

## TOLERANCE AND GENETIC RELATEDNESS OF THREE MEIOBENTHIC COPEPOD POPULATIONS EXPOSED TO SEDIMENT-ASSOCIATED CONTAMINANT MIXTURES: ROLE OF ENVIRONMENTAL HISTORY

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**Abstract**—Meiobenthic copepod populations (*Microarthridion littorale*) were collected from three South Carolina, USA, estuaries having different pollution stress histories (i.e., pristine sediments, high polycyclic aromatic hydrocarbon [PAH] sediments, high metals/moderate PAH sediments) and then assayed for survival and reproductive output in 14-d exposures to pristine and heavily PAH/metals-contaminated sediments. Whole-sediment reproduction bioassays were used to determine whether copepods exposed to a highly contaminated sediment mixture exhibited differential survival and reproductive outputs as a function of previous environmental histories and whether genetic relatedness among populations measured as DNA sequences of the mitochondrial gene, cytochrome apoenzyme *b*, were linked to copepod contaminant tolerance. Overall, adult survival and reproductive success in contaminated sediments were significantly reduced relative to controls for all three populations irrespective of environmental histories. Differential resistance to sediment-contaminant mixtures by the two copepod populations inhabiting the contaminated sites was not found, despite their previous exposures to mixed contaminants at  $\Sigma$ PAH and  $\Sigma$ Metal concentrations of 7,287 to 2,467 ng/g dry wt and 461 to 3,497  $\mu$ g/g, respectively. Significant genetic differentiation, however, was found between copepod populations from the control and the two contaminated sites. Generally, cross-population survival and reproductive outputs were not significantly different and could not be linked to genetic differentiation at the population level.

**Keywords**—Mixed contaminants Sediment bioassay Adaptation Population genetics Meiofauna

### INTRODUCTION

Estuarine sediments are sinks for contaminants, which may accumulate and persist at sublethal to lethal concentrations [1]. Organisms living in these sediments receive pollution exposures that may impair secondary production, decrease fitness, and potentially alter genetic diversity. Numerous studies have measured varying degrees of tolerance in populations of estuarine species exposed to sublethal concentrations of single or mixed sediment-associated contaminants [2,3]. Tolerance to contaminated sediments can be developed by either non-genetic (physiologic acclimation) or genetic (adaptation) mechanisms. Adaptation may come at a cost, however, including loss of alleles or modification of the population's genetic structure. Through toxicant-induced selection, the genetic variability in surviving and reproducing, toxicant-tolerant individuals can produce changes in population allelic frequencies [4,5]. Such changes may reflect adaptation to prevailing local environmental conditions [6], and they may explain how survival and reproductive success can occur during chronic exposures to contaminated sediments.

Many laboratory studies that have tested natural field populations for enhanced/diminished pollution tolerance have re-

lied on acute mortality tests with single toxicants [2,3,7,8]. These tests are informative, but they often do not measure sublethal reproductive endpoints and typically are of short duration, which may underestimate chronic field exposures [9–11]. Population-linked differential resistance to contaminants as a function of environmental history has been seen in single-toxicant laboratory bioassays with oligochaetes [4], springtails [12], chironomids [13], copepods [9], and estuarine nematodes [14]. Resistance and adaptation to in situ contaminant mixtures has been more difficult to measure, however, because many pollutants interact simultaneously [6,7,15]. Population-linked resistance to mixed toxicants has been found in nematodes [16] and oligochaetes [4,17] but not in more mobile chironomids [8,13] or darter gobies [15]. Little is known regarding benthic faunal tolerance to sediment contaminant mixtures as a function of genetic adaptation [2].

Meiofauna (benthic metazoans that pass through a 0.5-mm sieve but not a 0.063-mm sieve) receive continuous exposure to sediment-associated contaminants through burrowing and ingestion, and they are sensitive to mixed toxicant environments [3]. The meiobenthic copepod *Microarthridion littorale* has exhibited toxicant-reduced survival and reproductive output in both single- and mixed-contaminant toxicity tests [18,19]. It is known to bioaccumulate pollutants [20,21], and it has a relatively short life-cycle of egg to egg (30 d at 20°C) [22]. This copepod also comprises, on average, 18% of estuarine sediment-dwelling fauna in the North Inlet, South Carolina, USA, estuary [23], and it provides an important food source for many benthic feeding fish [24,25]. The ability of

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Table 1. Background chemical analysis of sediments from which the North Inlet Estuary (NIE), Diesel Creek (DLC), and Shipyard Creek (SYC) *Microarthridion littorale* copepod populations were collected<sup>a</sup>

Metal	NIE ( $\mu\text{g/g}$ dry wt)	DLC ( $\mu\text{g/g}$ dry wt)	SYC ( $\mu\text{g/g}$ dry wt)	PAH	NIE ( $\mu\text{g/g}$ dry wt)	DLC ( $\mu\text{g/g}$ dry wt)	SYC ( $\mu\text{g/g}$ dry wt)
Cadmium	<0.01	0.28	1.03	Anthracene	<2	481.8	64.6
Chromium	0.01	104.1	2,873	Benz[ <i>a</i> ]anthracene	<4	585.5	242.6
Copper	2.2	46.9	68.1	Benzo[ <i>a</i> ]pyrene	<5	431.3	201.7
Lead	4.2	101.3	109.4	Chrysene	<1	1,186.7	339.7
Mercury	0.01	0.07	0.14	Fluoranthene	<3	1,861.6	532.7
Nickel	4.4	13.2	50.7	Phenanthrene	2.3	526.9	230
Silver	<0.01	0.11	0.18	Pyrene	<2	1,724.8	516.4
Zinc	15.01	194.5	394.5	$\Sigma$ Total PAH	37.3	7,286.7	2,466.9

<sup>a</sup> Predominant polycyclic aromatic hydrocarbons (PAHs) are listed, and all measured effects-range low/effects-range median concentration-designated [30] PAHs are summed under  $\Sigma$ Total PAH. A < denotes a concentration below the instrument detection limit. Table adapted from Sanger [29], with permission.

*M. littorale* to form physiologically and genetically distinct populations within the same salt marsh complex [26,27], its rapid life cycle, and the adaptive capacities exhibited by other benthic copepods to chronic contaminant exposures [6,10,28] all suggest a strong potential for *M. littorale* to develop resistance locally in chronically polluted sediments.

The genetic population structure of *M. littorale* has been examined in a macrogeographic (100s of km) [27] and a microgeographic (10s of km) molecular study (Schizas et al., unpublished data). *Microarthridion littorale* collected from seven estuaries along the southeastern United States and Gulf of Mexico were strongly segregated into distinct phylogenetic units [27], whereas *M. littorale* collected from 10 creeks (mainly in South Carolina, USA) maintained high levels of gene flow even though populations from some creeks were significantly differentiated genetically (N.V. Schizas et al., unpublished data).

The objectives of this study were to determine, as a function of previous toxicant exposures (environmental history), the tolerance and reproductive response of three geographically distinct populations of *M. littorale* to high concentrations of a field-sediment contaminant mixture, and to use polymerase chain reaction-based measures of genetic differentiation among populations to correlate and predict relative population adaptive response (principally reproductive output).

#### MATERIALS AND METHODS

In this study, *M. littorale* collected from two heavily polluted and one pristine sediment site were exposed to both clean and heavily contaminated field sediments for 14 d in reproductive bioassay experiments that were replicated in time. Concomitantly, copepods collected from these same sites underwent estimation of population genetic parameters using DNA sequences of the mtDNA gene, cytochrome apoenzyme *b*.

##### Copepod collection

*Microarthridion littorale* were collected from pristine North Inlet Estuary (NIE), Georgetown, South Carolina, USA (33°20' N, 75°10' W); Diesel Creek (DLC; 32°49' N, 80°01' W), Charleston, South Carolina, USA; and Shipyard Creek (SYC), Charleston, South Carolina, USA (32°50' N, 79°57' W). North Inlet Estuary is largely undeveloped, and it has been designated as a U.S. National Estuarine Research Reserve. Based on the results of previous studies [29] and this research, concentrations of typical anthropogenic toxicants are either nondetectable or in the low ng/g range in NIE sediments. Diesel Creek is an industrial watershed adjacent to a U.S.

Environmental Protection Agency Superfund site. Sediment contaminant concentrations exceed the effects-range low concentration for 10 metals and nine high polycyclic aromatic hydrocarbons [PAHs] [29,30]. The SYC watershed drains a recently decommissioned U.S. naval base and metal-plating facility, which were active in ship repair and chromium electroplating. As a result, sediments have high levels of metals and hydrocarbons. Background contaminant concentrations for metals and PAHs in sediments inhabited by the test populations are reported in Table 1. Thirteen SYC sediment contaminants exceed their respective effects-range low concentration, and one contaminant (chromium) exceeds the effects-range median concentration [30]. For comparison, DLC sediments may contain 1.4- to 1.7-fold higher total PAH concentrations than SYC sediments but lower concentrations of metals, solvents, and polychlorinated biphenyls (solvents and polychlorinated biphenyls not listed) [29].

Copepods were collected from each site at low tide by manually scraping the uppermost 1 to 2 cm of exposed mud flat. Scrapings were then sieved through 500- and 125- $\mu\text{m}$  stainless steel sieves with seawater. Sediment retained on the 125- $\mu\text{m}$  sieve was transported to the laboratory, transferred to translucent containers, and aerated. Copepods were attracted from sediment to overlying water using fiberoptic lights and then captured using glass pipettes. Adult male and gravid female *M. littorale* were sorted under a dissection stereomicroscope. Sorted adults were held for 1 d in an environmental chamber at test conditions of 20°C and 12:12 h (fluorescent light:dark) before testing. For genetic characterizations, an average of 18 specimens per site were preserved in 95% ethanol and stored at -80°C until analysis.

##### Sediment collection and chemistry

Control sediments were collected from a 3 m<sup>2</sup> area of unimpacted, intertidal mud flat in the pristine NIE. Contaminated treatment sediments were collected from a heavily polluted 5 m<sup>2</sup> intertidal stretch within SYC. Sediments were collected using the same method described previously for copepod collection. Approximately 1 kg of the NIE 0- to 1-cm sediment layer was collected concurrently with copepods, homogenized using a Teflon<sup>®</sup> spatula, and placed into two solvent-washed, 500-ml glass jars. One jar of sediment was sealed and refrigerated at 4°C for 36 h to kill all meiobenthic copepods and most of the other sediment-dwelling meiofauna (G.T. Chandler, unpublished data.). The second jar was similarly sealed and frozen for later chemical analysis. Using identical techniques,

SYC sediments were collected concurrently with NIE sediments from the same SYC site in October and December 1997.

Within two months of sediment collection, trace metal and PAH concentrations in test sediments were measured by the U.S. National Oceanic Atmospheric Administration/National Ocean Service Laboratory (Charleston, SC, USA). Metals were extracted from sediments using an acid microwave digestion (CEM® Model MDS-2000, CEM, Matthews, NC, USA). Metals analysis was conducted using inductively coupled plasma spectroscopy (Perkin Elmer® Plasma 1000, Perkin Elmer, Norwalk, CT, USA) for a suite of metals. Graphite furnace atomic absorption spectrometry was used to measure Ag, As, Cd, Pb, and Se. Mercury was measured via cold-vapor atomic absorption spectroscopy using a Leeman Labs® PS200 Hg analyzer (Leeman Labs, Hudson, NH, USA). Polycyclic aromatic hydrocarbons were extracted from sediments using Soxhlet extraction and measured using capillary gas chromatography:ion-trap mass spectroscopy (Finnigan® MAT Magnum Ion Trap MS, Finnigan, Bremen, Germany) and high-performance liquid chromatography [31] with fluorescence detection (Gilson® Model 231, Gilson Medical Electronics, Milwaukee, WI, USA, and Waters® HPLC Pump Model 501, Waters Systems, Milford, MA, USA).

#### *Reproduction bioassay procedure*

Copepod whole-sediment bioassay procedures followed those described by Chandler and Green [32]. Each of the three copepod populations was exposed to control sediments from North Inlet, South Carolina, USA, or to heavily polluted sediments from a contaminated area of Shipyard Creek, South Carolina, USA. Each sediment type was divided into four replicates, exposing 25 adult males and 25 gravid females in each. The experiment was replicated in time (experiment I and experiment II in the general linear model, discussed later).

Test sediments were 31% to 41% sand, 59% to 69% silt:clay, and exhibited median grain diameters of 2.4 to 3.5  $\mu\text{m}$ . Total organic carbon as determined by C:H:N analysis was 3.2% to 4.3%. Fresh sediments, which were stored at 4°C for 36 to 48 h, were collected as described earlier and press-sieved through a 63- $\mu\text{m}$  stainless steel sieve before introduction into test chambers to remove all dead meiofauna and small macrofauna. Test chambers consisted of 50-ml Teflon® Erlenmeyer flasks with two opposing, 1-cm-diameter holes at a 2-cm height that were covered with Nitex® 63- $\mu\text{m}$  mesh (Aquatic Eco-Systems, Apopka, FL, USA). Each test chamber received 10 ml of press-sieved sediment (introduced as a compacted layer via a sterile, 10-ml glass syringe) and 25 to 30 mL of artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH, USA) at a pH of 8.0 and 30 ppt S. Test chambers were then placed under dripping flow in a single-pass, flowthrough seawater system [32] for 1 to 2 h. Copepods were loaded via glass pipettes into test chambers, and test chambers were then placed into an incubator at 20°C and 12-h light:dark cycle. Water-quality parameters were maintained at a pH of 8.0  $\pm$  0.1, 30  $\pm$  0.5 ppt S,  $\text{NH}_4$  less than 0.01 ppm, dissolved oxygen of greater than 6.0 mg/L, and total acid volatile sulfides less than 1  $\mu\text{mole/g}$  sediment. System water flowed once through each test chamber at a rate of 2 to 3 ml/min.

Salinity and pH were measured in the system daily, and ammonia and dissolved oxygen were measured every third day. In addition, all water-quality parameters were measured and recorded at test initiation and termination for a total of six parameter readings for each 14-d test. The pH was mea-

sured using an Orion® benchtop pH meter model 8104 Ross combination pH glass electrode meter (Orion, Beverly, MA, USA). Dissolved oxygen was measured using an Orion model 820 dissolved oxygen meter. Salinity was measured using a refractometer, and ammonia was measured by the salicylate method using a Hach® DR/700 Colorimeter (Hach, Loveland, CO, USA). Acid volatile sulfide was measured as described by Allen et al. [33]. Copepods were fed a cold 1:1 algal mixture of *Isochrysis galbana* Haines and *Dunaliella tertiolecta* Butcher every third day to yield  $10^7$  cells per chamber. Algal cell mixture concentrations were counted in triplicate on a Coulter® MultiSizer II particle analyzer (Coulter Electronics, Toronto, ON, Canada) with AccuComp® software (AccuComp, Brampton, ON, Canada).

At the end of each test, water-quality analyses were performed on each individual test chamber. For pH and dissolved oxygen, probes were carefully placed into the test chamber to avoid disturbing the sediments. Ammonia and salinity samples were taken using a glass pipette wrapped in 63- $\mu\text{m}$  Nitex mesh to prevent loss of copepods via the pipette. To measure ammonia, 12.5 ml of test chamber artificial seawater obtained from two replicates were pooled to yield a total sample volume of 25 ml.

At test termination, chamber sediments were individually sieved (63  $\mu\text{m}$ ), rinsed with artificial seawater into a 60-mm Petri plate, checked for dead individuals, and preserved in a 5% formalin and rose bengal solution. Copepods were enumerated for males, females, gravid females, copepodites, nauplii, and clutch sizes of gravid females.

#### *Statistical analyses*

Means and standard deviations were calculated for each test endpoint. An additional parameter—realized production—was calculated as ([copepodites + nauplii]/number of females alive at end of test) to normalize total offspring production to a per-surviving-female basis [32]. Data were analyzed as a three-way analysis of variance (Experiment  $\cdot$  Test Sediment  $\cdot$  Population) model under a general linear model. Arcsine square-root transformations were performed on all proportion or percentage values, and log transformations were performed on count data to meet analysis of variance parametric assumptions.

Because of significant three-way interactions among main effects (Experiment, Test Sediment, Population), a one-way analysis of variance was performed on the NIE *M. littorale* population across experiments to confirm the three-way interactions. Each endpoint for the NIE *M. littorale* population exposed to control sediments was designated a control response, and data were normalized to that response within each bioassay to eliminate significant experiment-to-experiment effects. Endpoint data were normalized within each replicate over the three populations by dividing each endpoint by the mean control response for that parameter in a given experiment (i.e., experiment I or II). For example, NIE and DLC naupliar production in the first experimental replicate in time (i.e., experiment I) for both sediment types was normalized by dividing by the mean naupliar production for the NIE *M. littorale* population in control sediments in experiment I. After normalization over both experiments, data were regrouped across experiments into one set, and general linear models were run to test for significant test-sediment and population-level effects using Tukey's Studentized Range test at  $\alpha = 0.05$ . A general linear model and orthogonal contrasts were employed to test

Table 2. Contaminant concentration in homogenized sediments used in whole-sediment bioassays<sup>a</sup>

Metal	SYS			PAH	SYC		
	NIE control ( $\mu\text{g/g}$ dry wt)	Collection I ( $\mu\text{g/g}$ dry wt)	Collection II ( $\mu\text{g/g}$ dry wt)		NIE control ( $\mu\text{g/g}$ dry wt)	Collection I ( $\mu\text{g/g}$ dry wt)	Collection II ( $\mu\text{g/g}$ dry wt)
Cadmium	<0.031	1.6	1.3	Anthracene	4.1	69	97
Chromium	73	1,800	1,900	Benz[ <i>a</i> ]anthracene	7.5	330	300
Copper	19	120	110	Benzo[ <i>a</i> ]pyrene	12.25	550	510
Lead	28	160	160	Chrysene	15.5	490	380
Mercury	0.08	49	50	Fluranthene	21.0	1,200	810
Nickel	25	0.28	0.25	Phenanthrene	14.1	230	270
Silver	<0.021	0.25	0.28	Pyrene	14.5	1,200	840
Zinc	82	510	490	$\Sigma$ Total PAH	222.1	4,184	5,257

<sup>a</sup> Shipyard Creek (SYC) sediments were collected at two times and correspond with experiments I and II in analysis of variance model. Predominant polycyclic aromatic hydrocarbons (PAHs) are listed, and all measured effects-range low/effects-range median concentration–designated [30] PAHs are summed  $\Sigma$ Total PAH. A < denotes a concentration below the instrument detection limit.

for significant differences between male and female percent survival among treatments and populations.

### Genetic analyses

Seventeen copepods from the NIE, 22 from the DLC, and 14 from the SYC (total, 53 individuals) were successfully extracted for DNA-level genetic analysis. The methodology for copepod DNA amplification by polymerase chain reaction and subsequent sequencing of the mitochondrial cytochrome *b* gene was developed by Schizas et al. [27,34]. Cytochrome *b* is an appropriate mtDNA molecular marker for population-level studies in harpacticoid copepods. In most animals, mtDNA-derived data are easier to interpret relative to nuclear DNA-derived data, because mtDNA is inherited in a matrilineal, nonrecombinant fashion [35]. In this study, a segment of 350 base pairs of cytochrome *b* was amplified from each individual and sequenced using either a LI-COR 4000L (LI-COR, Lincoln, NE, USA) or an ABI PRISM 377 sequencing system (Perkin Elmer). Sequences of DNA were aligned using Sequencer<sup>®</sup> (Gene Codes, Ann Arbor, MI, USA) software and analyzed with Arlequin software [36]. We calculated genetic distance [37] between populations using subroutines in Arlequin. The null hypothesis of “no genetic difference between populations” was evaluated using pairwise  $F_{ST}$  [38] tests as instructed in Arlequin. The pairwise  $F_{ST}$  values were used to estimate the degree of genetic differentiation between populations and were calculated using the pairwise-distance method [38].

## RESULTS

### Chemistry

Concentrations of metals and PAHs in test sediment samples are reported in Table 2. Concentrations of metals and PAHs in control sediments were five- to 20-fold lower than those in treatment sediments. Treatment-sediment metal and PAH concentrations were almost identical for both collections/experiments (experiments I and II).

### Copepod adult survival

Normalized *M. littorale* female adult survival was high for the NIE population, with greater than 89% survival in control and treatment sediments (Fig. 1a). In control sediments, DLC and SYC female survival was generally lower than that in the NIE population, but not significantly so. In contaminated sediments, a significant treatment effect on female survival was not observed for any population. Between-population differ-

ences, however, did occur where DLC and SYC female survival were significantly lower than that in the NIE population.

Male survival was lower and more variable than female survival for all three populations (Fig. 1b). In control sediments, male survival was highest for the NIE *M. littorale* (83%). Male survival for the DLC and SYC populations was lower than that for the NIE population in control sediments, but only the DLC sample was significantly reduced. In treatment sediments, NIE and DLC populations exhibited significant mortality in treatment sediments, but SYC males were not significantly reduced. Male survival in the DLC sample was significantly reduced compared with that for the NIE and SYC male populations in the treatment sediments.

Results of previous toxicity tests using copepods often have shown significantly higher female than male survival [19,39], but we observed no consistent patterns in this study. For the NIE and SYC populations, female survival was greater than male survival for both sediment types, but significant differences were not detected. For the DLC population, however, female survival was significantly higher than male survival in both sediment types.

### Copepod reproductive output

Mean copepodite production in control sediments was highest for the NIE population, but no significant differences were detected among the three populations (Fig. 2a). In treatment sediments, SYC copepodite production was greatest but still not significantly larger than copepod production by the other

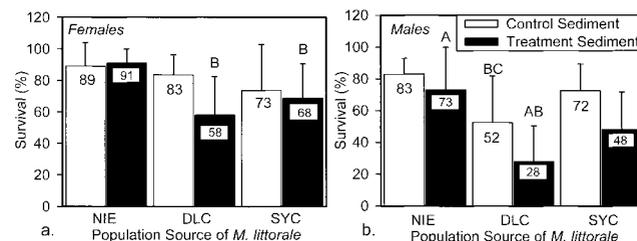


Fig. 1. Comparison of adult female (a) and male (b) mean survival for three populations of *Microarthridion littorale* exposed for 14 d to sediments from pristine North Inlet Estuary (NIE, Control) and contaminated Shipyard Creek (SYC; Treatment). Error bars represent 1 SD of the mean. A = significant treatment effects ( $\alpha = 0.05$ ) between control sediment and treatment sediment, B = significant population effects ( $\alpha = 0.05$ ) between pristine (NIE) and polluted (Diesel Creek [DLC] and SYC) copepod populations, C = significant population effects ( $\alpha = 0.05$ ) between the two polluted copepod populations (DLC and SYC).

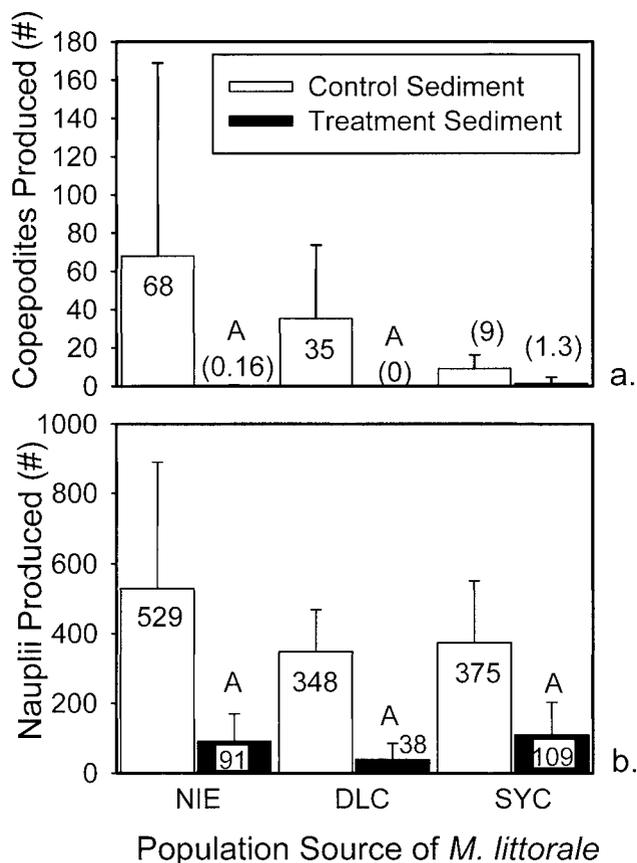


Fig. 2. Comparison of mean copepodite (a) and naupliar (b) production for three populations of *Microarthridion littorale* exposed for 14 d to sediments from pristine North Inlet Estuary (NIE; Control) and contaminated Shipyard Creek (SYC; Treatment). Error bars represent 1 SD of the mean. A = significant treatment effects ( $\alpha = 0.05$ ) between control and treatment sediment, DLC = Diesel Creek.

two populations. Copepodite production in the NIE and DLC populations was significantly reduced in the treatment sediments compared with that in the control sediments. No significant reduction in SYC copepodite production was observed in either sediment type (Fig. 2a).

For nauplii, the NIE production was similarly higher in control sediments than that for DLC and SYC populations, but not significantly so. In treatment sediments, naupliar production was significantly reduced for all three populations compared with the three-population production in control sediments. On average, the SYC naupliar production in treatment sediments was approximately 30% greater than the NIE and DLC population production, but this difference was not significant (Fig. 2b).

The number of gravid females at the end of the 14-d exposures was significantly greater for each population in control sediments than in treatment sediments. In control sediments, 56% of the NIE, 60% of the DLC, and 42% of the SYC females were gravid. In treatment sediments, 43% of the NIE, 33% of the DLC, and 24% of the SYC females were gravid. No significant differences among the three populations within either treatment were found. Average clutch size was similar for all three populations in control sediments, with the NIE females producing the largest clutches. In treatment sediments, clutch sizes of all three populations were significantly reduced compared with size in control sediments (Fig. 3a). The DLC and SYC females, however, produced larger clutch sizes than the

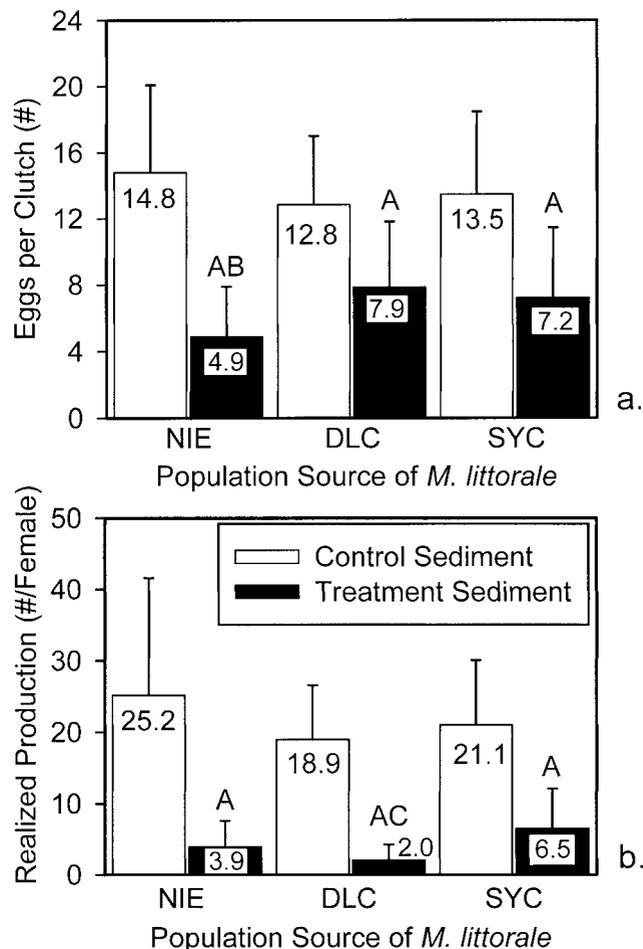


Fig. 3. Comparison of mean clutch sizes (a) and realized offspring production (b) for three populations of *Microarthridion littorale* exposed for 14 d to sediments from pristine North Inlet Estuary (NIE; Control) and contaminated Shipyard Creek (SYC; Treatment). Error bars represent 1 SD of the mean. A = significant treatment effects ( $\alpha = 0.05$ ) between control sediment and treatment sediment, B = denotes significant population effects ( $\alpha = 0.05$ ) between pristine (NIE) and polluted (Diesel Creek [DLC] and SYC) copepod populations, C = significant population effects ( $\alpha = 0.05$ ) between the two polluted copepod populations (DLC and SYC).

NIE females in treatment sediments. Only DLC clutch sizes were significantly larger than NIE clutch sizes.

All three populations exhibited significantly reduced realized offspring production in treatment sediments compared with those in control sediments (Fig. 3b). In control sediments, all three *M. littorale* populations exhibited similarly high realized offspring production. In treatment sediments, realized offspring production by the SYC *M. littorale* exceeded that by the other two populations, but the SYC production was only significantly higher than that of the DLC *M. littorale* population in treatment sediments (Fig. 3b).

#### Three-population genetic relatedness

Genetic analysis of the 53 *M. littorale* individuals from the NIE, DLC, and SYC yielded 17 distinct haplotypes. Haplotypes are unique DNA sequences that are shared by one or more individuals. Haplotype A was most common, and it was shared by 57% of specimens. Unique haplotypes (i.e., sequences represented by only one individual) were found in every collection site. Haplotype A was the most abundant DNA

Table 3. Distribution of the 17 cytochrome *b* haplotypes identified in *Microarthridion littorale* from North Inlet Estuary (NIE), Shipyard Creek (SYC), and Diesel Creek (DLC)<sup>a</sup>

Haplotype	NIE	SYC	DLC	Total
A	8	7	15	30
B	1	2	0	3
C	0	1	1	2
D	0	0	1	1
E	0	2	0	2
F	0	0	1	1
G	0	0	1	1
H	0	1	0	1
I	1	0	0	1
J	0	1	0	1
K	0	0	1	1
L	0	0	1	1
M	3	0	0	3
N	1	0	0	1
O	2	0	0	2
P	0	0	1	1
Q	1	0	0	1
Total	17	14	22	53
Frequency	0.32	0.26	0.42	1

<sup>a</sup> The DNA alignment file of the 53 sequences is available on request from NVS.

sequence in the three sites, but a certain degree of population differentiation was observed (Table 3). For example, haplotypes M, N, O, and Q belong in a different phylogenetic assemblage than the other observed haplotypes, and they contribute to the differentiation and high nucleotide diversity found in the NIE copepod population. This phylogenetic assemblage, however, is not completely unique to the NIE, because haplotype O was also sequenced from a single copepod collected from the DLC. Overall genetic diversity of the NIE and SYC populations was similar ( $0.7647 \pm 0.0943$  and  $0.7473 \pm 0.1114$ , respectively), and both were significantly more diverse than the DLC copepods ( $0.5455 \pm 0.1276$ ). The presence of 15 individuals (68%) of haplotype A in the DLC sample may suggest reduced genetic diversity in the DLC population. Pairwise  $F_{ST}$  tests, however, showed that the SYC and DLC populations were significantly different from the NIE *M. littorale* population but not significantly different from each other.

## DISCUSSION

The widespread, abundant occurrence of the copepod *M. littorale* in pristine to heavily contaminated sediments throughout U.S. Atlantic coast and Gulf of Mexico estuaries raises the question of whether they have evolved resistance to toxic conditions. In this study, we measured the sensitivities of three geographically separated populations of copepods to sediment contaminant mixtures through chronic reproduction bioassays. Genetic analysis showed significant genetic differentiation between the NIE population and the two populations with contaminant histories (i.e., the DLC and SYC). The population genetic similarities in *M. littorale* from the DLC and SYC might suggest a priori a similar toxicologic response of the SYC and DLC copepods to contaminated sediments that would be different from that of the NIE *M. littorale*. In these partial life-cycle bioassays, however, all three populations exhibited survival and reproductive outputs that largely were not significantly different from one another, irrespective of their previous histories of contaminant exposure or their patterns of population genetic differentiation. Overall, no significant dif-

ferences were found among any population's toxicologic responses to contaminated or uncontaminated sediments that could be considered to be commensurate with a history of previous toxicant exposure, selection of tolerant genotypes, and preadaptation. This failure to measure any historically linked tolerance in copepods exposed to mixed contaminants may be attributed to the physiologic and genetic difficulties involved in building resistance to whole suites of toxic contaminant mixtures and/or to population gene flow that might dilute selection/fixation of adaptive genes.

Contrary to our initial predictions, survival and reproductive success did not correlate with a previous history of sediment toxicant exposure for the DLC and SYC populations. Copepods collected from the NIE with little to no previous contaminant exposure were not the most sensitive population (i.e., exhibitors of lowest survival or of lowest reproductive success) to sediment contamination. The only endpoint for which a previous history of contaminant exposure appeared to be of benefit was the capacity to produce large clutch sizes (Fig. 3a). In contaminated treatment sediments, copepods from contaminated sites (SYC and DLC) produced almost twice as many eggs per clutch as the NIE copepods. Larger clutch sizes may be a pollution-induced trait to enhance the number of offspring potentially reaching sexual maturity under stressful conditions. In this single-generation bioassay, however, a larger clutch size did not ultimately benefit reproductive success (i.e., realized production) of the DLC and SYC populations relative to the NIE control. The previous contaminant burdens in the  $P_1$  of DLC and SYC may have led to nonviable eggs [18], prolonged embryonic development [10], poor hatching success, and poor larval quality/survival. The NIE *M. littorale* clutch sizes in control sediments were similar to the clutch sizes of field-collected NIE copepods (i.e.,  $15.02 \pm 0.86$  eggs) [22]; unfortunately, to our knowledge no field data are available from the other two population field sites for comparison. Any of these factors would counter the simple numeric benefits of the  $P_1$  producing larger clutch sizes.

Alternatively, whereas evidence of contaminant tolerance among the three *M. littorale* populations was not detected in the  $P_1$  and  $F_1$  generations, resistance may have been detected if the bioassays had been allowed to proceed to the  $F_2$  generation or beyond. Population-level resistance was acquired in three to five generations in two other studies that cultured harpacticoid copepods in the presence of single toxicants [10,28], but anthropogenically impacted sediments rarely are polluted with single chemicals. Rather, as a rule, a spatially and temporally dynamic mixture of contaminants persists for long periods at sublethal levels in muddy sediments of polluted estuarine settings. Physiologic and heritable resistance to mixed-contaminants may be difficult to achieve in dynamic habitats because of the immense number of individual compounds that could, either individually or synergistically, act on an organism's defense and detoxification systems. Development of resistance to one compound may facilitate/prohibit development of resistance to another. In this study, an exposure history-linked response might have been seen if the copepods had been chronically pre-exposed to a single contaminant or contaminant class (e.g., divalent metals only, PAHs only). Such scenarios have been demonstrated in several cases for oligochaetes [4], blue mussels [40], chironomid midges [13], freshwater snails [41], and mosquitofish [7]. Results of these investigations generally have shown that species inhabiting environments contaminated predominantly by one toxicant ex-

hibit enhanced resistance when tested in single-contaminant bioassays of that toxicant or toxicant class.

The presence of more diverse genotypes in a population increases the probability that certain genotypes will have higher fitness in a stressful environment and that certain stress-linked genotypes will be detectable in the environment [42]. Generally, however, this is true only for nuclear genes. Reduced mtDNA diversity also has been attributed indirectly to the presence of contaminants through stochastic genetic processes such as genetic drift [5,6]. Contaminants can reduce the effective population size (i.e., the breeding portion of the population) to such a degree that rare haplotypes are lost through chance events (i.e., genetic drift). Sediment contaminant levels were high for both the SYC and DLC populations, but significant differences in mtDNA haplotype frequencies were recorded only in the DLC population (relative to both the SYC and NIE populations). The estimated immense population size of *M. littorale* in each sampling site ( $\gg 10^6$ ; B.C. Coull, unpublished data) may render genetic drift a negligible evolutionary force relative to gene flow in these organisms. Furthermore, a pattern of reduced genetic diversity with increased pollution loading (sensu [6]) is not consistent with the pollution histories of our study sites, because the pristine site (NIE) and the heavily metals/PAH-contaminated site (SYC) showed similar levels of genetic diversity in *M. littorale*.

Surprisingly, copepods collected from all sites shared identical cytochrome *b* nucleotide sequences, thus providing evidence of relatively high levels of current or recent gene flow among our test populations (i.e., site to site). Even though *M. littorale* has benthic larvae, these larvae can move demersally into the water column and disperse locally with current flow [43]. If advantageous genotypes cannot become established in local environments because of high dispersal and subsequent gene flow (i.e., genetic dilution), then the development of genetic adaptation to local conditions (and its subsequent detection) may be limited [7]. The poor evidence of differential adaptive capabilities by the three populations to sediment contaminant mixtures in the bioassays could be attributed to gene flow among the study sites.

The SYC and DLC copepods were not genetically differentiated, but both populations were significantly different from the NIE copepods. The genetic relationship between the SYC and DLC copepods versus the NIE copepods may be attributed to similarities/differences in contaminant qualities of the population field sites, but the observed genetic relationships also may be confounded by their geographic locations. The two polluted sites (SYC and DLC) are geographically closer to each other (8.2 km) than they are to the NIE (92 km). Alternatively, the observed patterns of genetic relatedness among populations could be a function of sampling size (i.e., number of individuals and of assayed genes). Ideally, genetic relatedness should have been evaluated with one or more additional molecular markers, such as an independently evolved locus of the nuclear genome.

In general, genetic relatedness estimated from the copepod cytochrome *b* survey support the bioassay-based conclusion that a chronic history of sediment contaminant exposure conferred little or no adaptive advantage to *M. littorale* under mixed-toxicant stress. When concordant results arise between toxicity tests and concurrent genetic surveys, strong supporting evidence is provided for population-level development of resistance [44] or lack of resistance [14]. Conversely, conflicting results between the two methods may indicate that factors

currently unaccounted for (e.g., general organism insensitivity to toxicants, incorrect choice of genetic markers, genetic drift, gene flow, sibling species) are responsible for the observed patterns.

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## REFERENCES

- Chapman PM, Munday DR, Morgan J, Frink R, Kocan RM, Landot ML, Dexter RM. 1983. Survey of biological effects of toxicants upon Puget Sound biota II: Tests of reproductive impairment. NOS-102-0M21. Technical Report. U.S. National Oceanic and Atmospheric Administration, Rockville, MD.
- Klerks PL, Weis JS. 1987. Genetic adaptation to heavy metals in aquatic organisms: A review. *Environ Pollut* 45:173-205.
- Coull BC, Chandler GT. 1992. Pollution and meiofauna: Field, laboratory, and mesocosm studies. *Oceanogr Mar Biol Annu Rev* 30:191-271.
- Klerks PL, Levinton JS. 1989. Rapid evolution of metal resistance in a benthic oligochaete inhabiting a metal polluted site. *Biol Bull* 176:135-141.
- Murdoch MH, Hebert PDN. 1994. Mitochondrial DNA diversity of brown bullhead from contaminated and relatively pristine sites in the Great Lakes. *Environ Toxicol Chem* 13:1281-1289.
- Street GT, Montagna PA. 1996. Loss of genetic diversity in Harpacticoida near offshore platforms. *Mar Biol* 126:271-282.
- Klerks PL, Lentz SA. 1998. Resistance to lead and zinc in the western mosquito fish *Gambusia affinis* inhabiting contaminated Bayou Trepagnier. *Ecotoxicology* 7:11-17.
- Wentzel R, McIntosh A, Atchison G. 1978. Evidence of resistance to metals in larvae of the midge *Chironomus tentans* in a metal contaminated lake. *Bull Environ Contam Toxicol* 20:451-455.
- Moraitou-Apostolopoulou M, Verriopoulos G, Palla P. 1979. Temperature and adaptation to pollution as factors influencing the acute toxicity of Cd to the planktonic copepod *Acartia clausi*. *Tethys* 9:97-101.
- Moraitou-Apostolopoulou M, Kiortsis M, Verriopoulos V, Planastioti S. 1983. Effects of copper sulphate on *Tisbe holothuriae* Humes (Copepoda) and development of tolerance to copper. *Hydrobiologia* 99:145-150.
- Luoma SN, Ho KT. 1993. Appropriate uses of marine and estuarine sediment bioassays. In Calow P, ed, *Handbook of Ecotoxicology*, Vol. 1. Blackwell, London, UK, pp 193-226.
- Posthuma L, Verweij RA, Widanarko B, Zonneveld C. 1993. Life-history patterns in metal-adapted *Collembola*. *Oikos* 67:235-249.
- Postma JF, Kyed M, Admiraal W. 1995. Site specific differentiation in metal tolerance in the midge *Chironomus riparius* (Diptera, Chironomidae). *Hydrobiologia* 315:159-165.
- Millward RN, Grant A. 1995. Assessing the impact of copper on nematode communities from a chronically metal-enriched estuary using pollution-induced community tolerance. *Mar Pollut Bull* 30:701-706.
- Klerks PL, Leberg PL, Lance RF, McMillin DJ, Means JC. 1997. Lack of development of pollutant resistance or genetic differentiation in darter gobies *Gobionellus boleosoma* inhabiting a produced water discharge site. *Mar Environ Res* 44:377-395.
- Austen MC, Somerfield PJ. 1997. A community level sediment bioassay applied to an estuarine heavy metal gradient. *Mar Environ Res* 43:315-328.
- Klerks PL, Levinton JS. 1993. Evolution of resistance and changes in community composition in metal-polluted environments: A case study on Foundry Cove. In Dallinger R, Rainbow PS, eds, *Ecotoxicology of Metals in Invertebrates*. CRC, Boca Raton, FL, USA, pp 223-241.
- Chandler GT. 1990. Effects of sediment bound residues of the pyrethroid insecticide fenvalerate on survival and reproduction of meiobenthic copepods. *Mar Environ Res* 29:65-76.
- DiPinto LM, Coull BC, Chandler GT. 1993. Lethal and sublethal effects of the sediment-associated PCB Aroclor 1254 on a meiobenthic copepod. *Environ Toxicol Chem* 12:1909-1918.

20. Wirth E, Chandler GT, DiPinto LM, Bidleman T. 1994. Assay of polychlorinated biphenyl bioaccumulation from sediments by marine benthic copepods using a novel microextraction technique. *Environ Sci Technol* 28:1609–1614.
21. DiPinto LM, Coull BC. 1997. Trophic transfer of sediment associated polychlorinated biphenyls from meiobenthos to bottom feeding fish. *Environ Toxicol Chem* 16:2568–2575.
22. Palmer MA, Coull BC. 1980. The prediction of development rate and the effect of temperature for the meiobenthic copepod *Microarthridion littorale* (Poppe). *J Exp Mar Biol Ecol* 48:73–83.
23. Coull BC, Dudley B. 1985. Dynamics of meiobenthic copepod populations: A long-term study 1973–1983. *Mar Ecol* 24:219–229.
24. Feller RJ, Coull BC, Hentschel BT. 1990. Meiobenthic copepods: Tracers of where juvenile *Leiostomus xanthurus* (Pisces) feed? *Can J Fish Aquat Sci* 47:1913–1919.
25. McCall JN. 1992. Source of harpacticoid copepods in the diet of juvenile starry flounder. *Mar Ecol Prog Ser* 81:41–50.
26. Palmer MA. 1980. Variation in life history patterns between intertidal and subtidal populations of the meiobenthic copepod *Microarthridion littorale*. *Mar Biol* 60:159–165.
27. Schizas NV, Street GT, Coull BC, Chandler GT, Quattro JM. 2000. Molecular population structure of the marine harpacticoid copepod *Microarthridion littorale* along the southeastern and Gulf coasts of the United States. *Mar Biol* (in press).
28. Street GT, Lotufo GR, Montagna PA, Fleeger JW. 1998. Reduced genetic diversity in ameobenthic copepod exposed to a xenobiotic. *J Exp Mar Biol Ecol* 222:93–111.
29. Sanger DM. 1999. Physical, chemical, and biological environmental quality of tidal creeks and salt marshes in South Carolina estuaries. PhD thesis. University of South Carolina, Columbia, SC, USA.
30. Long ER, MacDonald DD, Smith SL, Calder FD. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine estuarine sediments. *Environ Manage* 19:81–97.
31. Krahn MM, Moore LK, Bogar RG, Wigren CA, Chan SL, Brown DW. 1988. High performance liquid chromatographic method for isolating organic contaminants from tissue and sediment extracts. *J Chromatogr* 437:161–175.
32. Chandler GT, Green AS. 1996. A 14 day harpacticoid copepod reproduction bioassay for laboratory and field contaminated muddy sediments. In Ostrander GK, ed, *Techniques in Aquatic Toxicology*. Lewis, Boca Raton, FL, USA, pp 23–39.
33. Allen HE, Fu G, Boothman W, Di Toro DM, Mahoney JD. 1991. Analytical method for determination of acid volatile sulfide and selected simultaneously extractable metals in sediment. U.S. EPA Office of Water 821/12–91. U.S. Office of Science and Technology, Washington, DC.
34. Schizas NV, Street GT, Coull BC, Chandler GT, Quattro JM. 1997. An efficient DNA extraction method for small metazoans. *Mol Mar Biol Technol* 6:381–383.
35. Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annu Rev Ecol Syst* 18:489–522.
36. Schneider S, Kueffer JM, Roessli D, Excoffier L. 1996. *Arlequin: A Software Package for Population Genetics*. University of Geneva, Geneva, Switzerland.
37. Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, NY, USA.
38. Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139:457–462.
39. Carman KR, Todaro MA. 1996. Influence of polycyclic aromatic hydrocarbons on the meiobenthic copepod community of a Louisiana salt marsh. *J Exp Mar Biol Ecol* 198:37–54.
40. Hoare K, Beaumont AR, Davenport J. 1995. Variation among populations in the resistance of *Mytilus edulis* embryos to copper: Adaptation to pollution? *Mar Ecol Prog Ser* 120:155–161.
41. Lam PKS. 1996. Interpopulation differences in acute response of *Brotia hainanensis* (Gastropoda, Prosobranchia) to cadmium: Genetic or environmental variance? *Environ Pollut* 94:1–7.
42. Nevo E, Noy R, Lavie B, Beiles A, Muchtart S. 1986. Genetic diversity and resistance to marine pollution. *Biol J Linn Soc* 29:139–144.
43. Chandler GT, Fleeger JW. 1983. Meiofaunal colonization of azoic estuarine sediment in Louisiana: Mechanisms of dispersal. *J Exp Mar Biol Ecol* 69:175–188.
44. Nevo E, Ben-Shlomo R. 1984. Mercury selection of allozymes in marine organisms: Prediction and verification in nature. *Proc Natl Acad Sci USA* 81:1258–1259.