactors. The different responses observed suggest that the specific mechanisms affecting resistance might differ among antibiotics, although data generally suggest cross-resistance with Zn. As the actual soluble Zn concentrations throughout the experiment were substantially less than added values, these interactions may also occur at Zn levels substantially lower than those tested here. In conclusion, we contend that resistance studies in real wastewater treatment systems need to performed and interpreted with great care because factors that influence resistance, such as moderate Zn levels, are not always obvious. Therefore, future studies on antibiotic resistance in treatment plants must be more inclusive relative to measured parameters in the waste and mixed liquor, particularly the ambient concentrations of both antibiotics and soluble metals.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.watres.2010.04.041.

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