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Sublethal effects of the antibiotic tylosin on estuarine benthic microalgal communities

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ABSTRACT

Pharmaceuticals are common chemical contaminants in estuaries receiving effluent from wastewater and sewage treatment facilities. The purpose of this research was to examine benthic microalgal (BMA) community responses to sublethal exposures to tylosin, a common and environmentally persistent antibiotic. Bioassays, using concentrations of 0.011–218 μmol tylosin l^{-1}, were performed on intertidal muddy sediments from North Inlet Estuary, SC. Exposure to tylosin resulted in a reduction in total BMA biomass and primary productivity. Furthermore, exposure seemed to retard diatom growth while having a minimal effect on cyanobacteria biomass. Estuarine systems receiving chronic inputs of trace concentrations of tylosin, as well as other antibiotics, may experience significant reductions in BMA biomass and primary productivity. Given the well-documented role of BMA in the trophodynamics of estuaries, these impacts will likely be manifested in higher trophic levels with possible impairments of the structure and function of these sensitive systems.

1. Introduction

Estuaries and salt marshes are critical habitats that provide a source and sink for nutrients, nurseries, foraging areas for wildlife, and a valuable recreational resource. Benthic microbial communities (bacteria and microalgae) are an essential component of these systems and alterations in their structure and/or function may have cascading impacts on the biota and processes essential for ecosystem services. Biogeochemical cycling, trophodynamics, sediment–water–atmosphere exchange, sediment stabilization, and primary productivity are just a few examples of the key roles this community plays in estuaries.

Antibiotics and pharmaceuticals are common chemical contaminants in estuaries receiving effluent from wastewater and sewage treatment facilities (Halling-Sørensen et al., 1998; Kolpin et al., 2002; Daughton, 2004; Benotti and Brownawell, 2007, 2009; Kemper, 2008; Nakada et al., 2008). Of particular concern are those compounds and their derivatives that exhibit long degradation half-lives and are thus persistent in the environment. Estuarine microbiota exposed to these chemical contaminants may be especially impacted since many of these compounds are, by their nature, effective antimicrobial agents (Halling-Sørensen et al., 1998; Kümmerer, 2003; Kostich and Lazorchak, 2008). The continued growth of the human population in the coastal zone will inevitably result in an increase in the prevalence and concentrations of pharmaceutical contaminants because standard treatment procedures are ineffective in removing them from sewage and wastewater.

Recent studies have conclusively demonstrated the presence and persistence of anthropogenic pharmaceuticals at effective concentrations in estuaries (Hirsch et al., 1999; Kolpin et al., 2002; Ashton et al., 2004; Benotti and Brownawell, 2007, 2009; Kemper, 2008). Antibacterial pharmaceuticals are a major environmental concern due to their potential ecological impacts on microbial communities. The negative effects of biocides on estuarine microbiota could have cascading consequences for both trophic transfer and biogeochemical cycling, possibly resulting in major changes in ecosystem structure and function (Halling-Sørensen et al., 1998; Ellis, 2006).

Tylosin is used worldwide as a veterinary prophylactic and growth factor. This macrolide antibiotic interferes with prokaryotic protein synthesis by binding to the 50S ribosomal subunit. Tylosin has been found in a wide variety of aquatic systems in concentrations ranging from 0.31 to 3.02 nmol l^{-1} (0.28–2.77 μg l^{-1}) in water and 2.84 nmol kg^{-1} (2.6 μg kg^{-1}) in sediments (Halling-Sørensen, 2000; Kolpin et al., 2002; Calamari et al., 2003; Kim and Carlson, 2007b). Tylosin is toxic to freshwater and marine microalgae at near-environmental concentrations (Ebringer, 1972; Halling-Sørensen, 2000; Eguchi et al., 2004; Yang et al., 2008; Swenson et al., 2012). Furthermore, tylosin has an affinity

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for sediment particles and a high sediment/water partitioning coefficient, with an active resident time of 100+ days (Kim and Carlson, 2007a,b).

Previous work in North Inlet Estuary, SC has demonstrated that benthic microalgae (BMA) are major primary producers with an annual of ca. 3423 tonnes C y^{-1} (Pinckney and Zingmark, 1993a,b,c). Based on production estimates for North Inlet estuary, BMA production is approximately 43% (3.4×10^9 g C y^{-1}) of the maximum estimated total *Spartina* production (7.9×10^9 g C y^{-1}) in this system. Any reduction in BMA primary productivity or change in community composition due to pharmaceutical exposure would likely have cascading impacts on benthic carbon cycling, trophodynamics, and the composition of higher food webs. The purpose of this research was to examine benthic microalgal community responses to sublethal exposures to tylosin, a common, environmentally persistent antibiotic.

2. Materials and methods

The North Inlet–Winyah Bay National Estuarine Research Reserve (NERR), South Carolina, USA (33.3500°N, 79.1902W) is a euhaline *Spartina* marsh system with minimal anthropogenic impacts. The nearly pristine conditions of this estuary minimize potential experimental artifacts due to acclimation of the local benthic communities to antibiotic exposure (Wirth et al., 1998; Sanger et al., 1999). Cores (9.6 $cm^2 \times 6$ cm) of unvegetated intertidal mud were collected on 13 February and 16 May 2011. The sediment at the core collection site was composed of very fine sand (62.5–125 μm) with 35% silt/clay by weight. Core tubes were sealed and returned to the laboratory for incubations.

Microcosms were constructed from low density polyethylene trays ($33 \times 12 \times 8$ cm) connected to individual water reservoirs (10 l) and diaphragm water pumps (Aqua Lifter®). The pumps were placed on a timer to simulate ebb and flow tidal conditions as well as high and low water at the collection site. Light was supplied using a 91 cm 4× 39 W Ocean Light T5 hood (10,000 K 39 W – TRU fluorescent bulbs) to achieve an *in situ* irradiance of ca. 1000 μmol quanta m^{-2} s^{-1} at the surface of the sediment in the core tubes. Light was cycled according times of sunrise and sunset on the dates the cores were collected.

Two separate bioassays were conducted in this experiment. In the first bioassay, tylosin (tylosin tartrate salt; MP Biomedical cat. no. 193454) was added to 10 l of sand-filtered seawater (35 ppt) contained in reservoirs for separate microcosms to achieve final concentrations of 2.18 and 218 μmol tylosin l^{-1}. A third reservoir was designated as the control (no tylosin added). In the second bioassay, tylosin was added at concentrations of 0.011 and 0.11 μmol tylosin l^{-1} with a third reservoir as a control. Each bioassay tray contained 10 replicate sediment cores. Incubations were terminated after 10 days and samples were collected for measurements of primary productivity and benthic microalgal photosynthetic pigments.

Gross primary production was measured with oxygen microelectrodes (Unisense, Denmark; 20 μm tip dia.) using the light/dark shift method (Revsbech and Jørgensen, 1986). Productivity measurements consisted of illuminating the sample with a fiber-optic halogen light (ca. 1200 μmol photons m^{-2} s^{-1}) and measuring the initial slope of oxygen decrease at 100 μm depth intervals within 1–2 s after darkening the sediment surface (Revsbech and Jørgensen, 1986; Pinckney and Zingmark, 1993c). All measurements were undertaken after oxygen concentrations achieved steady state as determined by microelectrode profiles in the sample. Contact between the tip of the microelectrode and the sediment surface was observed with a small magnifying lens (25×). Productivity was measured at successive 100 μm intervals until there was no detectable response within 5 s of the light/dark shift. Data were acquired and processed using Sloper software (Unisense). The measured rate at each depth interval was then integrated over all depth intervals to give a depth-integrated areal estimate of gross primary production (GPP). Five vertical profiles of production were obtained at random locations within each core. Depth-integrated GPP for all five profiles was averaged to provide an estimate of total community production for each core. Productivity measurements were obtained under subaerial (i.e., not submerged) exposure conditions to minimize the potential effects of gas diffusion constraints and therefore represent maximum potential rates of GPP for comparisons among the different treatments.

Five subsamples (1.00 $cm^2 \times 0.3$ cm) were collected from each incubation core for photopigment analysis, stored in 2 ml microcentrifuge tubes, and immediately frozen. High performance liquid chromatography (HPLC) was used to determine chemosystematic photosynthetic pigments for benthic microalgae. Samples were lyophilized for 24 h at −50° C, placed in 90% acetone (1.00 ml), and extracted at −20° C for 18–20 h. Filtered extracts (250 μl) were injected into a Shimadzu HPLC equipped with monomeric (Rainin Microsorb-MV, 0.46×10 cm, 3 μm) and polymeric (Vydac 201TP54, 0.46×25 cm, 5 μm) reverse-phase C18 columns in series. A nonlinear binary gradient consisting of the solvents 80% methanol:20% 0.50 M ammonium acetate and 80% methanol:20% acetone were used for pigment separations (Pinckney et al., 1996). Absorption spectra and chromatograms (440 ± 4 nm) were acquired using a Shimadzu SPD-M10av photodiode array detector. Pigment peaks were identified by comparison of retention times and absorption spectra with pure standards (DHI, Denmark). The synthetic carotenoid β-apo-8′-carotenal (Sigma) was used as an internal standard.

BMA biomass (chl *a*), with measured units of μg cm^{-2}, was converted to carbon units assuming a C:chl *a* ratio of 47.6 (de Jonge, 1980). Since the oxygen microelectrode technique measures gross photosynthesis (GPP), rates were converted to net photosynthetic rates (NPP) by assuming that NPP was 90% of GPP (i.e., NPP = 0.9 × GPP) (Pomeroy, 1959). Oxygen units were converted to carbon units using a conservative photosynthetic quotient (PQ) of 1.4 (Grant, 1986).

The results of both bioassays were combined and analyzed using a single-factor multivariate analysis of variance (MANOVA). Data were normalized by dividing measurements by the corresponding control values and expressed as a proportion (i.e., response relative to control) to allow the combination of both bioassays in a single analysis. For the MANOVA, treatment was the main factor (control, tylosin additions) with the control-normalized variables GPP, fucoxanthin, zeaxanthin, and chlorophyll *a* as variates. The assumptions for the MANOVA (e.g., normality, equality of covariance matrices, equality of error variances) were tested and satisfied. Responses of individual variables were assessed using a univariate analysis of variance ($\alpha \leqslant 0.05$) and *post hoc* treatment means were compared using the Ryan–Einot–Gabriel–Welsch *F*-test (REGW-F; $\alpha \leqslant 0.05$). Discriminant analysis was applied to further classify changes in benthic microalgal responses to tylosin exposure.

In the second bioassay only, microbial remineralization of organic matter was measured using fluorogenic analog substrates (Hoppe, 1983). For each core, the upper 5 mm of sediment was removed and weighed (~0.6 g), suspended at 1 g in 10 ml of 0.2 μm filtered supernatant water that had been boiled (BFSW) to denature any enzymes. Then the sample was vortexed, and the slurry used for the following ectohydrolytic enzymatic assays: α- and β-glucosidases (4-methylumbelliferyl (MUF)-α-D-glucopyranoside, 4-MUF-β-D-glucopyranoside), aminopeptidase (L-Leucine-4-methylcou-marinyl-7-amide) and alkaline phosphatase (4-methylumbelliferyl phosphate; Sigma). In triplicate, 40 μl of slurry was

diluted into 160 μl of substrate in BFSW with a final substrate concentration of 100 μM in a 96 well opaque plate. Hydrolysis rates were determined from change in fluorescence as measured on Molecular Devices SpectraMax Gemini EM (ex. 365 nm and em. 420 nm) over 30 min. Heat-killed controls were used to account for abiotic degradation. Final values were normalized to weight and standardized against known concentrations of MUF after accounting for the heat-killed controls.

3. Results

Values for the measured variables were divided by the values for the respective controls to combine the results of both bioassays for statistical comparisons (i.e., control-normalized variables) (Fig. 1). Benthic microalgal responses to tylosin exposure in both bioassays were significantly different from the respective controls (MANOVA, Pillai's Trace, $p < 0.01$). Univariate ANOVAs indicated that all four variables (GPP, fucoxanthin, zeaxanthin, chl *a*) were affected by tylosin exposure ($p < 0.01$). Gross primary production (GPP) in all tylosin addition treatments were significantly lower than the control ($p < 0.01$). Chl *a* concentrations were lower in all treatments except the 2.18 μmol tylosin l^{-1} addition ($p < 0.01$). Similarly, concentrations of the indicator pigment for cyanobacteria (zeaxanthin) and diatoms (fucoxanthin) were significantly different ($p < 0.05$) at all levels except the 2.18 μmol tylosin l^{-1} For all measured variables, responses to the 0.11 μmol tylosin l^{-1} treatment did not differ from the 0.011 μmol tylosin l^{-1} treatment.

Discriminant analysis was performed following the MANOVAs to build a predictive model of group membership based on the observed responses to the different concentrations of tylosin (Fig. 2). The variables used in the analysis were the control-normalized values for fucoxanthin, zeaxanthin, chl *a*, and GPP. The first two discriminant functions explained 86.9% and 8.8% of the variance, respectively (cumulative total = 95.6%) (Wilks' $\lambda = 0.111$, $p < 0.01$). Using these functions, 76.1% of the samples were correctly classified according to the concentration of tylosin added. The group centroids, derived from the canonical discriminant functions, suggest that BMA responses differed between the control and tylosin additions and that the concentration of tylosin also promoted differences in BMA responses. The differences between the control and other treatments were best explained by the first discriminant function (Fig. 2). The primary difference between the two functions was related to a change in sign (i.e., ±) for the coefficients for all factor levels except the control and the 218 μmol tylosin l^{-1} addition.

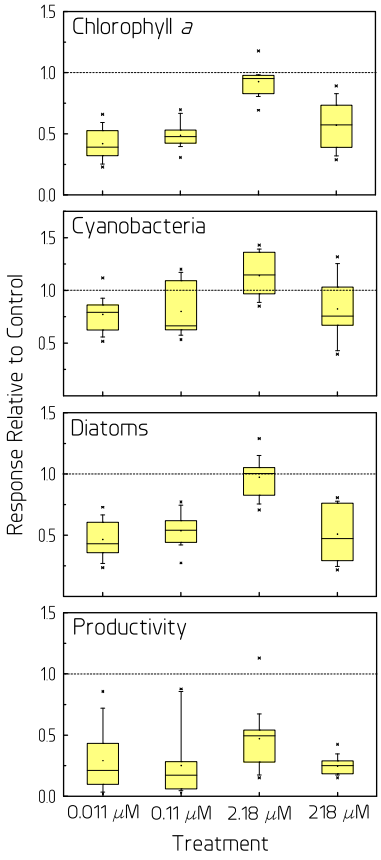

Fig. 1. Benthic microalgal responses to tylosin exposures of 0.011, 0.11, 2.18 and 218 FM. Values are ratio of the measured response relative to the mean value of the respective control. Boxes indicate the 25th and 75th percentiles, whiskers denote the 10th and 90th percentiles, and the median is shown by the horizontal line within each box. The horizontal dashed line denotes a value of 1.00 (control values).

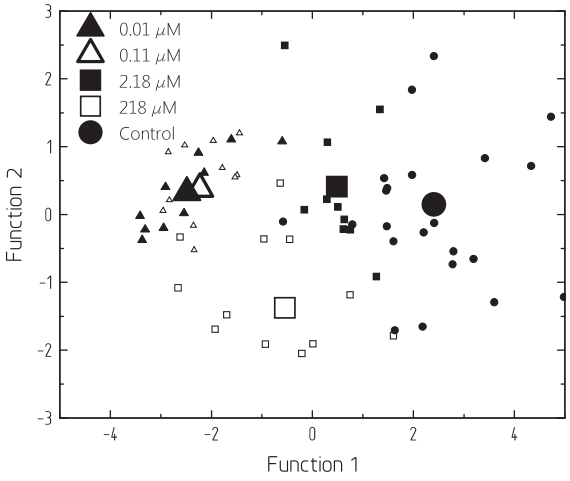

Fig. 2. Results of the discriminant analysis. Group centroids are indicated by the large symbols and corresponding data points are shown with the smaller symbols.

The ratio of primary production (NPP) to BMA biomass (B) provides a useful measure of the turnover rate for the BMA community. In the first bioassay, the turnover rates for the control, 2.18 and 218 μM treatments were 0.84, 0.43, and 0.37 d^{-1}, respectively. For the second bioassay, turnover rates were shorter and varied from 0.22 d^{-1} for the control, 0.16 d^{-1} for the 0.011 μM treatment, and 0.12 d^{-1} for the 0.11 μM treatment. Collectively, these results suggest that exposure to tylosin results in a near doubling of the BMA turnover time and a halving of BMA growth rates across a range of tylosin concentrations from 0.011 to 218 μmol l^{-1} .

With the exception of the leucine amino-peptidase (LAP) the microbial ectohydrolytic enzymes showed no significant differences between the controls and the treatments. There was a two to fourfold decrease of LAP activity in the tylosin treatments (*t*-test, $p < 0.001$). Alkaline phosphatase activity was below the limit of detection for all treatments.

4. Discussion

Studies of the biotic effects of antibiotics on natural marine systems are rare. Of the few published studies, most have targeted *Bacteria* as the sentinel organisms. In the present study, we demonstrate a significant effect of a commonly used antibiotic (tylosin) on eukaryotic microalgae within estuarine sediments at environmentally-relevant concentrations in the nanomolar range. The results of both bioassays indicate that tylosin exposure over a range of concentrations from 0.011 to 218 μM promotes a significant reduction in total benthic microalgal biomass and primary

productivity. Furthermore, exposure seems to retard diatom growth in these intertidal sediments. The results of the discriminant analysis further support the conclusion that tylosin has a significant overall effect on BMA community structure and function.

Tylosin effects on BMA were similar across the 2×10^4 magnitude range of concentrations suggesting that concentrations of tylosin greater than 11 nmol l^{-1} are above the threshold for significant negative effects. Although this study does not provide a mechanistic explanation for the mode of action for tylosin on BMA, one possibility is that tylosin affects bacterial processes with a cascading effect on BMA. Dose–response experiments with unialgal cultures from different algal classes suggest a direct effect of tylosin on microalgae (Hagenbuch and Pinckney, 2012). Halling-Sørensen (2000) and Eguchi et al. (2004) both found that tylosin was toxic to the freshwater chlorophyte *Selenastrum capricornutum* (= *Pseudokirchnerella subcapitata*). Halling-Sørensen (2000) also tested *Microcystis aeruginosa*, a freshwater cyanobacterium, with similar results. Swenson et al. (2012) demonstrated that tylosin and two other common antibiotics (ciprofloxacin, linkomycin) significantly depress division rates in the benthic marine diatom *Cylindrotheca closterium*. Hagenbuch and Pinckney (2012) show a similar trend using unialgal cultures of *C. closterium* and *Navicula ramosissima*. However, the unialgal culture experiments were not axenic and the potential cascade effects due to the impacts of tylosin on bacteria in the cultures cannot be discounted. Although tylosin exposure results in BMA mortality at relatively high concentrations (>22 μM), cell division and growth are greatly reduced at concentrations orders of magnitude lower.

The 2.18 μM tylosin exposure concentration did not exhibit the same effect as the higher or lower concentration exposures and is especially notable in the diatom and total chl *a* responses. This effect may be an example of hormesis, which occurs when a toxicant may work as a stimulant in small doses and an inhibitor in larger or smaller doses. Hormesis is a dose–response relationship characterized by low-dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2003). Further work is needed to determine if this response is indeed a hormesis effect.

The results of the enzyme activity measurements in the second bioassay showed a significant reduction in leucine amino-peptidase activity and suggests reduced nutrient remineralization of nitrogenous compounds in the tylosin exposed samples. The glucosidase measurements did not differ between the control and tylosin exposures and the rates fall within the range of *in situ* measurements at North Inlet (Thornton et al., 2010).

The microcosms used in this study were designed to simulate *in situ* conditions at the collection site. However, extrapolating the results from microcosms to field communities does have limitations (Carpenter, 1996; Drake et al., 1996; Kendrick et al., 1996; Porter et al., 2004). In our experimental setup, we could not assess the potential effects of grazing, bioturbation, resuspension, etc. on tylosin exposed BMA. Field trials of tylosin exposure were not practical or possible. However, the experimental results were evaluated relative to unexposed samples incubated under conditions identical to the tylosin treatments. Thus the relative responses are suggestive of significant impacts to natural communities at concentrations as low as 11 nmol tylosin l^{-1}.

Another potential limitation of the study is the use of a single water reservoir for each of the tylosin exposure levels. In analyzing the data, we have assumed that the responses would have been similar if the reservoirs had been replicated. Logistical limitations prevented "true replication" of the experimental treatments. Normalization of the measured variables to the respective control values allowed the combination of the results of the two bioassays into one statistical analysis. The consistent results across both bioassays suggest that the use of a single reservoir for each treatment probably had little impact on the experimental results. In nature, intertidal BMA are exposed to a common water source and conservative dissolved contaminants in the water would be assumed to have a nearly homogeneous distribution.

BMA and their associated bacteria offer many advantages as bioindicators of anthropogenic impacts in estuaries. Benthic microalgae are essentially permanent residents in the sediments of the estuary and thereby receive constant exposure to watershed inputs and integrate these cumulative effects over time scales of days to years. Chronic exposure to both waterborne and deposited pollutants, in combination with the huge collective surface area of individual cells, makes the benthic microbial community an ideal sentinel for critical estuarine processes and overall habitat quality. Estuarine systems receiving chronic inputs of trace concentrations of tylosin, as well as other antibiotics, may experience significant reductions in BMA biomass and primary productivity. Given the well-documented role of BMA in the trophodynamics of estuaries, these impacts will likely be manifested in higher trophic levels with possible impairments of the structure and function of these sensitive systems.

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