Estuaries and Coasts
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Seasonal and Spatial Effects of Wastewater Effluent on Growth, Survival, and Accumulation of Microbial Contaminants by Oysters in Mobile Bay, Alabama

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Abstract We measured seasonal effects of wastewater treatment plant (WTP) effluent on growth, survival, and accumulation of microbes in oysters near a major WTP in Mobile Bay, AL. Despite higher nutrients near the WTP, seasonal conditions rather than distance affected chlorophyll *a* concentration and oyster growth. In summer and fall, when oyster growth was higher, δ^{15}N‰ in oysters near the WTP changed through time to reflect δ^{15}N‰ in effluent (approx. −4‰). Microbial indicators (male-specific coliphage, fecal coliforms) were highest in oysters near the WTP in all seasons and correlated with δ^{15}N‰ in fall and summer. Increased riverine discharge and slower acquisition of δ^{15}N‰ likely confounded correlations in winter/spring. Although we did not detect gross ecological effects of wastewater exposure for oysters, data indicated wastewater-derived particles entered the local food web and accumulated in oysters. These data highlight the importance of using multiple indicators of wastewater exposure and considering both seasonal and spatial effects when defining wastewater influence on a system or species.

Keywords Sewage · Stable isotopes · Fecal coliform · Male-specific coliphage

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Introduction

Each year, billions of gallons of wastewater are discharged into near-shore waters in the USA, delivering nutrients and other contaminants that have negative effects on coastal ecosystems and human health (Alexander 1998). Input of nutrients, such as nitrogen (N), stimulates primary production that can initially feed fisheries, but in excess, may lead to eutrophication-associated hypoxic events and fisheries loss (Cloern 2001; Nixon et al. 2002). Even treated wastewater effluent may transport fecal-associated microorganisms such as hepatitis A virus and human norovirus, which are implicated in thousands of illnesses each year among people who consume contaminated shellfish (Burkhardt and Calci 2000; Shieh et al. 2000). To prevent illnesses, thousands of hectares of fishing areas in the USA are closed to shellfish harvest due to elevated concentrations of fecal indicator bacteria and other criteria that may not be reflected by fecal coliform indicators (Rippey 1994; Glasoe and Christy 2005). The resulting economic impact from lost fishery yield is considerable (Alexander 1998; Landrum and Ache 2000). Hence, there is substantial need for better detection and identification of wastewater influences on coastal systems to inform fisheries management and protect public health (e.g., Randall 2003; Savage 2005).

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The timing and spatial scale of exposure may determine how wastewater inputs affect shellfish. Seasonal differences in water temperature or salinity, for example, may mediate wastewater-stimulated shellfish growth by affecting the abundance of microalgae available as food for shellfish or altering bivalve feeding rates (Brown and Hartwick 1988; Carmichael et al. 2004). Seasonal temperature variation may also affect the abundance of bacteria and viruses (Rippey 1994; Mathias et al. 1995). Bacteria, such as coliforms, may proliferate in nutrient-rich waters during warmer months and survive in colder months (Anderson et al. 1983; Lipp et al. 2001) while viruses are relatively more stable in colder months (Burkhardt and Calci 2000). Seasonal variation in riverine discharge into local waters can also change the concentration and composition of microbes delivered to an embayment, further confounding interpretation of wastewater influences on accumulation of microbial contaminants by shellfish (Calci et al. 1998; Gregory and Frick 2001; Lipp et al. 2001). Similarly, distance from a wastewater source may reduce the concentration of N and microbes as they are diluted by receiving waters. This dilution has been detected within and among estuaries as shifts in N stable isotope ratios (an established indicator of wastewater-derived N) in suspended particles in the water column and in tissues of contaminated shellfish (e.g., Savage 2005; Daskin et al. 2008). No studies have quantified the influence of wastewater on shellfish ecology and pathogen accumulation seasonally and spatially, despite importance to defining ecosystem level effects as well as fisheries and wastewater management strategies.

To investigate the temporal and spatial effects of wastewater exposure on shellfish, we employed three independent measures of wastewater influence—traditional oyster ecology, N stable isotope analysis, and microbial indicators—across three seasonal periods (summer, fall and winter/spring) and at different distances from a major wastewater treatment plant (WTP). Season and distance from the WTP outfall had significant effects on environmental attributes, oyster growth, and bioindicators of wastewater exposure. Despite seasonal effects on oyster growth, assimilation of wastewater-derived particles was detectable to some extent in oysters year round using this suite of wastewater indicators. Fecal coliforms, the standard indicator used to assess the sanitary quality of shellfish harvest areas in the USA, were less useful to indicate wastewater exposure during the winter/spring when riverine discharge was higher. Concentrations of male-specific coliphage (MSC), a proxy for norovirus and thought to be more specific to pathogens of concern to human health (Dore and Lees 1995; Shieh et al. 1999), however, showed potential to function effectively regardless of season. Herein, we provide the first data regarding simultaneous influence of wastewater on ecology and microbe accumulation in a commercially harvested species.

Methods

Sampling Scheme

To determine spatial and temporal effects of WTP effluent on sentinel oysters, we transplanted oysters at increasing distance (0.07, 0.5, 2.18, and 5.68 km, referred to as sites 1–4, respectively) from the Clifton C. Williams WTP located on MacDuffie Island in the northern part of Mobile Bay (Fig. 1) during three seasonal periods. Seasonal periods were defined as summer (8 Jun–16 Jul 2007), fall (18 Oct–6 Dec 2007), and winter/spring (11 Feb–25 Mar 2008). Each seasonal period was approximately 6 weeks in duration with sampling every 2 weeks, weather permitting. Wastewater at the facility undergoes secondary treatment prior to discharge into Mobile Bay (Volkert and Associates, Inc. 2006). The nearby Mobile Bay ship channel may act as a conduit for waters from the upper bay to the mouth (Fig. 1), providing a potential north–south gradient of wastewater concentration as it mixes with and is diluted by surrounding bay water (Daskin et al. 2008; Huang et al. 2009).

Transplanted Oysters

Oysters (*Crassostrea virginica*) were deployed in cages measuring 33×33×10 cm deep and constructed from a 2.5-cm plastic-coated wire mesh. During the initial summer season, 18 oysters, measuring on average 54.5±0.4 mm height (longest shell dimension), were placed in each cage. During subsequent seasons, 30 oysters, measuring 55.0±0.3 (fall) and 57.3±0.3 mm (winter/spring) shell height were planted. The higher number better accommodated sample processing. Two replicate cages were placed 0.5 and 1.5 m below the surface at each site (n=4 per site). Oysters were obtained from stocks reared at the Auburn University Shellfish Laboratory on Dauphin Island, AL. Prior to transplanting, oysters were held in recirculating seawater aquaria at the Dauphin Island Sea Lab and gradually acclimated to ambient field conditions by adjusting salinity to 2–3 and temperature to 2–3°C per day. Oysters (n=4 in summer and 8 in fall and winter/spring) were collected from each cage biweekly to measure growth and stored at −20°C for subsequent biochemical analyses.

Oyster Growth and Survival

To measure growth, the leading edge of each shell was marked with permanent marker or paint pen before trans-

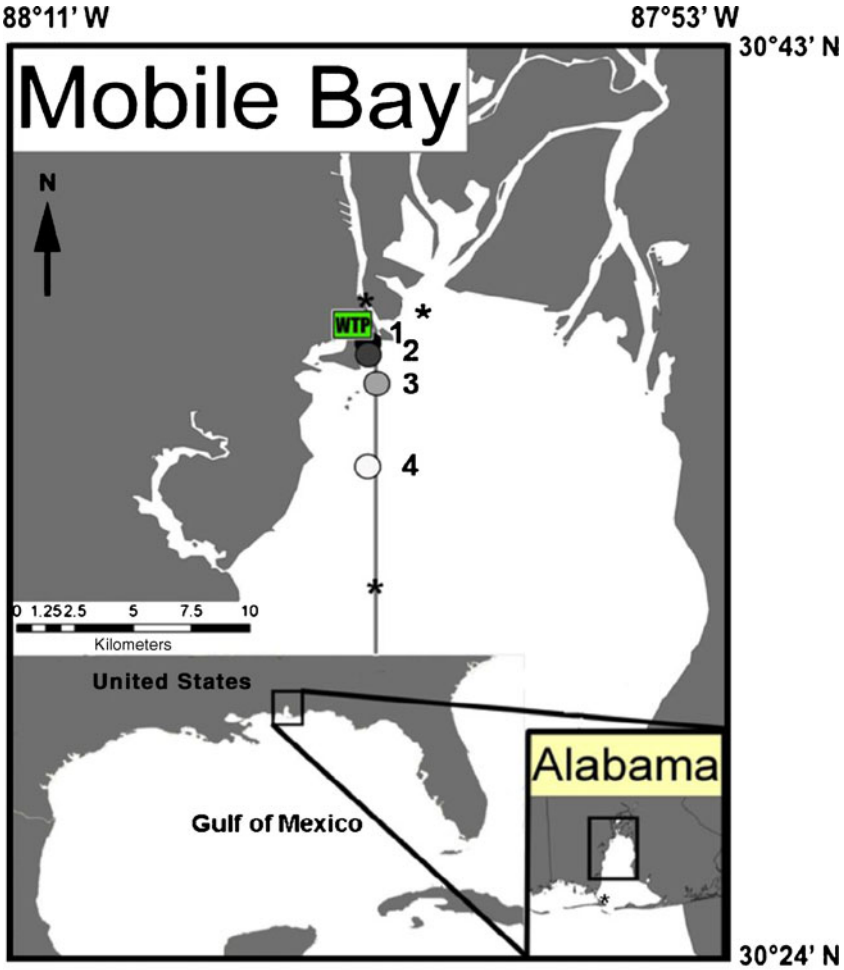

Fig. 1 Study sites in Mobile Bay, AL, located 0.07, 0.5, 2.18, and 5.68 km (*black*, *dark gray*, *light gray*, and *white dots*, respectively) from the Clifton C. Williams WTP outfall (*WTP*). The *gray line* indicates the Mobile Bay ship channel, and *stars* indicate four background control water sampling sites

planting. Upon collection, mortalities were noted and growth was measured to the nearest 0.1 mm as the difference between the marked edge and new growth along the axis of longest shell length (shell height). Growth was reported as the mean growth per day for each collection period and site. During the fall, cages at 2.18 km from the WTP outfall were lost due to theft or vandalism, and data from oysters at this site were omitted from growth, isotope, and microbial statistical analyses.

Ancillary Measurements

Water and Effluent Sampling Water samples were collected using a horizontal water sampler (Wilco) at the depth of transplant cages prior to collection of oysters on each collection date. WTP effluent was collected directly from the dechlorination contact chamber immediately prior to release into the outfall pipe at the start and end of the study. To determine the background conditions in Mobile Bay, water samples were collected from control sites north of the WTP outfall and south of the study sites. Samples were stored on ice in acid-washed opaque Nalgene bottles and processed within 8 h of collection to separate filtrate for nutrient analyses from suspended particles (SPM) for chlorophyll *a* (chl *a*) and stable isotope analysis. To do this, samples were pre-filtered through a 200-μm plastic mesh before vacuum filtration through 25 mm 0.7 μm pore-size Wattman GF/F glass fiber filters.

Nutrient and Chl a Analyses Filtrate was frozen at −20°C for N and phosphorus (P) analysis. Nutrient (NO_3^-, NO_2^-, and NH_4^+) analysis of the filtered water was performed on a Skalar San+Autoanalyzer according to the methods of Strickland and Parsons (1972). Chl *a* was extracted from filters using a 2:3 DMSO/90% acetone solution as described by MacIntyre and Cullen (2005). Filters for chl *a* were analyzed using a Turner Designs TD700 fluorometer. Three pseudo-replicate filters were processed to obtain one value for each water sample.

Environmental Attributes Temperature, salinity, and DO concentrations at each site were measured using a YSI 85 handheld meter at the depth of transplant cages. To determine the effects of freshwater discharge on microbial

concentrations, riverine discharge into Mobile Bay was estimated by adding daily discharge rates at two US Geological Survey (USGS) gauging stations, the Coffeeville Dam on the Tombigbee River (USGS site #: 02469765) and the Claiborne Dam on the Alabama River (USGS site #: 02429500) (USGS 2010). The mean discharge for 14 days prior to each sampling date was selected for analysis based on our sampling scheme (the time estimated for temporally explicit stable isotope and microbial data) and an estimated 5-day lag between readings at the river gauging stations and our sites in Mobile Bay (Schroeder 1979; Daskin et al. 2008). The resulting flow data from the 2-week increments were used for comparison to biweekly microbial data and to calculate seasonal means.

Stable Isotope Analysis

Two filters (prepared as described above) of SPM from each receiving water and effluent sample were dried at 60°C to a constant weight for stable isotope (SI) analysis. To obtain mean SI ratios for oysters at each site, and capture individual variation, three replicate samples were used; the samples were comprised of tissue from two individual oysters and an aggregate tissue sample from four oysters. Soft tissues were removed from the shell and washed with ultra-pure water (dual deionization followed by a microbial post filter). The adductor muscle was separated from the remaining whole tissues. Adductor muscle was dried at 60°C to a constant weight, ground to a powder using a mortar and pestle, and 1–3 mg was packed for analysis.

All stable isotope samples were analyzed at the UC 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.

Microbial Analysis

Oysters for microbial analysis were sampled at time zero and at the end of each seasonal period (n=8 for summer and n=10 for fall and winter/spring). Oysters seasonally sampled for microbial analysis were combined from four cages at each site to provide a sufficient quantity of oyster tissue for subsequent microbial assays. Whole oyster samples were stored on ice and processed within 24 h, including thorough washing, shucking, and aggregating whole tissues for analyses according to recommended Procedures of the Examination of Seawater and Shellfish (American Public Health Association 1970). Fecal coliform concentrations in oyster tissue and water were determined by a conventional five-tube multiple dilution most-probable number (MPN) procedure (American Public Health Association 1970). Briefly, lauryl tryptose broth (Difco) was chosen as the presumptive growth media, while confirmation was performed by inoculating liquid EC-MUG media (Difco) and incubating at 44.5°C for 18–24 h (Rippey et al. 1987). MSC densities were determined in oyster tissues and water by using a modified double-agar overlay method described by Cabelli et al. (1988). Water was analyzed similarly to oyster tissue supernatant. After incubation at 35°C for 18–24 h, viral plaques were enumerated and densities determined.

Statistical Analyses

Analysis of variance revealed no significant differences in growth or SI ratio between cages at each site. Hence, cages were considered replicates (n=4) for each site. Mean values for SI ratios of oyster tissue represent the mean of two individuals and one aggregate. All analyses were performed by Minitab 15®, using a general linear model for analyses of variance followed by Tukey's test of variability, unless otherwise indicated. Data were log-transformed for further analysis and error was propagated according to Valiela (2000). All linear correlations were tested using the Pearson's product-moment correlation coefficient. Principle component analysis (PCA) was applied to determine the combination of variables (i.e.*;* nutrients, temperature, salinity, and DO concentrations) that had the greatest effect on chl *a* concentration among sites.

Results

Seasonal and Spatial Variation in Environmental Attributes

Mean water temperatures ranged from 15°C to 29°C and showed predictable seasonal differences regardless of distance from the WTP (Table 1; ANOVA: $F_{2,\ 46}$=116.01, P<0.001; Tukey's post hoc test: for all seasonal period comparisons P<0.001). Mean DO values fell within the normoxic range at all sites in all seasons and generally had an inverse relationship with temperature (Table 1).

Salinity ranged from 4 to 20 during the study, showing significantly higher salinity in the fall and lower salinity in the winter/spring, but no difference relative to distance from the WTP (ANOVA season: $F_{2,\ 46}$=59.84, P<0.001;Tukey's test: P<0.001 for all seasonal comparisons). Lower salinity in winter/spring corresponded to significantly higher riverine discharge into Mobile Bay during that seasonal period (Table 1; ANOVA: $F_{2,\ 58}$=78.96, P<0.001; Tukey's test: P<0.001 for comparisons between winter/spring and summer or fall, P=0.91 for summer compared to fall).

Table 1 Riverine discharge, water temperature (Temp), dissolved oxygen concentration (DO), salinity, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), total dissolved nitrogen (TDN), phosphate (PO_4^{-3}), and chlorophyll *a* (chl *a*)±standard error measured during three sampling seasons at four sites increasingly distant from a WTP outfall in Mobile Bay, AL

Season	Site	Riverine Discharge ($m^3\ s^{-1}$)	Temp (°C)	DO ($mg\ L^{-1}$)	Salinity	NO_2^- (μM)	NO_3^- (μM)	NH_4^+ (μM)	TDN (μM)	PO_4^{-3} (μM)	Chl *a* ($mg\ L^{-1}$)
Summer	1	233±8	29.0±0.5	4.3±0.7	13.2±1.2	0.7±0.1	2±0	90±14	134±12	3.1±1.3	16.3±4.2
	2		28.5±0.8	4.7±0.6	11.7±1.3	0.3±0.1	1±0	13±7	50±11	1.2±0.4	21.8±7.3
	3		28.7±0.6	4.9±0.3	12.9±1.3	0.2±0.1	1±1	4±2	36±3	0.7±0.1	21.1±7.1
	4		27.6±1.0	4.9±0.3	14.0±1.5	0.2±0.0	1±0	4±1	35±3	0.8±0.2	21.5±8.2
Fall	1	191±9	20.7±2.0	5.4±0.3	20.2±1.0	1.0±0.2	3.0±0.6	122±30	178±34	4.0±1.3	7.8±1.5
	2		20.7±2.0	5.7±0.4	19.9±1.4	0.6±0.2	2.3±0.6	27±6	66±8	1.6±0.4	7.1±1.1
	3		20.6±2.0	6.1±0.2	18.6±0.9	0.3±0.1	2.0±0.6	5±1	35 ±2	0.9±0.2	7.9±1.3
	4		20.3±2.0	6.9±0.5	17.9±1.3	0.3±0.1	1.5±0.7	2±1	35±4	0.6±0.1	10.2±1.4
Winter/spring	1	1,691±120	15.6±0.7	7.0±0.3	6.6±2.1	1.9±0.3	8.7±1.8	121±27	213±43	2.5±0.4	9.2±1.5
	2		15.0±0.9	6.9±0.4	7.0±2.4	0.7±0.2	9.7±1.5	26±3	79±2	1.0±0.2	9.7±1.7
	3		14.7±1.0	8.1±0.4	5.8±3.6	0.3±0.1	9.3±1.9	3±1	47±4	0.4±0.0	12.6±1.5
	4		14.7±1.0	8.4±0.2	4.4±1.7	0.3±0.1	9.7±0.9	5±1	53±5	0.5±0.0	11.2±1.4
Effluent	–	–	–	–	–	2.8±0.1	2.7±0.4	936±160	1,223±309	57±36	–

Unlike other environmental variables, nutrient concentrations differed among season and among sites. NH_4 typically was the nutrient of greatest concentration across all sites and seasons. Concentrations of NO_3, NO_2, and total dissolved nitrogen (TDN) differed among seasons (ANOVA season: NO_3: $F_{2,\ 46}=86.40$, $P<0.001$; NO_2: $F_{2,\ 46}=6.89$, $P<0.01$; TDN: $F_{2,\ 46}=4.15$, $P=0.02$), with significantly lower concentrations in summer compared to winter/spring (Tukey's test: $P<0.02$ for all comparisons). Nutrient concentrations were significantly higher at the site nearest the WTP, in all seasons for all nutrients except NO_3 (not significantly higher in any season) and PO_4 (not significant in summer) (Tables 1 and 2).

Chl *a* concentrations ranged from 7 to 22 mg L^{-1} (Table 1) and were significantly higher in summer than in the fall or winter/spring, but did not differ among sites (Table 2; ANOVA season: $F_{2,\ 46}=10.61$, $P<0.001$; Tukey's: $P<0.01$ for both comparisons). Accordingly, PCA analysis indicated that nutrients and temperature made the greatest contribution to chl *a* concentration (Table 3). The PCA extracted three significant components with eigenvalues above 1, and the first two components explained more than 75% of the variance in the data (Table 3).

Oyster Growth and Survival

Oysters showed significant growth through time in all seasons (Fig. 2; ANOVA days in situ: summer: $F_{2,\ 148}=108.34$, $P<0.001$; fall: $F_{2,\ 244}=28.16$ $P<0.001$; winter/spring: $F_{2,\ 419}=66.41$, $P<0.001$), with the greatest growth measured on the last collection days (Fig. 2). Because oyster growth rates differed significantly among seasons (ANOVA season: $F_{2,\ 816}=334.99$, $P<0.001$), but not relative to distance from the WTP, data for all sites were combined in Fig. 2. The greatest oyster growth occurred in the summer (Fig. 2, top panel; Tukey's test: $P<0.001$ for all comparisons) when water temperature and chl *a* concentrations were highest. Summer growth was approximately

Table 2 ANOVA statistics for comparison of nutrient and chl *a* concentrations among study sites in each season

	Summer		Fall		Winter/spring	
Attribute	F_3	P	F_3	P	F_3	P
NO_3^-	2.07	0.16	1.06	0.40	0.10	0.96
NO_2^-	5.54	0.01	6.48	<0.01	16.46	<0.001
NH_4^+	28.51	<0.001	21.37	<0.001	34.59	<0.001
TDN	33.49	<0.001	21.37	<0.001	12.84	<0.001
PO_4^-	2.61	0.10	5.32	0.02	23.72	<0.001
Chl *a*	0.14	0.93	1.01	0.42	1.06	0.40

Table 3 Eigenanalysis and corresponding correlation matrix outputs for the two primary components from principal components analysis (PCA) of chlorophyll *a* concentration and environmental data

Variable	PC1	PC2
Eigenvalue	3.51	2.51
Proportion	0.44	0.31
Cumulative	0.44	0.75
TDN	0.51	−0.07
PO_4^{-3} (μM)	0.42	−0.23
NH_4^+ (μM)	0.50	−0.17
NO_2^- (μM)	0.47	−0.02
NO_3^- (μM)	0.24	0.33
DO (mg L^{-1})	0.07	0.59
Temperature (°C)	−0.16	−0.57
Salinity	−0.04	−0.37

2.5 times faster than growth in the fall and nearly ten times faster than growth in winter/spring (Fig. 2, insets; ANOVA: fall: $F_{2,\ 244}$=8.78, P<0.001; winter/spring: $F_{3,\ 419}$=3.91, P<0.01). During all seasons oyster mortality was less than 5%, and mortality was not related to number of days in the field or distance from the WTP outfall.

Stable Isotopes

$\delta^{15}N$ in SPM Nitrogen-stable isotopes in SPM (available food for oysters) differed with season and distance from the WTP. $\delta^{15}N$ values in SPM of receiving waters ranged from −5.5‰ at site 1 in the fall to 8.1‰ at site 4 in the summer. Overall, $\delta^{15}N$ values in SPM were heavier in the summer than in fall (ANOVA: $F_{2,\ 94}$=5.13, P<0.01; Tukey's test; P=0.01), but $\delta^{15}N$ values in winter/spring were not different from other seasons. $\delta^{15}N$ values of SPM in receiving waters were lighter at site 1, closest to the WTP, than at other sites in the summer and winter/spring (ANOVA: summer: $F_{2,\ 30}$=14.52, P<0.001; winter/spring: $F_{2,\ 30}$=50.14, P<0.001; fall: $F_{2,\ 30}$=2.26, P=0.10; Tukey's test: P<0.01 for Site 1 comparisons). The isotopic differences among sites are consistent with lighter $\delta^{15}N$ values of suspended particles in WTP effluent (−4.2±0.03‰) compared to surrounding waters of Mobile Bay (7.0±0.5‰; Fig. 3). A control site sampled immediately north of the WTP on an incoming tide in winter/spring showed a mean $\delta^{15}N$ value of 2.6±1.0‰, which is close to values at the outfall and consistent with tidal flow moving lighter, wastewater-derived particles northward on an incoming tide in contrast to heavier particles in surrounding waters. In the fall, $\delta^{15}N$ values were similar among sites, driven by unusually light values at sites 3 and 4 (Fig. 3, middle panel). Similarly, light $\delta^{15}N$ values (approx. −3‰) were found at the control site south of site 4 during this same sampling period.

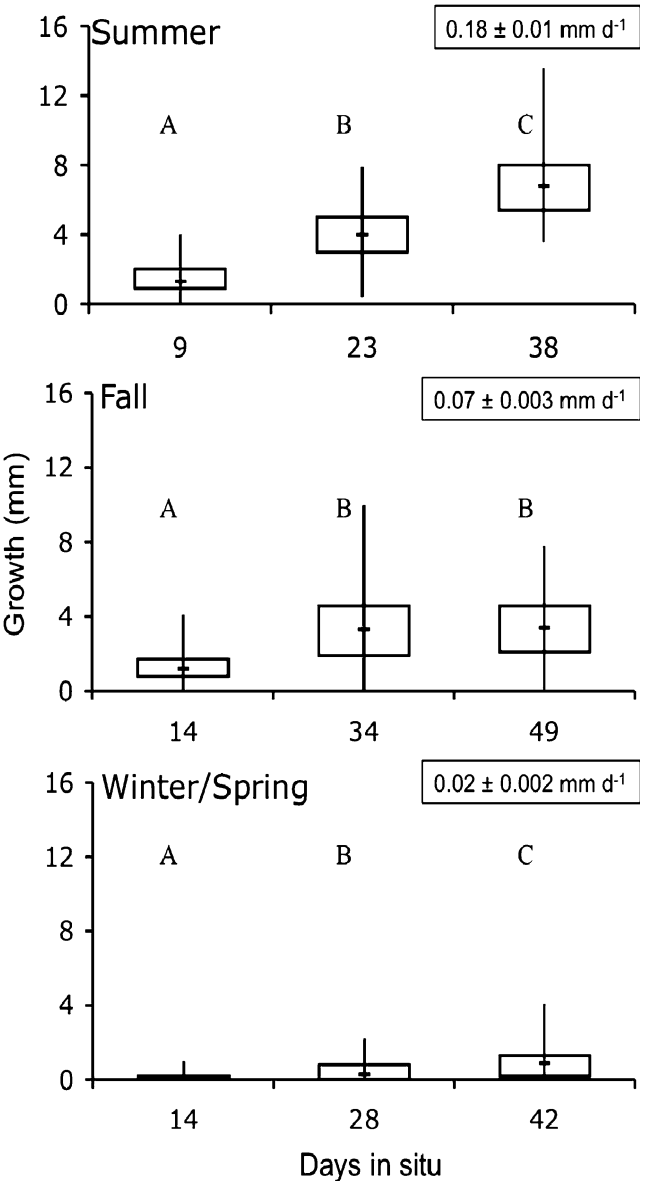

Fig. 2 Oyster growth compared to days in situ for the three seasons of study. The *vertical line* in each plot indicates the range of growth. *Top and bottom box boundaries* show upper and lower quartiles, and the *hash mark crossing each vertical line* represents median growth. Mean (±standard error) growth per day for the season is shown in the insets. *Letters* indicate results of Tukey's post hoc tests: P<0.001 for all summer and winter/spring comparisons; P<0.001 for day 14 compared with other days in fall

$\delta^{15}N$ in Oyster Tissue $\delta^{15}N$ values in oyster tissues differed relative to days in situ, season, and distance from the WTP. The mean $\delta^{15}N$ values in oyster tissues decreased with increasing days in situ as oysters grew in summer and fall (Fig. 4; ANOVA days in situ: summer: $F_{2,\ 38}$=8.42, P=0.001; fall: $F_{2,\ 107}$=34.43, P<0.001). The initial hatchery-derived isotope ratio (10.0±0.5‰) was replaced by ratios specific to each transplant site (Fig. 4; Tukey's test: summer: P<0.001 for day 38 compared to day 10; fall: P<0.001 for

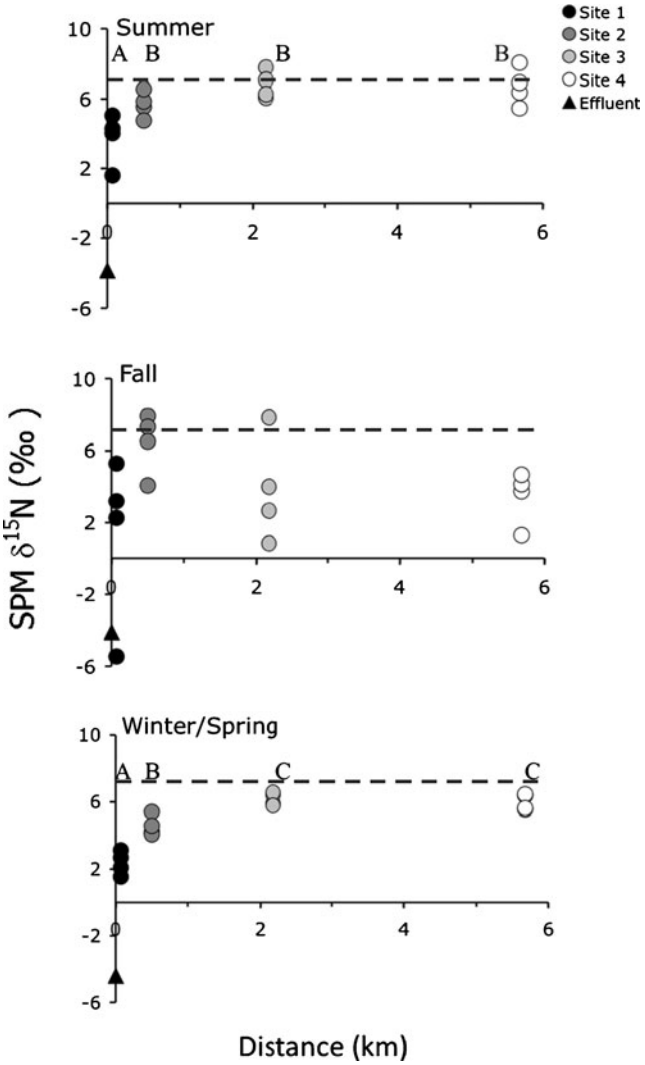

Fig. 3 $\delta^{15}N$ values in suspended particulate material (*SPM*) from receiving waters at four sites, increasingly distant from the WTP. *Triangles* represent the $\delta^{15}N$ values of WTP effluent. The *dashed lines* represent mean (±standard error) background $\delta^{15}N$ values of SPM in Mobile Bay. Note that the *y*-axis passes through '0' km distance, indicating the location of the outfall

all comparisons to day 14). Overall, $\delta^{15}N$ values in oyster tissues were lightest in summer, reflecting the greatest acquisition of ambient $\delta^{15}N$ values (Tukey's test: $P<0.01$ for all comparisons). Accordingly, $\delta^{15}N$ values in oyster tissue positively correlated with but were heavier than $\delta^{15}N$ values in SPM from surrounding waters in summer (Fig. 5, Pearson's correlation $r=0.80$, $P=0.001$).

Microbial Concentrations

MSC and fecal coliform concentrations showed similar patterns in water and oyster tissues, but MSC was a more specific indicator of wastewater influence among seasons. MSC and fecal coliform concentrations were consistently higher in tissues than in water (Table 4), and neither indicator differed in concentration among seasons (ANOVA season: MSC: Water: $F_{2,\ 6}=3.99$, $P=0.14$; Tissue: $F_{2,\ 9}=1.09$, $P=0.40$; Fecal coliform: Water: $F_{2,\ 6}=2.24$, $P=0.23$; Tissue: $F_{2,\ 9}=2.73$, $P=0.16$). During the winter/spring, however, fecal coliform concentrations in oyster tissue were greater than the maximum limit of detection, corresponding to the period of highest riverine discharge into Mobile Bay (Table 1). Because riverine discharge may have contributed to the high microbial load during this period, we opted to eliminate winter/spring data from subsequent analyses. MSC and fecal coliform concentrations in water and tissues did not

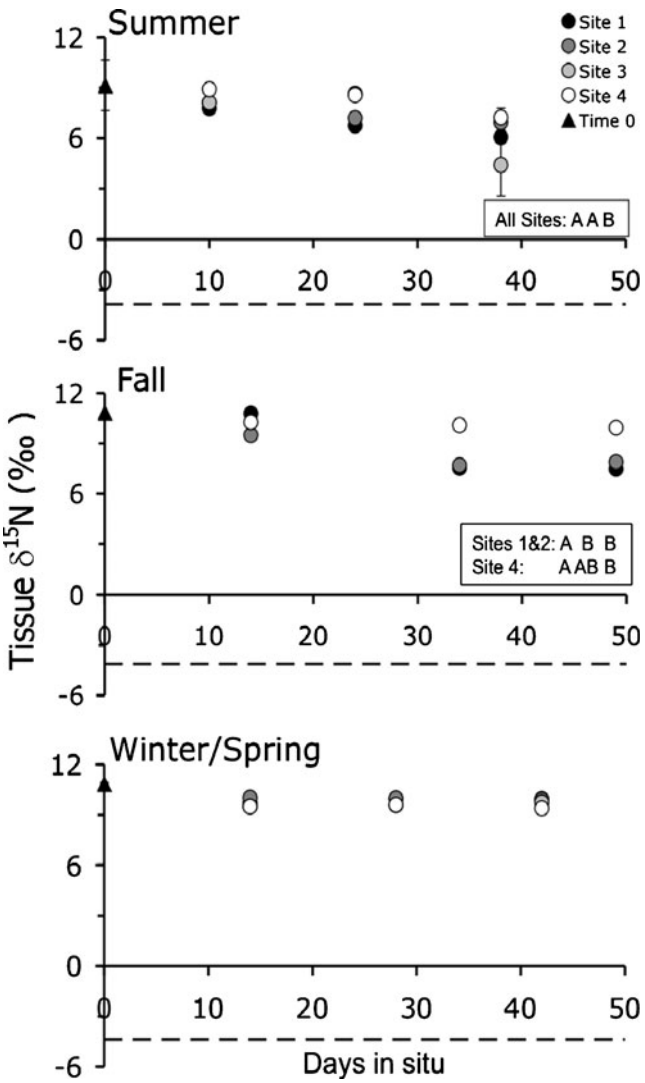

Fig. 4 $\delta^{15}N$ values in oyster tissue compared with the number of days in situ during three seasonal periods of the study. *Triangles* indicate the $\delta^{15}N$ value in oyster tissues at time 0 (hatchery based). The *dashed lines* represent the $\delta^{15}N$ value of suspended particles in WTP effluent. Note that the *y*-axis passes through day '0', indicating the day oysters were deployed at field sites

Table 4 MSC and fecal coliform concentrations in receiving waters and oyster tissues at four sites increasingly distant from a WTP in Mobile Bay, AL, separated by sampling season

		MSC (PFU 100 g^{-1})		Fecal coliforms (MPN 100 g^{-1})	
Season	Site	Water	Tissue	Water	Tissue
Summer	1	100	2,296	79	540
	2	20	1,643	13	350
	3	10	197	17	130
	4	10	171	8	350
Fall	1	160	27,503	490	9,200
	2	60	1,368	140	3,500
	3	20	–	49	–
	4	10	5	2	130
Spring	1	–	2,633	–	>16,000
	2	–	3,112	–	>16,000
	3	–	126	–	1,700
	4	–	13	–	120

MSC male-specific coliphage, *MPN* most-probable number

differ among sites (ANOVA—MSC: Water: $F_{2,\ 6}$=4.20, P=0.09; Tissue: $F_{2,\ 9}$=1.14, P=0.42; fecal coliform: Water: $F_{2,\ 6}$=1.81, P=0.32; Tissue: $F_{2,\ 9}$=0.73, P=0.23), but the highest absolute concentrations of each indicator were found at site 1 (Fig. 6). When distance was considered a continuous variable, MSC concentrations in tissues showed a significant logarithmic decrease relative to distance from the WTP (Fig. 6, bottom left panel, Regression: F_{reg3}=22.19, P<0.01, R^2=0.752, y=−27.60ln(x)+43).

Stable Isotope and Microbial Correlations

MSC and fecal coliform concentrations in oyster tissues were correlated with δ^{15}N values in oyster tissues (Pearson's correlation—MSC: r=−0.38, P=0.01; fecal coliform: r=−0.42, P=0.01; Fig. 7, bottom panels). In contrast, neither MSC nor fecal coliforms correlated with δ^{15}N values in water (Fig. 7, top panels).

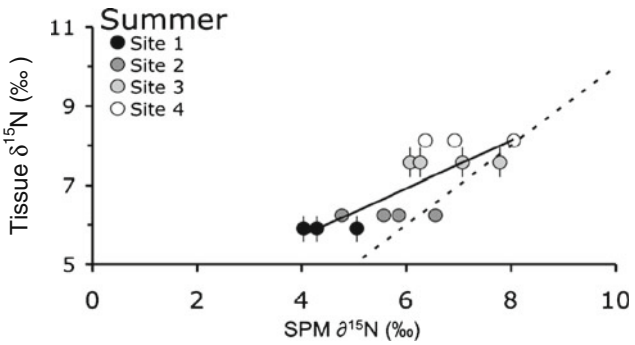

Fig. 5 δ^{15}N values in oyster tissues compared with δ^{15}N values in SPM from receiving waters at four sites increasingly distant from the WTP. The *dashed line* shows a 1:1 fit

Discussion

Season and distance from the WTP outfall had significant effects on environmental attributes, oyster growth, and other bioindicators of wastewater exposure. Season affected water temperature, salinity, chl *a* concentrations, oyster growth, and in turn, the integration of wastewater-specific stable isotopes into oyster tissue. Seasonal variation in riverine discharge may also have affected total fecal coliform load to receiving waters because upstream riverine and terrestrial inputs convey fecal coliforms to estuaries (Calci et al. 1998; Gregory and Frick 2001; Lipp et al. 2001). In contrast, distance from the WTP affected the concentration of nutrients in receiving waters, the magnitude of δ^{15}N values in SPM and oyster tissues, and MSC concentrations in oysters. Although these seasonal and spatial effects were distinct, they were not entirely independent. For example, chl *a* concentration (a measure of food supply for oysters) was mediated by wastewater-dependent nutrient concentrations and season-dependent water temperature. Similarly, δ^{15}N values in oyster tissues were determined by proximity to the wastewater source but also mediated by growth rates, which varied with seasonal shifts in water temperature and food supply. These data highlight the importance of considering both seasonal and spatial effects when defining wastewater influence on a system or species.

Implications for Estuarine Ecology Despite seasonal and spatial variation, stable isotope ratios, MSC, and fecal coliforms reflected wastewater exposure in oysters and their habitat when ecological indicators did not. Previous research has found distinct habitat changes such as reduced DO, increased primary production, and increased secondary

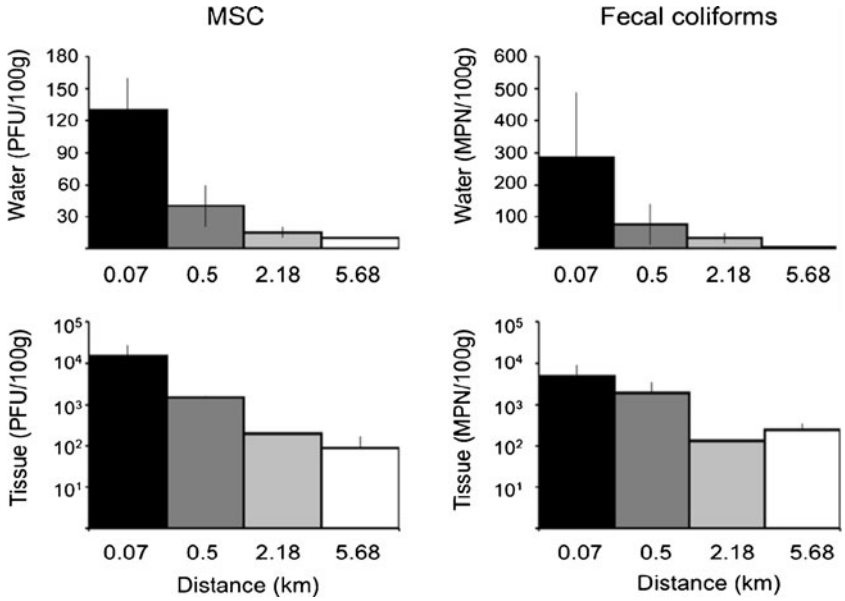

Fig. 6 Concentrations of male-specific coliphage (*MSC*) and fecal coliforms in water (*top*) and oyster tissue (*bottom*) at four distances from the WTP outfall (values represent the mean± standard error of summer and fall sampling periods)

production or species loss in areas with high wastewater input (e.g., Carmichael et al. 2004; Nixon et al. 2002; Valiela 2006). While our study is consistent with others in finding elevated nutrients in water closer to the wastewater source (Bowen and Valiela 2001; Savage 2005), nutrient enrichment did not translate to reduced DO, increased chl *a* concentrations, enhanced oyster growth, or reduced survival with proximity to the WTP outfall. Stable-N isotopes as well as MSC and fecal coliform concentrations in water and oyster tissues, however, showed that the oysters had been exposed to and incorporated particles from WTP effluent. These data point out that wastewater-derived N and microbial contaminants can enter, contribute to, and potentially pass through estuarine food webs without measurably changing habitat, growth and survival of primary consumers. Hence, the relative importance of wastewater-derived particles to estuarine trophic structure or contamination of commercial fishery stocks may be underestimated in areas where the net influence of wastewater input has not prompted substantial changes in habitat quality or species biomass and abundance.

Indicator Function While water samples reflected short-term variation in wastewater exposure, oyster tissues were a better indicator of wastewater contamination through time. For example, the temporally discrete, unusually light $\delta^{15}N$ values we captured in SPM during fall at sites 3 and 4 (farthest from the WTP) were not reflected in oyster tissues, suggesting that whatever source contributed to those values was episodic. Water samples, therefore, provided a "snap-shot" of local conditions, representing a moment in time. In

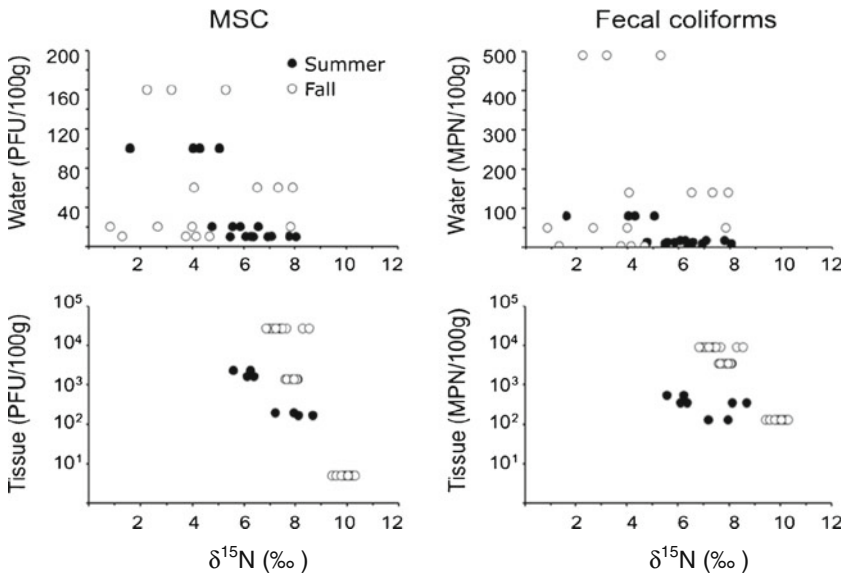

Fig. 7 MSC and fecal coliform concentrations in water (*top*) and oyster tissues (*bottom*) compared with $\delta^{15}N$ values in SPM and tissue, respectively, for the summer and fall sampling periods

contrast, oysters assimilated N stable isotopes and accumulated microbial indicators through time, which will provide integrative information on conditions in situ on a scale of days to weeks (Burkhardt et al. 1998; Burkhardt and Calci 2000; Daskin et al. 2008). For this reason, stable isotope ratios best indicated wastewater exposure in the summer and fall when oysters were actively feeding on local SPM and growing.

Fecal coliforms also best reflected WTP-derived contaminants during fall and summer, when riverine discharge and the potential for microbial input from alternative sources were low. Consistent with our findings, winter/spring is typically the season of highest riverine discharge to Mobile Bay (Park et al. 2007; USGS 2010). The long-term mean discharge to the bay is ~1,800 m^3 s^{-1}, more than 100 m^3 s^{-1} greater than measured during this study, which occurred in a period of drought (Park et al. 2007; US Drought Monitor 2007; USGS 2010). Riverine discharge did not have a significant effect on N stable isotope ratios or MSC concentrations as evidenced by the similarity among $\delta^{15}N$ values in SPM and MSC concentrations across seasons (Table 4). There is little doubt that riverine discharge contributes microbial load to the downstream estuary year round and more study is needed to quantitatively define the relative contribution of wastewater inputs and riverine discharge. Given that fecal coliform concentrations increase and then drop rapidly after rainfall and discharge events (in less than the biweekly periods we sampled; Chigbu et al. 2005) and the Mobile Bay area was experiencing a period of moderate to severe drought during the summer and fall periods sampled (Clark et al. 2008; US Drought Monitor 2007), it seems likely that we captured among the lowest possible background riverine contributions possible in the Mobile Bay system. Importantly, these data suggest that the reliability and utility of fecal coliforms to serve as an indicator of point municipal WTP failures may be confounded under typical discharge conditions in Mobile Bay and other water bodies that regularly receive high inputs of riverine discharge.

Unlike the other indicators, MSC showed potential to function effectively to indicate wastewater exposure regardless of season. Like fecal coliforms, MSC is not human specific. Contributions of MSC from municipal wastewater, however, far outweigh animal contributions in most estuaries (Calci et al. 1998). Moreover, when used in conjunction with a source-specific indicator such as N stable isotope ratios, the relationship between microbial contamination and a point wastewater source is strengthened. The positive correlation between MSC concentrations and stable isotope ratios in summer and fall, suggests that combining MSC and stable isotope analysis provided the better option for determining oyster exposure to WTP effluent from specific sources. N stable isotope ratios may be particularly useful to distinguish different types of wastewater because different levels of processing and delivery result in different isotopic signatures, ranging from light values in effluent from direct discharge facilities to heavier values in groundwater-derived septic inputs (Spies et al. 1989; Wayland and Hobson 2001; Savage 2005).

By combining three independent measures of wastewater influence—traditional oyster ecology, N stable isotope analysis, and microbial indicators—we provide the first data regarding the simultaneous influence of wastewater on ecology and microbe accumulation in a commercially harvested species. Although gross ecological effects of wastewater exposure may be too subtle to detect, wastewater-derived particles may still affect local food webs and the suitability of shellfish stocks for human consumption. Use of multiple indicators of wastewater exposure that are selected to best fit the temporal and spatial scales of interest can significantly improve detection of contaminated shellfish and ecological effects. These improvements are important because direct detection methods for pathogens require multiple specific tests and in many cases remain cumbersome, time consuming, and cost prohibitive while providing no ecological information and little predictive value (Lemarchand et al. 2005; Girones et al. 2010). In contrast, the multiple indicator approach we describe will inform municipal waste management and urban planning, refine boundaries for harvest area closures, reduce harvest of contaminated shellfish, and provide data for ecological assessments.

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