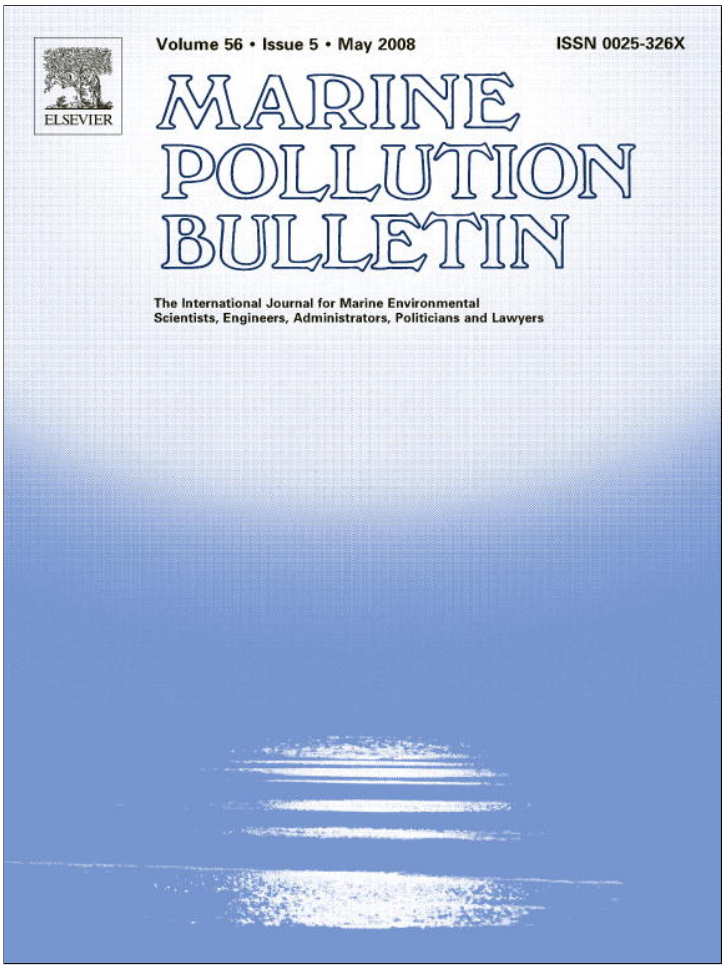

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Use of N stable isotope and microbial analyses to define wastewater influence in Mobile Bay, AL

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Abstract

We assessed short-term ecological and potential human health effects of wastewater treatment plant (WTP) effluent by measuring $\delta^{15}N‰$ and microbial concentrations in oysters and suspended particulate matter (SPM). We also tested male-specific bacteriophage (MSB) as an alternative to fecal coliforms, to assess potential influence of wastewater contamination on shellfish. WTP effluent did not affect oyster growth or survival, but SPM and oysters acquired wastewater-specific $\delta^{15}N‰$. $\delta^{15}N$ values were depleted near the WTP, typical of low-level processed wastewater. Fecal coliform and MSB concentrations were higher in samples taken closest to the WTP, and MSB values were significantly correlated with $\delta^{15}N‰$ in oyster tissues. Overall, oysters demonstrated relatively rapid integration and accumulation of wastewater-specific $\delta^{15}N‰$ and indicator microorganisms compared to water samples. These data suggest oysters were superior sentinels compared to water, and MSB was a more reliable indicator of wastewater influence on shellfish than fecal coliforms.

Keywords: Fecal coliforms; Male-specific bacteriophage; MSB; Eastern oysters; Virus; Bacteria; Growth

1. Introduction

Wastewater treatment plants (WTP) and related sewage overflows account for more than 30% of all shellfish area closures in the US and are a primary source of viral and bacterial pollution in estuarine environments (Alexander, 1998; Calci et al., 1998; Shieh et al., 2003). Shellfishing areas near WTPs are typically closed to fishing because of increased concentrations of fecal coliform bacteria that may indicate human health risk (Calci et al., 1998; Burkhardt et al., 2000; US Food and Drug Administration, 2005). Despite these attempts to minimize harvest of wastewater-contaminated shellfish, millions of shellfish-borne viral illnesses are estimated to occur each year in the US (Mead et al., 1999; Butt and Sanders, 2004). These data and other recent studies suggest fecal coliforms are not reliable indicator organisms of sanitary quality (LaBelle et al., 1980; Griffin et al., 2001). Hence, some shellfish area closures may be inaccurate, not sufficiently protecting public health or unnecessarily restricting harvest by watermen (LaBelle et al., 1980; Rippey, 1994; Alexander, 1998; Griffin et al., 2001).

Recent studies have investigated the effectiveness of a new suite of viral pathogen indicator organisms, male-specific bacteriophages (MSB) (Doré and Lees, 1995; Lucena et al., 1996; Griffin et al., 2001). MSB has emerged as a promising indicator organism, presumably because it has similar physiochemical properties to human enteric viruses, hepatitis A virus and norovirus, which are of greatest concern to shellfish consumers (Doré and Lees, 1995; Doré et al., 2003; Calci et al., 1998; Burkhardt et al., 2000). In contrast, the mode of infection, survival, and sequestration of bacterial indicators, such as fecal coliforms, in estuarine

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waters and shellfish tissues are quite different from the viruses of concern (Burkhardt et al., 2000; Doré and Lees, 1995). Hence, MSB may prove more reliable than fecal coliforms as an indicator to better assess the risk of wastewater-borne viral contamination of shellfish.

Wastewater treatment plant effluents are a source of nutrients, including nitrogen (N), which has prompted eutrophication and alteration of marine ecosystems around the world (Bowen and Valiela, 2001; Khan and Ansari, 2005). Increased N loads from WTPs can increase shellfish growth by increasing production of algae that shellfish feed upon (Granéli and Sundbäck, 1985; Carmichael et al., 2004a,b), but over-enrichment may reduce shellfish survival through increased hypoxia (Cloern, 2001; Gray et al., 2002). The combination of N enrichment and low dissolved oxygen (DO) may affect microbial growth, further influencing microbial contamination of shellfish (Burkhardt and Calci, 2000; De Zwann and Babarro, 2001; Babarro and de Zwaan, 2002). To better define wastewater-related effects on ecosystem and human health, we must be able to clearly link effects on fisheries and pathogen concentrations to wastewater sources using biologically meaningful measures (Peterson et al., 1994; Alexander, 1998; NSTC, 2007).

Stable isotope analysis (SIA) is a powerful tool capable of tracing material through ecosystems and trophic webs (Peterson and Fry, 1987; Fry, 2006). Use of SIA to trace the flow of wastewater-derived N through watersheds and into the tissues of organisms, including shellfish, is well established (McClelland et al., 1997; Carmichael et al., 2004a,b; Tucker et al., 1999; Savage, 2005; Piola et al., 2005; Martinetto et al., 2006). These linkages are possible because N stable isotope ratios are fractionated with biological and physical processing. Hence, N from raw or directly discharged sewage is relatively light compared to N conveyed through groundwater from septic systems or adjacent WTPs (Tucker et al., 1999; Carmichael et al., 2004a; Savage, 2005). Accordingly, the $\delta^{15}N$ values of producers and consumers in receiving estuaries shift, with appropriate fractionation, to reflect the influence of a given wastewater source (McClelland et al., 1997; Carmichael et al., 2004a; Savage, 2005).

In this study, we assessed potential short-term ecological and human health effects of WTP effluent in Mobile Bay, Alabama, relative to distance from a major WTP. We defined ecological effects by measuring growth and survival of transplanted sentinel commercially important bivalves (*Crassostrea virginica*) and comparing these data to dissolved inorganic N and chlorophyll *a* (chl *a*) concentrations, along with changes in DO, temperature, and salinity in water samples at each site. We defined the spatial extent of wastewater influence across study sites by measuring N stable isotope ratios in oysters and SPM at each site. This approach, in turn, allowed us to link biological changes in oysters and estuarine attributes to wastewater-derived N. We then compared concentrations of fecal coliforms and MSB to N stable isotope ratios to determine whether these indicators were correlated with wastewater exposure.

2. Methods

2.1. Field transplants

We transplanted hatchery-reared oysters (50–70 mm) at four sites distally located 0.07, 0.50, 2.18 and 5.68 km south of the Clifton C. Williams WTP outfall in Mobile Bay in Alabama (Fig. 1). Sampling locations were chosen to best capture variation in dilution due to hydrology and level

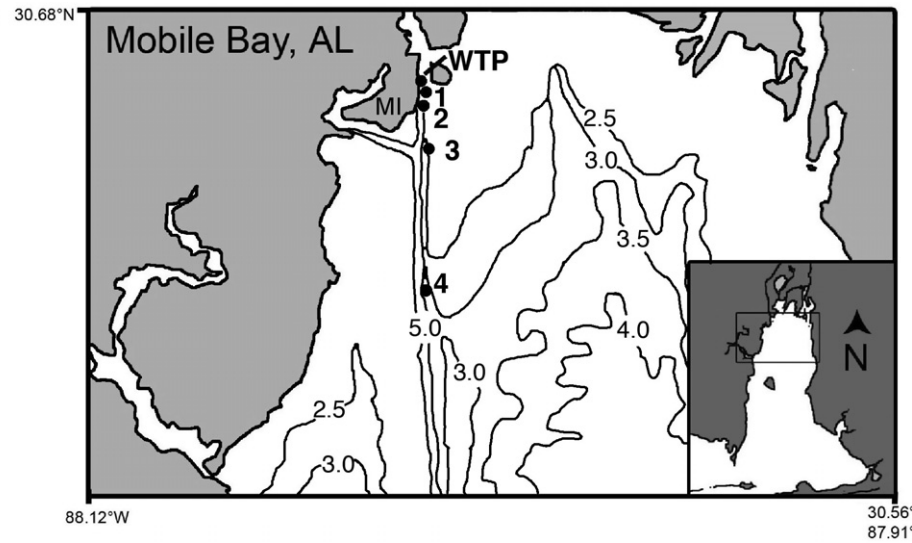

Fig. 1. Sampling sites at four locations in Mobile Bay, AL, relative to the effluent discharge site (WTP) from the Clifton C. Williams Wastewater Treatment Plant on McDuffie Island (MI). Sites 1, 2, 3, and 4 were located 0.07, 0.50, 2.18 and 5.68 km from the outfall, respectively. Contours show mean water depth in meters (Chen et al., 2005).

of WTP processing during the 6-week study period from early June to mid-July 2007. We used hatchery-reared oysters to ensure bivalves at each site originated from a common seed stock. Oysters obtained from the Auburn University Shellfish Hatchery on Dauphin Island were maintained under ambient seawater conditions (26.0 °C, 31.8‰ salinity, and 5.62 mg/L DO). Three days prior to transplant, oysters were transferred to a holding tank where salinity was reduced approximately 3‰ each day to prepare oysters for ambient salinities (~14‰) at the brackish transplant sites in Mobile Bay (Table 1). Oysters ($N = 18$) were placed in modified aquaculture cages (2.5 cm plastic-coated wire mesh), measuring 33 cm wide × 33 cm long × 10 cm high. Two pairs of cages were deployed at each site on fixed moorings. Pairs of cages were connected by 1.3 cm polypropylene rope so that a top cage hung ~60 cm from the water surface and a bottom cage was ~1.0 m below (1.6 m from the surface) in 3–5 m of water at mean low water. This approach allowed us to remove oysters and brush clean cages (biweekly) with minimal disturbance. It also provided more direct access to fresher wastewater effluent, which may stay near the surface in the strongly stratified waters of Mobile Bay (Chen et al., 2005, Fig. 1).

2.2. Oyster growth and survival

To capture temporal variation in oyster growth and survival, we removed oysters ($N = 4$ on days 10, 24; $N = 10$ on day 38) from each cage every 2 weeks (three times) during the study and noted any mortality. To determine oyster growth, we measured shell length by marking the outer shell edge of each oyster with waterproof ink before transplanting (Carmichael et al., 2004a,b). We then recorded the longest length of each oyster at each site ($N = 10$ on days 0 and 10; $N = 12$ on day 24; and $N = 18$ on day 38) to the nearest 0.1 mm to the ink mark and to the new outer edge of the shell. Growth was calculated as the difference between final and initial lengths. Ten additional randomly selected oysters from hatchery-reared control stock were measured to confirm initial average length. Growth rates were determined as the slope of the line best fit to the growth data through time at each site. To account for potential effects of handling stress on oyster survival, if any, a subset of marked and salinity acclimated oysters was maintained and monitored at ambient conditions in the laboratory throughout the study.

2.3. Water column attributes

Water samples were collected on each sampling date (every 2 weeks) using a horizontal water sampler at locations immediately adjacent to transplant cages (~1 m from the water surface). To measure nutrient and chl *a* concentrations, water was pre-filtered through a 200 μm mesh, collected in 1 L opaque Nalgene bottles, and stored on ice until processing. Water samples were vacuum filtered onto pre-ashed 25 mm diameter 0.7 μm glass fiber filters. Filtrate was frozen at −20 °C before analysis on a Skalar San+ Autoanalyzer after analysis using standard methods (Strickland and Parsons, 1972). Specifically, NO_2^- and NO_3^- concentrations were measured using colorimetric methods after cadmium reduction to NO_2^- and subtracting the resulting NO_2^- concentration from the $NO_3^- + NO_2^-$ complex. NH_4^+ and TDN concentrations were measured using alkaline phenol and persulfate oxidation methods, respectively (Strickland and Parsons, 1972). To measure chl *a* concentrations, we extracted filters with DMSO/90% acetone and analyzed on a Turner Designs TD-700 fluorometer, according to MacIntyre and Cullen (2005). Two or three replicate filters were analyzed to capture variation in handling and sample processing.

Water temperature, salinity, and DO were measured at each site prior to water sampling, using a handheld YSI 85 meter, at 1 m and at 2 m from the surface to capture potential differences in the water column attributes at top and bottom cages.

2.4. Stable isotope analyses

To capture temporal variation in N stable isotope ratios in oyster tissues, we analyzed tissues of transplanted oysters on each sampling date and an additional 10 oysters from hatchery-reared control stock. Recovered oysters were immediately frozen on shell at −20 °C. For stable isotope analysis, oysters were thawed, thoroughly washed in ultrapure water, and soft tissues were separated from shell. Adductor muscle was then separated from the remaining tissues to ensure no contamination by unassimilated food particles, which may be present in the siphon and gut

Table 1
Mean values (±SE) for water temperature, salinity, dissolved oxygen and inorganic nutrient concentrations at each oyster transplant site and for nutrients only in WTP effluent (Eff)

Site	Temperature (°C)	Salinity (ppt)	DO (mg/L)	Chl *a* (μg/L)	NO_2^- (μM)	NO_3^- (μM)	NH_4^+ (μM)	TDN (μM)
Eff.	–	–	–	–	2.3 ± 0.9	2.8 ± 0.2	1213.7 ± 37.4	1533.5 ± 63.5
1	29.0 ± 0.3	13.2 ± 1.1	4.3 ± 0.5	16.2 ± 2.7	0.7 ± 0.1	2.1 ± 0.4	90.5 ± 13.6	134.5 ± 11.9
2	28.5 ± 0.6	11.7 ± 1.1	4.7 ± 0.5	21.8 ± 4.8	0.4 ± 0.1	1.3 ± 0.4	13.2 ± 7.5	49.6 ± 10.6
3	28.7 ± 0.4	12.9 ± 0.9	4.9 ± 0.3	21.1 ± 4.7	0.2 ± 0.1	1.0 ± 0.5	3.8 ± 2.1	36.1 ± 2.5
4	27.6 ± 0.9	14.0 ± 1.0	4.9 ± 0.3	21.5 ± 5.4	0.2 ± 0.0	0.7 ± 0.2	3.9 ± 1.1	35.1 ± 3.3

Locations relative to the WTP are shown in Fig. 1.

region. Preliminary analyses also showed adductor muscle most consistently reflected recent diet compared to whole tissues, as suggested for other bivalves (Lorrain et al., 2004). At time 0 and 10 days, tissues of two oysters from each cage ($N = 8$ per site) were analyzed individually and compared to stable isotope ratios in tissues aggregated from multiple animals. Since no significant differences were found among these samples, on subsequent sampling dates, tissues were aggregated to yield a single sample from each cage for each sampling date ($N = 4$ per site). Tissues were dried to a constant weight at 60 °C, and ground to a powder with a mortar and pestle.

To determine stable isotope ratios in SPM available as food to shellfish, we collected and filtered 1 L of water from locations adjacent to each transplant cage as described above. To establish a N stable isotope endpoint for wastewater effluent, we directly collected 1 L of effluent from the WTP at a holding tank just prior to release. Effluent was collected (without prefiltration) and processed as described for water samples. Filters were dried to a constant weight at 60 °C. At least two replicate filters from each sample were analyzed on each date to capture variation in sample handling and analysis.

All samples were analyzed at the U. C. Davis Stable Isotope Facility by continuous flow isotope ratio mass spectrometry (20–20 mass spectrometer, PDZ Europa) after sample combustion to CO_2 and N_2 in an online elemental analyzer (PDZ Europa Automatic N and C Analyzer-Gas Solid Liquid). Gases were separated on a Carbosieve G column (Supelco) before introduction to the isotope ratio mass spectrometer (IRMS). This process also yields% N and C content of tissue and SPM.

2.5. Microbial analyses

To determine fecal coliform and MSB concentrations in oysters and water, samples collected on day 38 were immediately stored on ice and delivered to the US FDA Gulf Coast Seafood Laboratory on Dauphin Island. Oysters were held in clean plastic bags and water samples (250 ml) were collected into acid-washed bottles as described above without prefiltration. Samples were stored for no more than 8 hours at 4 °C prior to analysis. Oysters ($N = 8$ for each site) were thoroughly washed, shucked, aggregated to obtain sufficient material for analysis, and processed according to Recommended Procedures for the Examination of Seawater and Shellfish (American Public Health Association, 1970).

Fecal coliform concentrations were determined by the conventional five-tube multiple dilution most-probable number (MPN) procedure. Lauryl tryptose broth (Difco) was chosen as the presumptive growth media, while confirmation was performed in EC-MUG media (Difco) media (Rippey et al., 1987).

MSB densities were determined in oyster tissues and water by using a modified double-agar-overlay method described by Cabelli (1988). To analyze oyster tissues, following shellfish homogenization, a 20–25 g aliquot of each sample was removed and centrifuged for 15 min at 9000*g*, 4 °C. The supernatant was collected and analyzed for MSB with a modified double-agar-overlay method using *E. coli* HS(pFamp)R as the suitable host strain. Four 2.5 ml aliquots of the oyster supernatant were mixed with the bacterial host strain and double strength soft agar. Water was analyzed similarly to oyster tissue supernatant. After incubation at 35 °C for 18–24 h viral plaques were enumerated.

2.6. Statistical analyses

Because there was no significant difference between cages with depth, data from top and bottom cages were considered replicates so that each site had two sets of replicate cages. Mean values reflect the mean of two aggregate oyster samples for each of the two replicates and one water sample at each location on each sampling date for stable isotope analyses. Regression analyses and subsequent {*F*}-tests were performed in Microsoft Excel 11.3.7. Analyses of correlation, variance and covariance, and post-hoc tests were performed in Stat View 5.0.1. Analyses of Variance were preceded by tests of homogeneity of slopes according to Sokal and Rohlf (1981). A significance value of $P < 0.05$ was used for all tests. Error is reported as standard error and was propagated as necessary according to Valiela (2000).

3. Results

3.1. Ecological influence of wastewater effluent

Oyster growth rates ranged from 0.15 (±0.01) to 0.26 (±0.06) mm d^{-1} (Fig. 2), and were consistent with previous reports for oysters grown in the field and lab (Grizzle et al.,

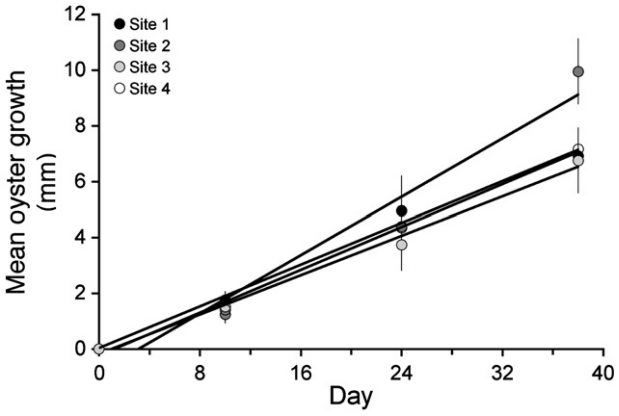

Fig. 2. Mean growth of oysters in mm of shell length compared to day since transplant. Where error bars are not visible, standard error was smaller than the size of the point. (Site 1: $y = 0.19x + 0.04$, $r^2 = 0.99$, $F_{reg3} = 221.96$, $P < 0.01$; Site 2: $y = 0.26x - 0.81$, $r^2 = 0.95$, $F_{reg3} = 40.09$, $P = 0.02$; Site 3: $y = 0.18x - 0.17$, $r^2 = 0.99$, $F_{reg3} = 273.74$, $P < 0.01$; Site 4: $y = 0.19x - 0.22$, $r^2 = 0.99$, $F_{reg3} = 418.82$, $P < 0.01$).

1992; Ortega and Sutherland, 1992; Wallace et al., 2001; Carmichael et al., 2004c). The rate and magnitude of oyster growth was similar among sites during the experiment (Fig. 2; test for homogeneity of slopes: $F_{3,8} = 2.16$, $P = 0.17$; ANCOVA: $F = 1.03$, $P = 0.42$). In general, greater oyster growth was related to higher chl *a* concentrations and lower salinity (data not shown).

NO_2^-, NH_4^+ and TDN showed significantly higher concentrations at Site 1, closest to the WTP, than at more distal locations (Table 1, NO_2^- ANOVA: $F_{3,12} = 5.56$, $P = 0.01$, Fisher's PLSD: $P < 0.05$; NH_4^+ ANOVA: $F_{3,12} = 28.52$, $P < 0.001$, Fisher's PLSD: $P < 0.001$; TDN ANOVA: $F_{3,12} = 33.49$, $P < 0.001$, Fisher's PLSD: $P < 0.001$). These findings are consistent with the higher nutrient concentrations in WTP effluent (Table 1). Despite higher nutrient concentrations nearest the WTP, there were no significant corresponding differences in chl *a* or salinity across sites (Table 1, chl *a* ANOVA: $F_{3,12} = 0.15$, $P = 0.93$; salinity ANOVA: $F_{3,28} = 0.85$, $P = 0.48$). There also were no significant differences in temperature or DO among sites, and accordingly no relationship between these variables and oyster growth (Table 1). Oyster survival was high during the study (>99%) at all sites, with a single dead oyster found at Site 4 (most distant from the WTP). Similarly handled control oysters, monitored in the lab, experienced 100% survival.

3.2. Stable isotope analyses

The mean $\delta^{15}N$ ratio in WTP effluent was −3.87‰ (Fig. 3). Accordingly, $\delta^{15}N$ ratios in SPM increased significantly with increasing distance from the WTP outfall. $\delta^{15}N$ ratios in SPM did not vary significantly with time, maintaining mean values of 3.8 (±0.3)‰, 5.7 (±0.5)‰, 6.8 (±0.2)‰, and 6.7 (±1.1)‰ at sites 1–4, respectively (Fig. 4, dashed lines).

$\delta^{15}N$ values in oyster tissues decreased significantly through time (Fig. 4). Oyster tissues showed a gradual shift from their initial hatchery values (9.1‰) to approach values in SPM available as food at each location (Fig. 4, dashed lines). $\delta^{15}N$ values in oysters at Sites 1 and 2 decreased at similar rates, but more rapidly than at Sites 3 and 4 (Fig. 4; test for homogeneity of slopes: $F_{3,23} = 8.34$, $P < 0.001$; Fisher's PLSD Sites 1 and 2: $P = 0.18$; Fisher's PLSD Sites 3 and 2: $P = 0.10$; Fisher's PLSD: $P < 0.001$ for all other comparisons). By day 38, oyster tissues acquired their location-specific isotope ratios, showing a mean difference of 1.2 ± 0.4‰ from SPM (Fig. 4), consistent with the trophic shift previously reported from SPM to oysters (Carmichael et al., 2004c; Valiela, 2006). Accordingly, $\delta^{15}N$ values in oyster tissues increased significantly with increasing distance from the WTP, similar to the response in SPM (Figs. 5 and 3).

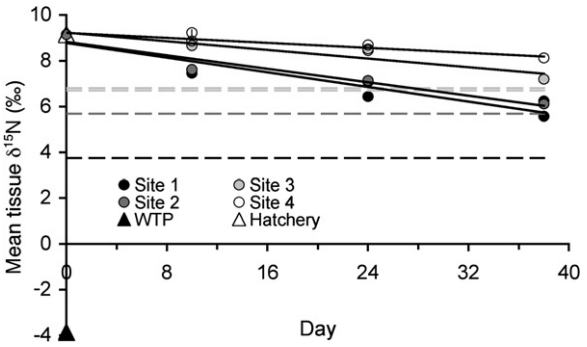

Fig. 4. Mean $\delta^{15}N$ values in oyster tissues compared to day since transplant at each site. Dashed lines represent mean $\delta^{15}N$ values in SPM at each site (from Fig. 3), with color corresponding to the site legend (sites 3 and 4 are shown in light grey). The open triangle shows the initial $\delta^{15}N$ ratio of the hatchery-reared oysters. The black triangle shows the N stable isotope ratio of wastewater effluent. Oysters from top and bottom cages were aggregated to yield two replicates at each site. Where error bars are not visible, standard error was smaller than the size of the point. Where two data points are not visible for each site, values overlapped. (Site 1: $y = -0.08x + 8.78$, $r^2 = 0.90$, $F_{reg7} = 53.84$, $P < 0.001$; Site 2: $y = -0.07x + 8.82$, $r^2 = 0.93$, $F_{reg7} = 77.92$, $P < 0.001$; Site 3: $y = -0.05x + 9.22$, $r^2 = 0.88$, $F_{reg7} = 18.27$, $P < 0.01$; Site 4: $y = -0.027x + 9.22$, $r^2 = 0.87$, $F_{reg6} = 34.38$, $P < 0.01$).

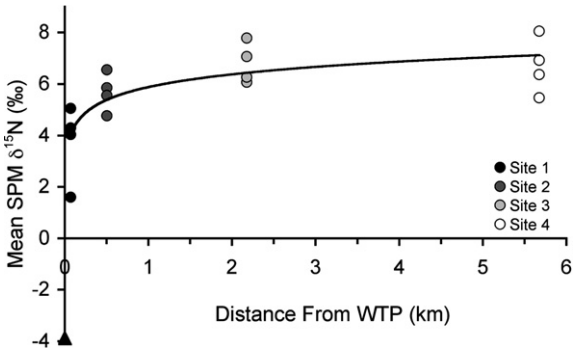

Fig. 3. Mean N stable isotope ratios in total suspended particulate matter (SPM) on four sampling dates compared to distance of each sampling site from the wastewater treatment plant outfall. The black triangle shows the N stable isotope value of wastewater effluent. Where error bars are not visible, standard error was smaller than the size of the point ($y = 0.71\ln(x) + 5.88$, $r^2 = 0.59$, $F_{reg15} = 20.11$, $P < 0.001$).

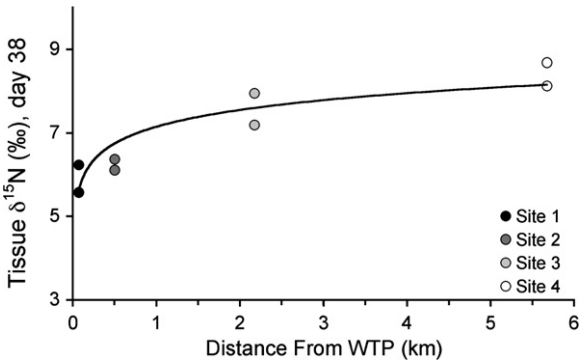

Fig. 5. Mean $\delta^{15}N$ values in oyster tissue at the end of the study (day 38) compared to distance of each sampling site from the wastewater treatment plant outfall. Oysters from top and bottom cages were aggregated to yield 2 replicates at each site ($y = 0.58\ln(x) + 7.15$, $r^2 = 0.83$, $F_{reg} = 29.31$, $P < 0.01$, $n = 9$).

3.3. Microbial assays

The highest concentrations of MSB and fecal coliforms were found in water and oyster samples from Site 1, closest to the WTP (Fig. 6). By day 38, MSB and coliform concentrations were 10–80 times higher in oyster tissues compared to water at sites near the WTP; showing concentrations of 10–100 pfu 100 ml^{-1} and 8–79 MPN 100 ml^{-1}, respectively in water compared to 171–2296 pfu 100 g^{-1} and 130–540 MPN 100 g^{-1}, respectively in oysters (Fig. 6). MSB levels in oyster tissues decreased significantly with increasing distance from the WTP outfall (Fig. 6, bottom). In contrast, no significant relationship was evident between coliform concentrations in oysters and their distance from the WTP (Fig. 6, bottom).

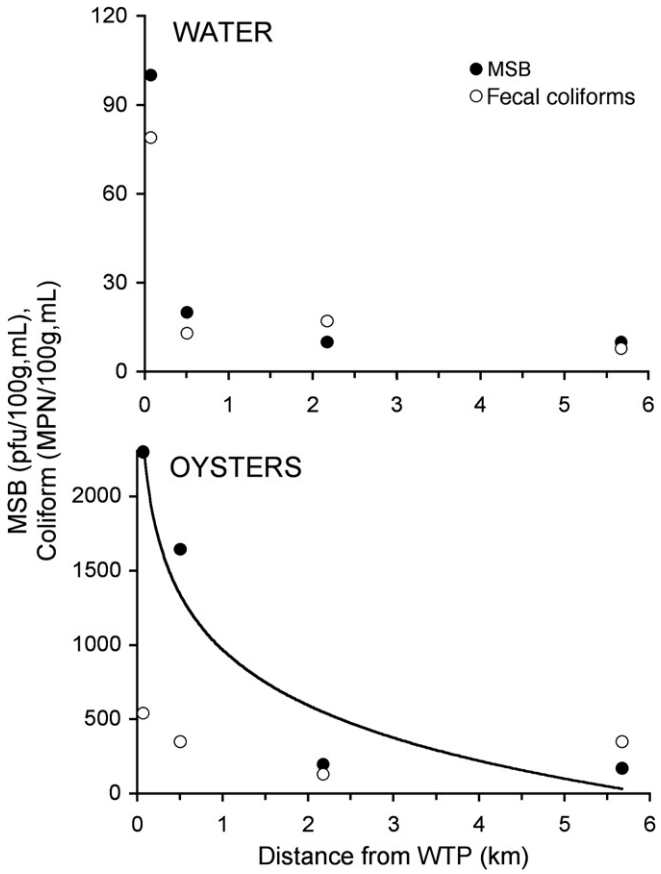

Fig. 6. Concentrations of male-specific bacteriophage (MSB) and fecal coliforms in water (top) and oyster tissues (bottom) relative to distance from the wastewater treatment plant outfall on day 38 ($y = -537.64 \ln(x) + 966.38$, $r^2 = 0.93$, $F_{reg3} = 25.78$, $P = 0.036$).

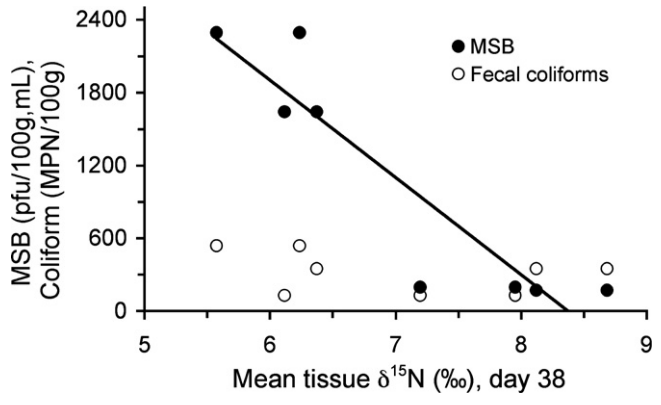

Fig. 7. MSB and fecal coliforms concentrations compared to final (day 38) mean δ^{15}N values in oyster tissues (MSB: $y = -802.02x + 6715.6$).

MSB concentrations also were significantly correlated with N stable isotope ratios in oysters (Fig. 7; $r = -0.91$, $Z = -3.46$, $P < 0.001$). Oyster tissues showing lighter δ^{15}N ratios, which reflect greater wastewater influence (Fig. 5), had higher MSB concentrations (Fig. 7). In contrast, coliform concentrations in oyster tissues were not correlated with mean δ^{15}N values (Fig. 7).

4. Discussion

4.1. Ecological influence of wastewater

4.1.1. Oyster growth and survival

To determine the potential ecological effects of wastewater on shellfish, we first measured growth and survival of transplanted sentinel oysters and compared changes in water column attributes among sites differing in proximity to the WTP outfall (Fig. 1). Although we found no significant effects of wastewater effluent on oyster growth or survival during the short duration of our experiment (Fig. 2), over longer periods, the significantly higher concentrations of NO_2^-, NH_4^+, and TDN at Site 1, could lead to changes in food supply and habitat for shellfish (Table 1; Carmichael et al., 2004a,b). Mean DIN (NO_2^-, NO_3^-, NH_4^+) concentrations at sites more distant from the WTP were within the range previously reported for Mobile Bay (Pennock et al., 1994; MacIntyre pers. Comm.), and overall, were relatively low compared to typically eutrophic embayments (Smith et al., 1999). Higher TDN values, engendered by the combination of wastewater-derived DIN and DON (Table 1, Eff.), however, may contribute to local periodic phytoplankton blooms, fueling hypoxic events already common to the Bay (Pennock et al., 1994). These changes can have conflicting effects on shellfish by increasing growth, but reducing survival (Gray et al., 2002; Carmichael et al., 2004a,b). Future experiments conducted over a broader spatial and temporal scale are needed to more clearly define the net effects of wastewater effluent on shellfish and their habitat in Mobile Bay.

4.1.2. Stable isotope analyses

To define the spatial extent of wastewater influence across study sites, we measured N stable isotope ratios in oysters and SPM at each site. This approach, in turn, allowed us to link biological changes in oysters and estuarine attributes to wastewater-derived N. The depleted δ^{15}N values in WTP effluent (−3.9‰; Fig. 3) were similar to those reported elsewhere for lower-level processed wastewater (directly released effluent as opposed to groundwater

derived) and provided a clearly identifiable endpoint for comparison with marine sources (Valiela et al., 1992; Tucker et al., 1999). The significantly lighter $\delta^{15}N$ values in SPM and oysters with increasing proximity to WTP effluent indicate that oysters and their available food resources assimilated wastewater-derived N relative to their specific location in the Bay. The logarithmic change in isotope ratios with distance may be due to relatively rapid changes in SPM composition at low N loads (Carmichael and Valiela, 2005) or dilution of wastewater N as it moves away from the WTP. These data in combination with the significantly higher nutrient concentrations (NO_2^-, NH_4^+, and TDN) at sites nearest the WTP validate our use of distance from the WTP outfall as a reasonable proxy for increasing exposure to WTP effluent.

The gradual change in $\delta^{15}N$ values in oyster tissues through time is characteristic of consumers feeding and growing after a change in diet composition (Tieszen et al., 1983; Carmichael, 2004). The 1.2‰ difference between $\delta^{15}N$ values in SPM and oysters is consistent with fractionation from oysters to SPM reported elsewhere (Carmichael et al., 2004c; Valiela, 2006). This difference is somewhat smaller than the 2–4‰ fractionation typically reported for a single trophic shift (Peterson and Fry, 1987) and may reflect differential assimilation or selection by oysters of specific components within bulk SPM (Carmichael, 2004). The more rapid decline in $\delta^{15}N$ values at Sites 1 and 2 compared to Sites 3 and 4 reflects the greater change in diet (more depleted $\delta^{15}N$ values in SPM) available to oysters at sites near the WTP. This difference produced a subsequently greater decline in $\delta^{15}N$ values in tissues despite similar growth rates (Figs. 4 and 2). Most importantly, these relationships unambiguously demonstrate that wastewater-derived N was available to and assimilated by oysters. This finding further suggests potential for assimilation of other wastewater-derived particles such as pathogens into local food webs.

4.2. Implications for human health

Concentrations of male-specific bacteriophage and fecal coliforms in water and oysters were determined to assess the influence of wastewater effluent on exposed shellfish. Higher concentrations of MSB and fecal coliforms in water and oyster tissues at sites nearest the WTP, and the significant logarithmic increase in MSB in oysters with proximity to the WTP (Fig. 6), implicate wastewater effluent as the likely source of fecal coliforms and MSB at these sites. Furthermore, MSB concentrations were significantly correlated with $\delta^{15}N$ ratios in oysters, indicating oysters that assimilated more wastewater-derived particles also had higher MSB counts (Fig. 7). Fecal coliform and MSB concentrations at all sites were within the range previously reported for water and oyster samples near Mobile Bay (Shieh et al., 2003).

These data suggest three important points. First, increased exposure to wastewater may increase the likelihood of shellfish contamination by other human pathogens (Burkhardt and Calci, 2000; Shieh et al., 2003). Second, oysters are superior sentinels of wastewater influence compared to water samples. Oysters not only rapidly acquired $\delta^{15}N$ ratios in tissues, to demonstrate entry of wastewater N to coastal food webs, they also bioaccumulated MSB and fecal coliforms to show longer-term influence compared to water (Fig. 6). Accumulation ratios (the ratio of indicator organism concentration in shellfish compared to water) found in this study were consistent with previous reports for oysters in nearby waters (Burkhardt and Calci, 2000; Shieh et al., 2003). Third, MSB was a better indicator organism than fecal coliforms to assess the impact of wastewater exposure at our study locations in Mobile Bay. Because SIA provides an effective tracer of wastewater material (Carmichael et al., 2004c; Savage, 2005; Piola et al., 2005), correlation of MSB concentration with $\delta^{15}N$ values (while fecal coliform counts were not correlated; Fig. 7), indicates MSB better correlates with overall exposure to and assimilation of wastewater particles by shellfish. Overall, these data demonstrate that the efficacy of fecal coliforms is limited and should continue to be reevaluated as an indicator of potential human health risk from wastewater influence on shellfish.

We are enthusiastic about the potential utility of MSB in combination with stable isotope ratios to serve as indicators of wastewater influence on ecosystem and human health. Stable isotope ratios provide a rapid and reliable method to demonstrate entry of wastewater N to coastal food webs, including commercially important bivalves, while MSB has proven reliable to trace wastewater-derived pathogens, even in the short term of this study. We also point out that this study was performed during the summer when viruses such as MSB are least stable in estuarine waters (Burkhardt et al., 2000). Hence, MSB may prove more effective if evaluated in tandem with $\delta^{15}N$ analyses year-round, and these relationships should be defined. Furthermore, this study was performed in an area closed to shellfishing and where shellfish resources are low. If applied where closed areas include harvestable shellfish resources, this approach may have broad implications for resource management. Used in combination, $\delta^{15}N$ ratios and MSB may provide a powerful tool to improve delineation of shellfish area closures, refine determinations of market-safe shellfish, and reduce the risk of harvesting contaminated shellfish.

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