EFFECTS OF ZINC ON MICROALGAL BIOFILMS ON INTERTIDAL AND SUBTIDAL HABITATS

M. Mayer-Pinto[1,2], R.A. Coleman, A.J. Underwood and T.J. Tolhurst[3]

Centre for Research on Ecological Impacts of Coastal Cities

School of Biological Sciences

Marine Ecology Laboratories A11

University of Sydney

NSW 2006, Australia

Fax number: +61 – 2 - 9351-6713

[1] – Corresponding author: mariana.mayer@sydney.edu.au

[2] – Current address: RPS Group, Level 2, Colin St. West Perth, WA 6005, Australia

[3] – Current address: School of Environmental Sciences, University of East Anglia, Norwich, NR4 7TJ.

Abstract (152 words)

Biofilms are sensitive to environmental conditions. Impacts of contaminants on assemblages are often investigated in laboratories or in meso-cosms. Such experiments are rarely representative of effects of contaminants on biofilms under natural conditions. Studies in real field situations, with enough power to detect impacts, are necessary to develop better understanding of the effects of contaminants on ecological processes. Metals are a common estuarine contaminant and can cause disturbances to the assemblages. Using a new technique to experimentally deliver contaminants to micro-algal assemblages, we tested hypotheses about the effects of zinc on micro-algal biofilm growing on settlement panels in subtidal and intertidal habitats. Control panels deployed for one month in each habitat had significantly greater amounts of biofilm than those exposed to zinc. After three months of deployment, results varied with location. The observed effects on the biofilm did not, however, cause significant changes in the fouling assemblages that developed on the panels.

Key-words: metal, microbial films, micro-algal, pollution, contaminants, assemblages, field-based experiment, Sydney Harbour.

Introduction

Bacteria and micro-algae are among the first organisms to colonize hard surfaces submersed in the sea, forming a film, hereafter referred as microbial films or biofilm. Biofilms, especially bacteria, are sensitive to changes in environmental conditions. Thus, the physiology and composition of a biofilm can reflect the local environmental conditions for a given substratum (Wieczorek et al. 1996 and references therein). Herbicides and metals, for example, have long-term effects on chlorophyll-*a* in biofilms, because the latter tend to react to toxicants by changing their composition, usually favouring more tolerant taxa (Sabater et al. 2007). Biofilms have also been suggested to contribute to the cycling of metals, such as zinc, in aquatic systems (e.g. Morris et al. 2005, 2006). Little is known , however, about the effects of contaminants on biofilms, but it is believed that lethal or chronic effects of contaminants throughout a trophic web depend on the specific effects of such contaminants on the biofilms in addition to the characteristics of the biofilms themselves (Sabater et al. 2007).

Microbial films may strongly influence benthic assemblages, providing cues for the settlement of many species of invertebrates (Henschel and Cook 1990, Wieczorek and Todd 1998). Larval responses to biofilms may be species-specific and may be in response to facilitatory or inhibitory processes (e.g. Holmstrom and Kjelleberg 1994, Todd and Keough 1994, Wieczorek and Todd 1998). In the early stages of colonization, larvae of certain species of macro-fouling organisms are able to differentiate between biofilms of different compositions (e.g. Henschel and Cook 1990, Holmstrom and Kjelleberg 1994), densities (e.g. Henschel and Cook 1990, Lau et al. 2005), ages (e.g. Wieczorek and Todd 1997, 1998), origins (Keough and Raimondi 1995) or physiological conditions (Wieczorek and Todd 1998). Effects of contaminants on biofilms can potentially, therefore, have indirect effects on benthic assemblages, with consequences to entire systems.

Coastal and estuarine environments have long received metals from industrial and mining wastes (McLean et al. 1991, Watzin and Roscigno 1997, Matthai and Birch 2000, Johnston et al. 2002). For many marine invertebrates, zinc is one of the most toxic metals after copper, mercury and silver (Bryan 1971). Zinc has been shown to have a negative effect on recruitment and early survival of many marine organisms (Watzin and Roscigno 1997). This could be the result of indirect effects of this metal on biofilms. Very little has been done, however, regarding the effects of zinc on biofilm (but see review by Sabater et al. 2007) and, most of current knowledge on the effects of metals on assemblages, in general, is based on poorly-designed experiments (reviewed by Mayer-Pinto et al. 2010).

Possible impacts of contaminants on assemblages are often investigated in laboratories or in meso-cosms to control extraneous variables. Such studies can never be representative of natural conditions (see e.g. Underwood and Peterson 1988, Mayer-Pinto et al. 2010). Biofilms in a laboratory will be considerably different from those naturally occurring in the field. In addition, organisms in laboratory may be stressed by unnatural conditions, causing artificially enhanced effects of contamination (Underwood and Peterson 1988). Since interactions between contamination and ecological processes such as competition can modify responses of organisms to a toxicant (Johnston and Keough 2003), laboratory toxicological studies also lack the ability to predict indirect effects of toxicants on organisms mediated through other processes (Underwood and Peterson 1988). Thus, these types of studies often have little ecological relevance.

Changes in assemblages caused by contaminants, for instance, are often confounded by the natural changes to which these organisms and/or assemblages are subjected. Many field studies of contaminant effects only describe patterns (e.g. Armstrong et al. 1980, Schratzberger et al. 2004, Lee and Correa 2005). Manipulative experiments are, however, necessary to demonstrate any cause-effect relationship between the contaminant(s) and the observed pattern(s). These need to be done using appropriate experimental designs (see e.g. Underwood

1997). Transplant experiments, for instance, are commonly used but rarely done with all the necessary procedural controls to avoid leaving their results open to the interpretation that artefacts of the experiment may well have determined the patterns seen (see extensive discussion in Chapman 1986).

The effects of contaminants may also differ among habitats. Thus, it is important to evaluate whether the effects of zinc, if any, are general (i.e. the same organisms and/or processes are affected in the same way) or if effects are dependent on the type of habitat.

It is also important to be sure that any experiment would be able to detect any impact due to contaminants. Thus, preliminary studies are usually necessary because they allow, among other things, an estimative of the effect sizes of a contaminant on assemblages, which, in turn, allows experimenters to design experiments with sufficient replication to properly test hypotheses. Power analyses done before a study commences, preferably using data obtained in preliminary studies, are extremely important to ascertain that the experiment will identify predicted effects of contaminants. Here, we did two preliminary studies, first to test the new methodology used and then second to calculate the effect-size of zinc and, consequently, the appropriate number of replicates needed (see Methods).

The main objective of our experiments was to evaluate the effect of zinc on assemblages of biofilm on hard-substrata, in two habitats and in different stages of development. The hypotheses tested were:

i. zinc will affect micro-algal biofilms

ii. biofilm assemblages in different habitats will be affected in different ways.

iii. biofilm assemblages in different stages of development will be affected in different ways (independently of the amount of time of exposure to zinc).

Methods

Study Area

Multiple locations and sites were done in each habitat to test whether the results found were consistent in different spatial scales.

Intertidal estuary habitat

The experiment was done in two randomly-chosen sites on a sheltered mudflat in a mangrove forest in Sydney Harbour at Looking Glass Bay (151.07º E, 33.50º S) and at 2 randomly chosen sites at Tambourine Bay (151.09˚ E, 33.49˚ S). The sites were at least 100 m apart.

Subtidal coastal habitats

The experiment was done in two sites inside pools enclosed by shark nets in Sydney Harbour at Chowder Bay (151.15˚ E, 33.5˚ S) and in two similar sites at Balmoral (151.2˚ E, 33.5˚ S). The sites were at least 50 m apart.

Experimental design

Plaster blocks have been used to deliver contaminants to assemblages in experimental tests of hypotheses about pollution because they release contaminants slowly and continuously into sediments (e.g. Morrisey et al. 1996, Lindegarth and Underwood 1999) and on hard substrata (e.g. Johnston and Keough 2000, Cartwright et al. 2006). Plaster blocks were made of 100 g of dental plaster mixed with 70 ml of water. Blocks contaminated with zinc (Zn) had 30 g of $ZnSO_4*7H_2O$ added to the mixture. Settlement panels (11 cm x 11 cm x 0.5 cm of roughened black perspex) were glued to plastic jar lids. Plaster blocks contaminated (or not) with zinc were then placed inside the jars. Prior to installation, 8 holes of 6 mm diameter were drilled in a circular pattern in the lids of the jars and in the panels to allow the gradual release

of contaminants through the panels. The holes were cleaned, when necessary, every two weeks to allow continued release of zinc.

A pilot experiment, done in a subtidal habitat (two sites), showed that there was a greater concentration of zinc in the water near zinc treatments than near the controls, indicating that the contaminant was being released into the water-column over the settlement panels (electronic appendix Figure 1). The pilot experiment also suggested that the main release of this contaminant probably occurred during the first two weeks of deployment. For the work reported here, plaster blocks were therefore replaced every two weeks to maintain greater concentrations of zinc in the appropriate treatments.

Subtidal panels were placed 1 m below MTL, attached to shark-nets by cable-ties. In the intertidal habitats, settlement panels were attached to sandstone panels attached to wood sticks because the sticks were too thin for the direct attachment of the plastic jars. The sticks were inserted into the sediment, in the area covered with pneumatophores between the edge of the mangrove forest canopy and open mudflats.

Panels were deployed at the same time and left in the sites for 1 or 3 months, from May to August 2006. Twelve panels of each treatment were placed in each of two sites at each of two locations of each habitat.

Sampling

Panels were collected, placed in separate sealed plastic bags and transported to the laboratory where they were analysed (within hours).

We used a Diving PAM (WALZ, Germany) as a non-destructive, non-invasive and rapid proxy for amount and physiological state of biofilm (Consalvey et al. 2005). Measurements were done at four random points on each replicate panel, with the measuring head positioned on top of the panel. Their average was considered a replicate. Before analyses, the measured area was dark-adapted for 15 minutes. The measurements used in this study were

F_o and F_v/F_m. F_o is the minimal fluorescence and can be considered an estimate of biomass of photosynthetic biofilms (e.g. Schmitt-Jansen and Altenburger 2008). F_v/F_m, the maximal light utilization efficiency measured in the dark, has been shown to be a useful indication of the physiological state of phytoplankton (Consalvey et al. 2005). Therefore, this may also be used, with care, as a proxy for the physiological status of micro-algal populations.

Statistical Analyses

Univariate analyses (ANOVA) were used to test null hypotheses of no differences in treatments effects. When necessary, *post-hoc* Student-Newman-Keuls (SNK) tests were done *a posteriori* of analyses of variance to separate out significant means. Factors in the ANOVAs were pooled when $p > 0.25$ (for detailed discussion see Underwood 1997).

The number of replicates needed to test null hypotheses was determined using a power analysis, with a power of 0.95, (according to Winer et al. 1991, Underwood 1997) to minimize type II errors. The effect-size, i.e. "how large a difference there is between the null hypothesis and the specified alternative hypothesis" (Underwood 1997), was calculated based on the differences found in pilot experiments in the biomass of biofilm on panels exposed to zinc and on control panels (see electronic appendix Figure 2). The pilot experiment used to calculate the effect-size was done in mangrove habitats in two sites.

When one replicate of a treatment was missing, an average of the remaining replicates of the treatment was used to maintain the balance of the analyses. The degrees of freedom of the residual were reduced accordingly and the probabilities were then recalculated (Underwood 1997).

Results

In both intertidal and subtidal habitats, the presence of zinc significantly reduced the amount of biofilm compared to control panels (Table 1; Fig. 1). The amount of biofilm on

control panels seemed to be more variable than on zinc panels, in both habitats (Fig. 1). In the intertidal habitat, the amount of biofilm was at least half of those on controls, in every site (Fig. 1). In the subtidal habitat, the amount of biofilm varied between locations independently of treatments (Table 1). Since location was a random factor, no importance is associated with such differences. On panels deployed for 3 months, PAM measurements were only taken in the intertidal habitat because, in the subtidal habitat, panels were colonized by a diverse assemblage of macro-algae and invertebrates (unpublished data). PAM measurements would, therefore, no longer be an estimate of biofilm, but of other variables that were not part of the initial models and hypotheses. In the intertidal habitat, there was significantly more biofilm on control panels submerged for 3 months than on those exposed to zinc in only one of the locations studied (Table 2; Fig. 2).

Yield was, generally, low, ranging from approximately 0.1 to 0.4 in all sites and locations studied. On panels deployed in the subtidal habitat, there were no significant differences in the Yield between treatments (Table 1; Fig. 3). At the mangrove habitat, the Yield from control panels was greater than from panels exposed to zinc for 1 month in 3 of the 4 sites (Table 1; Fig. 3). No significant differences between treatments were found in the Yield of biofilm on panels submerged for 3 months at the intertidal habitat (Table 2; Fig. 4). Tambourine Bay had greater Yield values than Looking Glass Bay, on both of zinc and control treatments (Fig. 4).

Discussion

Zinc reduced biomass of biofilm as measured by PAM, but the effects on its Yield were dependent on location and length of time of submersion of the panels. Algal biofilms, besides influencing settlement of many species (Henschel and Cook 1990, Wieczorek and Todd 1998), are an important food source for grazers (e.g. Underwood 1979). In fresh-water systems, for example, they can accumulate organic matter and metals and, therefore, have been proposed as

indicators of contamination in riverine systems (Barranguet et al. 2000). The yield of marine phytoplankton has been shown to decrease significantly when micro-algae are exposed to metal solutions of zinc, copper and cadmium (Miao et al. 2005). Disturbances can also change composition of assemblages, enabling more tolerant species to dominate benthic biofilms (Blanck 2002), resulting in restored yield and primary production (Alsterberg et al. 2007).

Here, no consistent differences were observed regarding the yield of the biofilm in this study in response to application of zinc. It is, however, possible that there were, in fact, changes in the yield in the early days of contamination and a subsequent recovery, which would not have been detected here because sampling was always done at least one month after submersion of panels. It is not possible though, with the methodology used in the study (PAM), to know whether there were or were not changes in the composition of the assemblages of biofilm. The differences in yield (if they exist) can be due to a shift in the composition of species (yield can differ among taxa; Consalvey et al. 2005) or can be due to an actual change in the efficiency of the photosynthetic apparatus due to the presence of the contaminant. A limitation of PAM is that it measures only pigments that have fluorescence, so the reduced biomass observed on panels exposed to zinc could be due to changes in the composition of species. Autotrophic species could have been replaced by non-autotrophic species, causing the reduced measured of biomass observed. Despite these limitations, this study has shown that PAM does provide a quick and easy assessment of contaminant effects on photosynthetic biofilms. This information can then be used to inform further studies, using more specific, but also laborious, time consuming or destructive techniques, such as HPLC or spectroradiometry.

The assumption made by PAM method, that most of the fluorescence emanates from the photosystem II, is not valid for cyanobacteria (Consalvey et al. 2005). A method that enables to determine the composition of species and its possible shifts (such as field spectrometry; see Murphy et al. 2005) would an useful addition to this type of study, rather than just the observation of changes in the photosynthetic apparatus.

On panels deployed for three months, zinc reduced the amount of biofilm, but only in one of the intertidal locations (subtidal locations were not analysed). Effects of contaminants may depend on any previous disturbances, the type of disturbance and the ecological history of the assemblage (Sankaran and McNaughton 1999, Fukami 2001). The assemblages manipulated here could potentially have become more (or less) vulnerable to other disturbances such as increased temperatures and other types of contaminants, resulting in the measured differences. To test this hypothesis, other manipulative experiments using multiple stressors and appropriate design would need to be done.

Tolerance of assemblages to disturbances may also result from the elimination of sensitive species and successive replacement by less sensitive species (Odum 1985). Assemblages that had been subjected for a period of time to a certain type of disturbance (including contaminants) can be tolerant to that type of disturbance, but may not be to other types of disturbances (or contaminants).

In any case, the observed effects on the biofilm, in general, varied in space and time. Ecologists try to find general models that can explain patterns observed in nature, but are criticized because most of the current theories have local contingency (Lawton 1999, but see Simberloff 2004). The fact that there are few generalities in ecology is of concern for some, but accepting generalities without sufficient evidence is an even greater problem (Underwood & Denley 1984). In eco-toxicological studies, generalizations are frequent. For example, experiments done in laboratory, meso or micro-cosms that show that a concentration X of a certain type of contaminant caused mortality in Y percentage of a certain species is used as a basis for environmental laws or management of systems (e.g. MAFF 1994, ISO 2005). Such experiments lack many factors/variables and interactions that occur in natural systems (for discussion see Underwood and Peterson 1988). It is sensible, then, to assume that organisms may respond differently under field conditions and environmental laws or management strategies based on laboratory work are not necessarily appropriate.

We showed that there is variability in the effects of zinc on biofilm assemblages. To be able to predict, with a degree of reliability, not only these effects, but also the consequences of such effects, is necessary that ecological based studies, using robust designs, are done. Management and conservation of habitats will only be effective if generalizations stop being made and local contingencies became part of the solution (Simberloff 2004). This means impact assessments are required for each place and time, using data obtained from experiments done under natural conditions.

Acknowledgements

We thank Ezequiel Marzinelli, Giordana Cocco, Carolina Zagal, Vicky Cole and Pedro Range for assistance with fieldwork and colleagues from EICC for useful comments and discussion on this manuscript, in special E. Marzinelli. We thank Basil Panayotakos for help with the panels. MMP was funded by an International Endeavour Postgraduate Research Scholarship. AJU was supported by an Australian Professorial Fellowship from the Australian Research Council. Additional support for this project was given by ARC special Research Centre funding to AJU.

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Table 1 – Analyses of variance of mean amounts of biomass of biofilm (F_o) and yield of biofilm (F_v/F_m) on panels submerged for 1 month in two habitats. Locations (Lo) and Sites (Si) were random, each with 2 levels, sites being nested in location. Treatment (Tr) was fixed with 2 levels. $n = 12$. Variances were homogeneous (Cochran's test - $p > 0.05$). Factors were pooled when $p > 0.25$. ns = not significant; * = $p < 0.05$; ** = $p < 0.01$.

Table 2 – Analyses of variance of the mean biomass (F_o) and yield (F_v/F_m) of biofilm on panels submerged for 3 months in intertidal mangrove forests. Locations (Lo) and Sites (Si) were random, each with 2 levels, sites being nested in location. Treatment (Tr) was fixed with 2 levels. $n = 11$. Variances were homogeneous (Cochran's test - $p > 0.05$). Factors were pooled when $p > 0.25$. ns = not significant; * = $p < 0.05$; ** = $p < 0.01$.

Figure 1 –

Figure 2 – Mean amounts (+ SE; $n = 12$) of biomass of biofilm in each treatment submerged for one month in the subtidal habitat (A) or in the intertidal mudflat (B). Black bars = Zinc; White bars = Control. LG = Looking Glass Bay; T = Tambourine Bay; S1 = Site 1; S2 = Site 2.

Figure 3 – Mean amounts (+ SE; $n = 11$) of biomass of biofilm on panels exposed to zinc (black bars) and on control panels (white bars) submerged for three months in the intertidal habitat. LG = Looking Glass Bay; T = Tambourine Bay. Sites were averaged within locations according to results found in the analyses of variance.

Figure 4 – Mean amounts (+ SE; $n = 12$) of yield (F_v/F_m) of biofilm in each treatment submerged for one month in the subtidal habitat (A) or in the intertidal mudflat (B). Black bars = Zinc; White bars = Control. B = Balmoral; C = Chowder Bay; LG = Looking Glass Bay; T = Tambourine Bay; S1 = Site 1; S2 = Site 2. At the subtidal habitat, sites were averaged within locations according to results found in the analyses of variance.

Figure 5 – Mean amounts (+ SE; n = 11) of yield (F_v/F_m) of biofilm on panels exposed to zinc (black bars) and on control panels (white bars) submerged for three months in the intertidal habitat. LG = Looking Glass Bay; T = Tambourine Bay; S1 = Site 1; S2 = Site 2.