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Accumulation of nonessential trace elements (Ag, As, Cd, Cr, Hg and Pb) in Atlantic horseshoe crab (*Limulus polyphemus*) early life stages

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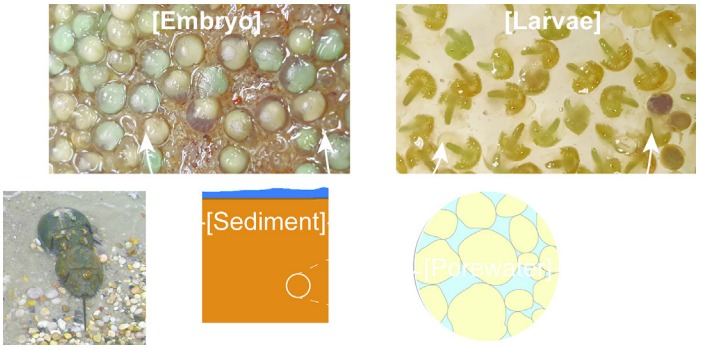
HIGHLIGHTS

- All nonessential trace elements were detected in the embryos and larvae
- Accumulation resulted from exposure to nonessential trace elements in sediment and pore water
- The concentration of Cr and Hg significantly increased from embryo to larvae at most sites
- Further laboratory studies are required to understand the bioavailability of trace elements in horseshoe crab early life stages

GRAPHICAL ABSTRACT



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ABSTRACT

During early development, benthic organisms can accumulate nonessential trace elements through aqueous and particulate sources. This study investigated the accumulation of Ag, As, Cd, Cr, Hg and Pb in Atlantic horseshoe crab (*Limulus polyphemus*) pre-spawned eggs, embryos, and developing larvae collected from 5 sites on Long Island, NY and compared these concentrations to that found in sediment, pore water, and overlying water. All investigated elements were detected in embryos and larvae at all sites. Arsenic was found at the highest concentration in each life stage across all 5 sites, followed by Ag, whereas Cd, Hg and Pb concentrations varied between sites. Chromium was not detected in pre-spawned eggs, but was present in embryos and larvae at all sites, however, along with Hg, significantly increased from embryo to larvae at most sites. We conclude that observed accumulation patterns are likely a result of abiotic factors, differences in uptake pathways between life stages and the rate of excretion. Future laboratory studies are required to understand the factors influencing the aqueous and dietary uptake of nonessential trace elements in the early life stages of Atlantic horseshoe crabs.

1. Introduction

The uptake of nonessential trace elements by marine organisms during embryonic development is of particular concern since early life stages are more susceptible to the impact of environmental pollutants

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than adults (Weis and Weis, 1991). In contrast to essential trace elements (e.g. Cu, Fe and Zn) which are required for embryonic development and are only toxic at high concentrations, nonessential trace elements (e.g. Hg and Pb) are toxic at low concentrations (Rainbow, 1985). For instance, exposure to nonessential metals, particularly Cd and Hg has been shown to result in abnormal embryogenesis, inhibited reproduction and molting, and increased mortality in aquatic organisms (Thain, 1985; Beiras and His, 1994; Guerin and Stickle, 1995; Moreno et al., 2003).

The early life stages (embryos and larvae) of aquatic organisms are exposed to nonessential trace elements in two ways; firstly, through maternal transfer (Kubota et al., 2002; Hopkins et al., 2006; Bakker et al., 2017), and secondly, through direct environmental exposure via particulate (ingested food and sediment) and aqueous pathways (Wang and Fisher, 1999; Williams et al., 2010). While we have established the relative importance of these two exposure pathways for essential elements in Atlantic horseshoe crab (*Limulus polyphemus*) early life stages (Bakker et al., 2016), this has yet to be determined for nonessential elements.

The Atlantic horseshoe crab is a marine benthic arthropod found along the eastern coast of the United States from Maine to Florida and throughout the Gulf of Mexico (Shuster et al., 2003). Its egg, embryos, and larvae are a vital food source for many migratory birds, arthropods and fish (Berkson and Shuster, 1999; Walls et al., 2002). Adult horseshoe crabs are an important resource as well; they are widely used as bait for eel and conch in commercial fisheries (Berkson and Shuster, 1999) and as a source of the blood protein, *Limulus* amebocyte lysate, (LAL) which is used for detecting the presence of pathogenic gram-negative bacteria in intravenous drugs, vaccines, and medical devices (Novitsky, 1991; Mikkelsen, 1988; Berkson and Shuster, 1999). Thus, exposure to nonessential trace elements in locations where horseshoe crabs spawn is of general concern.

Horseshoe crabs reproduce between April and August when females spawn on intertidal beaches during spring high tides. The eggs develop into embryos and hatch into first instar larvae in the sediment within three to four weeks post-fertilization (Botton, 2000). During these early life stages, developing horseshoe crabs are semi-immobile (i.e. unable to shift locality within beach sediment without tidal forces and sediment displacement) and are therefore exposed to nonessential trace elements in the surrounding environment (sediment and water), which at sublethal concentrations could result in reduced hatching success or cause developmental abnormalities. In a series of laboratory experiments, deleterious effects were observed when horseshoe crab embryo and larvae were subjected to various concentrations of nonessential trace elements (Itow et al., 1998a, 1998b; Botton, 2000); for example, during an aqueous exposure to Hg and Cd at 3.2 mg/l and 39.5 mg/l, respectively, embryos showed developmental abnormalities including abnormal eyes and defective limbs, as well as increased mortality (Itow et al., 1998a). Horseshoe crab embryos and larvae may uptake these elements through membrane absorption (chorion or gill, respectively) in pore water (water found within the sediment), sediment (from indirect biogeochemical cycling), or, to some degree, overlying water (water that covers the sediment surface). Larvae may also uptake elements through the diet, however this is dependent on life stage, as first instar larvae do not ingest particulates (Botton et al., 2010), but second instar larvae may assimilate nutrients from phytoplankton and potentially fine sediments (Gaines et al., 2002).

The accumulation of nonessential trace elements during early life stages is, to an extent, dependent on the concentrations that these developing embryos begin with at spawning (i.e. maternal transfer). We previously showed that for the six nonessential elements assessed (Ag, As, Cd, Cr, Hg and Pb) in horseshoe crabs in the present study, all but Cr was maternally transferred to the eggs (Bakker et al., 2017). Therefore, any assessment of nonessential trace element accumulation based on environmental concentrations in sediment and water should take element specific maternal transfer rates into account.

The objective of the present study was to investigate the concentration, in addition to what is already present in pre-spawned eggs, of six nonessential trace elements (Ag, As, Cd, Cr, Hg and Pb) in Atlantic horseshoe crab embryos and larvae at five beach beaches on Long Island, New York. We predicted that there would be an uptake of nonessential trace elements in embryo and larval stages. The concentration of each investigated element in early life stages was compared to the concentration in surrounding sediment, pore water and overlying water to determine the potential for accumulation from the surrounding environment. We did not directly compare concentrations between sites as differential fluctuations in local abiotic factors (e.g. temperature, salinity, and pH), and the differential fluctuations these factors experience at each site. However, we did use a bioconcentration factor and biota-sediment accumulation factor to help establish differences in accumulation between elements and sites. In the current study, the concentration of nonessential elements in eggs is taken from a previous study investigating maternal transfer (Bakker et al., 2017) and the concentrations reported here within developing embryonic and larval stages are thus due to the influence from the local environment. While prior studies have investigated the accumulation of nonessential trace elements in horseshoe crab embryos and larvae using laboratory based experiments (Itow et al., 1998a, 1998b; Botton, 2000), to our knowledge this is the first field based study to investigate the impact of natural environmental exposure on nonessential trace element uptake during Atlantic horseshoe crab early life stages while accounting for maternal transfer.

The investigated trace elements are found at elevated concentration in coastal areas with a heavy anthropogenic presence and are often found close to locations where horseshoe crabs are known to spawn. Furthermore, they are all listed on the United States Environmental Protection Agency Priority (U.S. EPA) Pollutant List under the Clean Water Act.

2. Methods

2.1. Study sites and horseshoe crab collection

Horseshoe crab eggs, embryos and larvae were collected from five beaches on Long Island, New York during evening/nighttime high tides in June and July 2015 (Fig. 1). All required federal and states permits were obtained prior to collection. Two locations were on the north shore of Long Island [Beekman Beach, Oyster Bay (40°52′34.3″ N, 73°32′26.3″ W) and a private beach in Manhasset Bay (40°50′01.9″ N, 73°43′37.2″ W)] and three locations were on the south shore [Jamaica Bay, Brooklyn (40°34′53.3″ N, 73°54′52.0″ W); West Jones Beach Coast Guard Station, Wantagh (40°35′24.5″ N, 73°32′57.2″ W); and Pikes Beach, Westhampton Bay (40°46′55.0″ N, 72°42′19.3″ W)]. The north shore sites are located on the Long Island Sound, an estuary bordering New York and Connecticut on all sides except the eastern end where it connects to the Atlantic Ocean. In contrast, the south shore sites border the Atlantic Ocean. Sites were chosen because they are high spawning density locations for horseshoe crabs on Long Island; however, this allows for a unique opportunity to investigate beach habitats close to large human population centers and industrial activities (i.e. including coal-fired power plants, industrial activities, urban and agricultural runoff, boating activities and wastewater treatment plants) where horseshoe crabs spawn, potentially exposing early life stages to elevated levels of nonessential trace elements due to anthropogenic activities.

In the present study, eggs were extracted from the carapace and were therefore unfertilized. Thus, the concentration of nonessential trace elements in eggs are due to maternal transfer only (Bakker et al., 2017). The accumulation or excretion of trace elements during the embryonic and larval stages can be determined by comparing the concentration at these later stages to the concentration of trace elements in the eggs. An increase in element concentration between successive stages would then be the result of environmental exposure (ingestion of

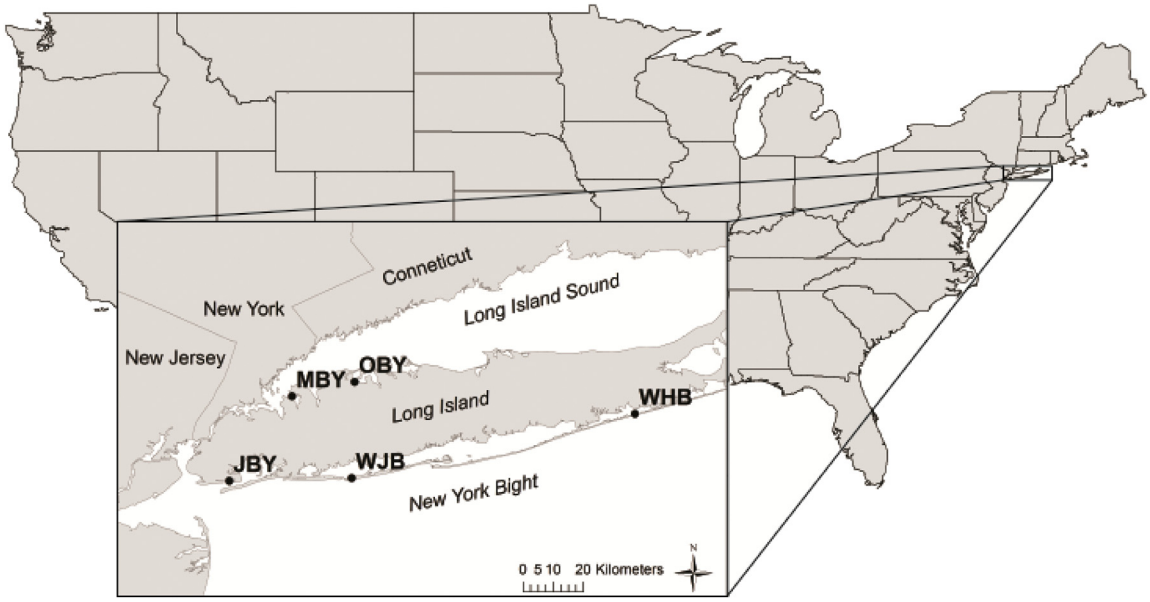

Fig. 1. Map of Long Island, NY showing the 5 Atlantic horseshoe crab (*Limulus polyphemus*) collection sites. Site abbreviations are as follows: JBY - Jamaica Bay; WJB - West Jones Beach; MBY - Manhasset Bay; OBY - Oyster Bay; and WHB - Westhampton Bay.

particulates and aqueous exposure), whereas a decrease in concentration between stages would indicate the element is either not readily accumulated through environmental exposure, is rapidly turned over and excreted, or is a result of growth dilution.

With the exception of West Jones Beach, all of the egg concentration values were from a previous study (Bakker et al., 2016). For West Jones Beach, eggs were collected using the method described in Bakker et al. (2016). Briefly, eggs were collected from females on spawning nights using a "drill-siphon" technique. A hole was drilled into the cavity using a 5/8th bit, eggs were siphoned from the cavity using a 60 cm^3 syringe attached to a 4.76 mm (3/16 in. standard) vinyl tube and placed in a 50 ml tube, and the hole was plugged using a nontoxic adhesive (ZAP®) and a disinfected rubber patch. The eggs were then transported on ice to Hofstra University for processing.

Embryos and larvae were collected directly from the sediment. Samples were collected from the south shore sites using random plots at the high tide line that were spread between 1 and 3 m apart. Clutches were less abundant at the north shore sites so plots were approximately 0.5 m apart. Embryo and larval stages were separated from sediment using plastic utensils and sieves, as described in Botton et al. (2006), transported on ice in 50 ml tubes to Hofstra University, rinsed in deionized water to remove any attached sediment, and separated by stage (embryos and larvae) using a dissection scope. Only late stage embryos (stage 20–1) were used in this study and were identified based on the loss of the outer chorion egg shell layer (Botton et al., 2010). Due to their small size, samples were pooled together by the clutch in which they were found in order to achieve an adequate sample weight for analysis. Samples were then dried at 60 °C for 48 h, homogenized into a fine powder using a mortar and pestle, and sent to the Trace Element Analysis Core Laboratory at Dartmouth College (Hanover, NH) for microwave digestion and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis. To allow for comparison between wet weight and dry weight, the water content for each life stage was calculated as 73% for eggs, 91% for embryos, and 87% for larvae.

2.2. Sediment and water collection

All sediment and water sample collections follow the methods as reported in Bakker et al. (2016). Sediment samples ($N = 3$) were collected at random at each site close to where embryos and larvae were found. Horseshoe crab embryos and larvae typically develop in sediment between 15 and 25 cm deep (Loveland et al., 1996), therefore sediment was collected from the next 5 to 20 cm depth using a plastic shovel and placed into Ziploc® bags. The first 5 cm surface layer of sediment was removed from the analysis because this layer is not in contact with the developing stages. Samples were dried at 60 °C for 48 h and passed through a 2 mm sieve (Wentworth scale; Wentworth, 1922); only sediment <2 mm in diameter was analyzed using ICP-MS analysis.

Given that >97% of the beach sediment was composed of medium to coarse sand, granules and pebbles (Wentworth scale; Wentworth, 1922) at all sites, pore water samples were collected as seep water through excavated troughs at depths between 15 and 20 cm, once tidal water had seeped through the sediment. Pore water and overlying water samples ($N = 3$) were collected in 50 ml tubes at the same time as the embryo collections. Water temperature, pH, and salinity were also recorded and are shown in Table 1. All water samples were passed through a 0.45 μm filter (Whatman 6970–2504) and preserved with 50 μl of trace metal grade nitric acid. Sediment and water samples were also sent to Dartmouth College for microwave digestion (sediment only) and ICP-MS analysis.

2.3. Trace element analysis

All horseshoe crab, sediment and water samples were collected, processed and analyzed using a trace metal clean technique to avoid contamination. To analyze the embryos and larvae samples, 0.25 g of sample was digested in 5 ml of acid (9:1 nitric acid to hydrochloric acid ratio) in a CEM MarsXpress microwave (Matthews, NC) for 55 min (ramp time: 20 min to 210 °C, hold time: 15 min at 210 °C, cool down: 20 min), after which the sample was diluted with 45 ml of deionized water for a total sample volume of approximately 50 ml (dilution factor ~ 200). To analyze the sediment samples, 0.25 g of sample was also digested in 5 ml of acid (9:1 nitric acid to hydrochloric acid ratio) using an open vessel microwave procedure (ramp time: 15 min to 90 °C, hold time: 45 min at 90 °C), after which the sample was diluted with 45 ml of deionized water. All of the horseshoe crab and sediment samples were further diluted 10-fold before trace element analysis using ICP-MS (Agilent 7700×). All of the horseshoe crab and sediment data are reported as μg/g dry weight.

Table 1
Nonessential trace element concentrations (mean ± 1 standard deviation) in sediment (μg/g dry weight), pore water (μg/l) and overlying water (μg/l). Temperature (°C), pH, and salinity (parts per thousand) values for water are also included. $N = 3$ for all elements and samples except for Cr in sediment at West Jones Beach ($N = 2$) and Cr and in overlying water at Jamaica Bay, West Jones Beach, and Oyster Bay [$N = 2$ for each site; due to BDL (below detection limit) values which were excluded from the mean and standard deviation calculations].

	Temperature (°C)	pH	Salinity (‰)	Ag	As	Cd	Cr	Hg	Pb
Jamaica Bay (JBY)									
Sediment				BDL	0.86 ± 0.05	BDL	2.41 ± 0.09	BDL	3.36 ± 0.68
Pore water	24.6	7.26	20.5	BDL	3.37 ± 0.22	0.05 ± 0.01	BDL	BDL	BDL
Overlying water	24.8	8.31	27.1	BDL	2.15 ± 0.29	0.02 ± 0.001	0.16 ± 0.16	BDL	BDL
West Jones Beach (WJB)									
Sediment				BDL	0.38 ± 0.07	BDL	1.20 ± 0.23	0.18 ± 0.04	2.42 ± 0.79
Pore water	25.4	7.27	35.3	0.24 ± 0.17	1.81 ± 0.11	0.07 ± 0.03	BDL	BDL	0.31 ± 0.06
Overlying water	23.7	8.02	34.8	BDL	1.97 ± 0.32	0.01 ± 0.01	BDL	BDL	BDL
Manhasset Bay (MBY)									
Sediment				BDL	0.78 ± 0.17	BDL	2.39 ± 0.61	0.31 ± 0.08	3.73 ± 0.67
Pore water	28.5	6.92	29.9	BDL	1.25 ± 0.12	0.15 ± 0.01	BDL	BDL	BDL
Overlying water	27.4	8.3	30.7	BDL	2.21 ± 0.17	0.04 ± 0.001	BDL	BDL	BDL
Oyster Bay (OBY)									
Sediment				BDL	0.47 ± 0.05	BDL	1.69 ± 0.36	BDL	2.13 ± 0.31
Pore water	27.7	6.93	16.3	BDL	1.20 ± 0.32	0.04 ± 0.01	BDL	BDL	BDL
Overlying water	28.5	7.41	9.9	BDL	1.12 ± 0.11	0.02 ± 0.01	0.32 ± 0.16	BDL	BDL
Westhampton Bay (WHB)									
Sediment				BDL	0.39 ± 0.21	BDL	BDL	BDL	0.24 ± 0.09
Pore water	25.9	7.09	34.2	BDL	7.48 ± 2.60	0.03 ± 0.01	BDL	BDL	BDL
Overlying water	24.2	8.42	33.6	BDL	5.89 ± 0.76	0.01 ± 0.002	BDL	BDL	BDL

2.4. Quality Assurance/Quality Control (QA/QC)

Blanks, certified or standard reference material (DORM-4 certified fish protein, National Research Council Canada; SRM 2711 Montana II soil, National Institute of Standards and Technology (NIST); SRM 1640a trace elements in natural waters, NIST), duplicate samples and spiked samples were used for quality control ($N = 5$ for embryos and larvae; $N = 3$ for sediment; $N = 2$ for water). Quality control procedure followed the USEPA protocol SW 846 of 1 set of QA/QC per 20 samples. The blanks were BDL (below detection limit) for all elements and sample type. For the horseshoe crab embryos and larvae samples, the recovery of DORM-4 ranged between 86 and 115%, spiked samples ranged between 84 and 115%, and the percentage difference between analysis duplicates averaged between 1.9 and 29% (1.9 to 4.7% for Ag, As, Pb and Se; 20.9 and 28.9% for Hg and Cd, respectively) for all elements. For sediment samples, the recovery of SRM 2711 ranged between 88 and 102%, spiked samples ranged between 85 and 115%, and the percentage difference between analysis duplicates averaged between 3.3 and 27% for all elements. For pore water and overlying water samples, the recovery of SRM 1640a ranged between 90 and 108%, spiked samples ranged between 85 and 125%, and the percentage difference between analysis duplicates averaged between 1.2 and 30% for all elements.

2.5. Statistical analysis

As stated previously, accumulation differences between individual sites were not assessed statistically because abiotic factors as well as pollution sources vary between study areas and this was outside the scope of the present study. In addition, our between-group factor was life-stage. Neither raw nor transformed egg, embryo, and larvae trace element data satisfied parametric assumptions, thus non-parametric Kruskal Wallis tests were used. If an overall significant effect was observed ($p < 0.05$), pairwise comparisons of eggs to embryos and embryos to larvae using a Dunn post hoc test with a Bonferroni correction for multiple comparisons was used. If the overall Kruskal Wallis was significant with no significant post hoc test ($p > 0.05$) then we concluded that the concentration progressively changed from egg to larvae. Because egg Cr concentrations for West Jones Beach ($N = 10$) were below the detection limit (BDL; <0.10 μg/g) for >50% of the samples they were removed from the analysis; in this case, embryo and larvae stage concentrations were compared using a Mann Whitney-*U* test. Following the methods of Hopkins et al. (2006), concentrations that were BDL for <50% of samples were replaced with a value equal to 50% of the detection limit. This was done for Cd in overlying water (Jamaica Bay, West Jones Beach, and Oyster Bay; BDL ≤ 0.01 μg/g), sediment for Cr (West Jones Beach; BDL ≤ 0.60 μg/g) and, as previously reported (Bakker et al., 2017), for egg samples for Cd ($N = 4$), Hg ($N = 5$), and Pb ($N = 4$) where the detection limits were 0.125 μg/g for Cd, 0.042 μg/g for Hg and 0.012 μg/g for Pb. Egg Cr concentrations in that study for the other four sites were BDL for 93% of samples ($N = 40$; <0.12 μg/g) and therefore removed from the analysis. All statistical analysis was performed using SPSS version 22.0 (IBM Armonk, NY, 2013).

Lastly, we calculated a ratio of trace element concentration in embryos and larvae to the concentration of trace elements in sediment and pore water, in order to help determine the relative uptake of each trace element from the surrounding environment. The bioconcentation factor (BCF) was calculated based on pore water concentrations for embryo and larvae while the biota-sediment accumulation factor (BSAF) was only calculated for larvae since they are the only stage to ingest particulates. The BCF was calculated as $BCF = C_B / C_W$, where C_B is the concentration found in the embryo or larvae and C_W is the concentration found in the pore water (Arnot and Gobas, 2006). The BSAF was calculated as $BSAF = C_B / C_S$, where C_B is the concentration in the larvae and C_S is the concentration found in the sediment.

3. Results

3.1. Environmental factors

The concentration of nonessential trace elements in the sediment is shown in Table 1. Lead was found in the highest concentration at each site (0.24–3.73 μg/g), followed by Cr [1.20–2.41 μg/g; although Cr was BDL (<0.60 μg/g) for Westhampton Bay] and then As (0.38–0.86 μg/g). Silver and Cd was not detected in the sediment at any site (BDL; Ag ≤ 0.04 μg/g, Cd ≤ 0.04 μg/g) and Hg was only detected in the sediment at two sites (BDL ≤ 0.10 μg/g). Pore water and overlying water concentrations are also shown in Table 1. Arsenic was found at the highest concentration in pore water across all sites, followed by Cd at all sites except

Table 2
Nonessential trace element concentrations (μg/g dry weight) in Atlantic horseshoe crab (*Limulus polyphemus*) eggs, embryos, and larvae. Values represent the mean ± 1 standard error and the range is provided in parentheses. All BDL (below detection limit) values were excluded from the mean and standard error calculations. $N = 10$ for all elements and life stages. Egg data for all sites, with the exception of West Jones Beach, is from a previous maternal transfer study (Bakker et al., 2017).

	Ag	As	Cd	Cr	Hg	Pb
Jamaica Bay (JBY)						
Egg	1.04 ± 0.11 (0.67–1.73)	7.85 ± 0.90 (4.03–12.29)	0.02 ± 0.004 (0.01–0.04)	BDL	0.08 ± 0.01 (0.05–0.13)	0.03 ± 0.01 (0.01–0.10)
Embryo	1.79 ± 0.87 (0.53–2.14)	8.80 ± 0.49 (7.24–12.42)	0.003 ± 0.001 (0.003–0.01)	0.05 ± 0.03 (0.01–0.10)	0.43 ± 0.32 (0.02–0.05)	0.19 ± 0.12 (0.10–0.34)
Larvae	0.98 ± 0.10 (0.60–1.68)	11.14 ± 0.63 (7.61–13.57)	0.006 ± 0.002 (0.002–0.003)	0.04 ± 0.01 (0.01–0.07)	0.03 ± 0.01 (0.07–0.85)	0.21 ± 0.08 (0.09–0.49)
West Jones Beach (WJB)						
Egg	1.16 ± 0.15 (0.61–1.94)	6.37 ± 0.76 (3.99–10.84)	0.01 ± 0.002 (0.01–0.03)	BDL	0.05 ± 0.01 (0.04–0.09)	0.05 ± 0.01 (0.01–0.14)
Embryo	1.63 ± 0.99 (0.65–1.77)	11.02 ± 0.80 (8.23–16.74)	0.007 ± 0.004 (0.004–0.10)	0.16 ± 0.12 (0.15–0.40)	0.04 ± 0.01 (0.01–0.07)	0.43 ± 0.07 (0.13–0.79)
Larvae	2.39 ± 0.85 (0.68–3.04)	13.09 ± 0.84 (8.83–17.11)	0.02 ± 0.03 (0.001–0.01)	0.28 ± 0.08 (0.05–0.45)	0.42 ± 0.02 (0.37–1.11)	0.50 ± 0.22 (0.35–0.58)
Manhasset Bay (MBY)						
Egg	1.34 ± 0.18 (0.76–2.49)	9.83 ± 0.85 (6.07–16.03)	0.04 ± 0.01 (0.01–0.13)	BDL	0.07 ± 0.01 (0.04–0.16)	0.06 ± 0.01 (0.02–0.12)
Embryo	1.42 ± 0.17 (0.79–2.15)	6.40 ± 0.65 (4.27–9.73)	0.05 ± 0.01 (0.03–0.10)	0.08 ± 0.01 (0.03–0.13)	0.02 ± 0.004 (0.004–0.04)	0.34 ± 0.05 (0.19–0.51)
Larvae	1.51 ± 0.12 (1.06–2.06)	5.92 ± 0.46 (4.49–9.24)	0.05 ± 0.02 (0.03–0.0)	0.28 ± 0.10 (0.13–1.14)	0.05 ± 0.01 (0.001–0.12)	0.59 ± 0.05 (0.41–0.92)
Oyster Bay (OBY)						
Egg	1.09 ± 0.06 (0.89–1.51)	10.49 ± 0.76 (6.77–15.08)	0.05 ± 0.02 (0.01–0.17)	BDL	0.06 ± 0.01 (0.04–0.09)	0.07 ± 0.02 (0.02–0.22)
Embryo	0.83 ± 0.06 (0.62–1.07)	7.93 ± 0.94 (5.56–12.14)	0.03 ± 0.004 (0.02–0.04)	0.05 ± 0.03 (0.01–0.09)	0.02 ± 0.003 (0.004–0.03)	0.23 ± 0.04 (0.13–0.40)
Larvae	0.91 ± 0.08 (0.75–1.32)	6.47 ± 0.31 (5.17–7.42)	0.01 ± 0.003 (0.004–0.01)	0.19 ± 0.02 (0.13–0.30)	0.02 ± 0.003 (0.01–0.03)	0.32 ± 0.03 (0.23–0.41)
Westhampton Bay (WHB)						
Egg	1.44 ± 0.34 (0.71–3.68)	15.44 ± 1.32 (7.02–22.24)	0.03 ± 0.01 (0.01–0.07)	BDL	0.07 ± 0.01 (0.04–0.11)	0.02 ± 0.01 (0.01–0.07)
Embryo	0.84 ± 0.10 (0.48–1.49)	12.98 ± 1.10 (8.34–21.28)	0.004 ± 0.001 (0.001–0.01)	0.02 ± 0.01 0.004–0.06	0.02 ± 0.002 (0.01–0.04)	0.05 ± 0.01 (0.02–0.14)
Larvae	1.07 ± 0.12 (0.57–1.73)	13.64 ± 1.11 (9.25–20.17)	0.004 ± 0.001 (0.001–0.01)	0.06 ± 0.01 0.03–0.12	0.15 ± 0.01 (0.09–0.19)	0.07 ± 0.01 (0.04–0.10)

for West Jones Beach. Silver and Pb were only detected in the pore water at West Jones Beach (BDL; Ag ≤ 0.02 μg/g, Pb = 0.18 μg/g) and Cr and Hg were not detected in the pore water at any site (Cr ≤ 0.29 μg/g, Hg = 0.49 μg/g). Arsenic was also found at the highest concentration in the overlying water followed by Cr when it was detected (Jamaica Bay and West Jones Beach) and then Cd. Silver, Hg, and Pb were not detected in the overlying water at any site.

3.2. Life stage differences

The average concentrations of nonessential trace elements in Atlantic horseshoe crab eggs, embryos and larvae are shown in Table 2. For each early life stage, As was found at the highest concentration, followed by Ag at all sites; in comparison, Cd, Hg and Pb were found at varying concentrations between sites. Chromium concentrations between embryos and larvae were different for four of the five sites (West Jones Beach: Mann Whitney U = 83.00, $p = 0.011$; Manhasset Bay: 79.00, $p = 0.001$; Oyster Bay: U = 49.00, $p = 0.001$; and Westhampton Bay: U = 93.00, $p = 0.001$; see Table 2 for life stage means), while Jamaica Bay showed no difference between life stages (Jamaica Bay: U = 47.00, $p = 0.853$).

The concentration of each nonessential element in eggs (data from Bakker et al., 2017), embryos, and larvae allows for a visual comparison of each element due to maternal transfer and between early developmental life stages (Fig. 2). Silver showed no statistically significant difference across stages for four of the five sites (Fig. 2, Table 3); however, a statistically significant decrease in concentration was observed between egg and embryo for Oyster Bay. For 2 of the sites (West Jones Beach and Manhasset Bay), the Ag concentration increased with successive life stage (Fig. 2, Table 2). For one site (West Jones Beach), there was a statistically significant increase from egg to embryo for As, while there was a statistically significant decrease for another site (Manhasset Bay). Other sites showed an increase (Jamaica Bay) or decrease (Oyster Bay) for As across all successive stages (Fig. 2, Table 2). Cadmium showed a statistically significant decrease in concentration between successive stages for four of the five sites (Table 3); decrease occurred between eggs and embryos at Westhampton Bay, while a decreases occurred between embryos and larvae at West Jones Beach, Manhasset Bay, and Oyster Bay (Fig. 2). Chromium showed statistically significant increases from embryo to larvae for all sites except West Jones Beach (Fig. 2). Mercury showed a statistically significant decrease from eggs to embryos for all sites except West Jones Beach (Fig. 2, Table 3). However, Hg showed significant increases from embryos to larvae for three sites (Jamaica Bay, West Jones Beach, and Westhampton Bay). Lastly, Pb showed a statistically significant increase from egg to embryo for all sites except Westhampton Bay (Fig. 2).

3.3. BCF and BSAF

Arsenic had the highest BCF for both embryos and larvae stages at most sites except for Westhampton Bay where Ag had the highest BCF for both embryos and larvae (6.79 and 9.95 respectively; Table 4). Westhampton Bay was also the only site where Ag was detected in the pore water. Lead was only detected at West Jones Beach where the embryo and larvae concentrations appeared to be in a simple equilibrium with the pore water given the values were all slightly over 1 at

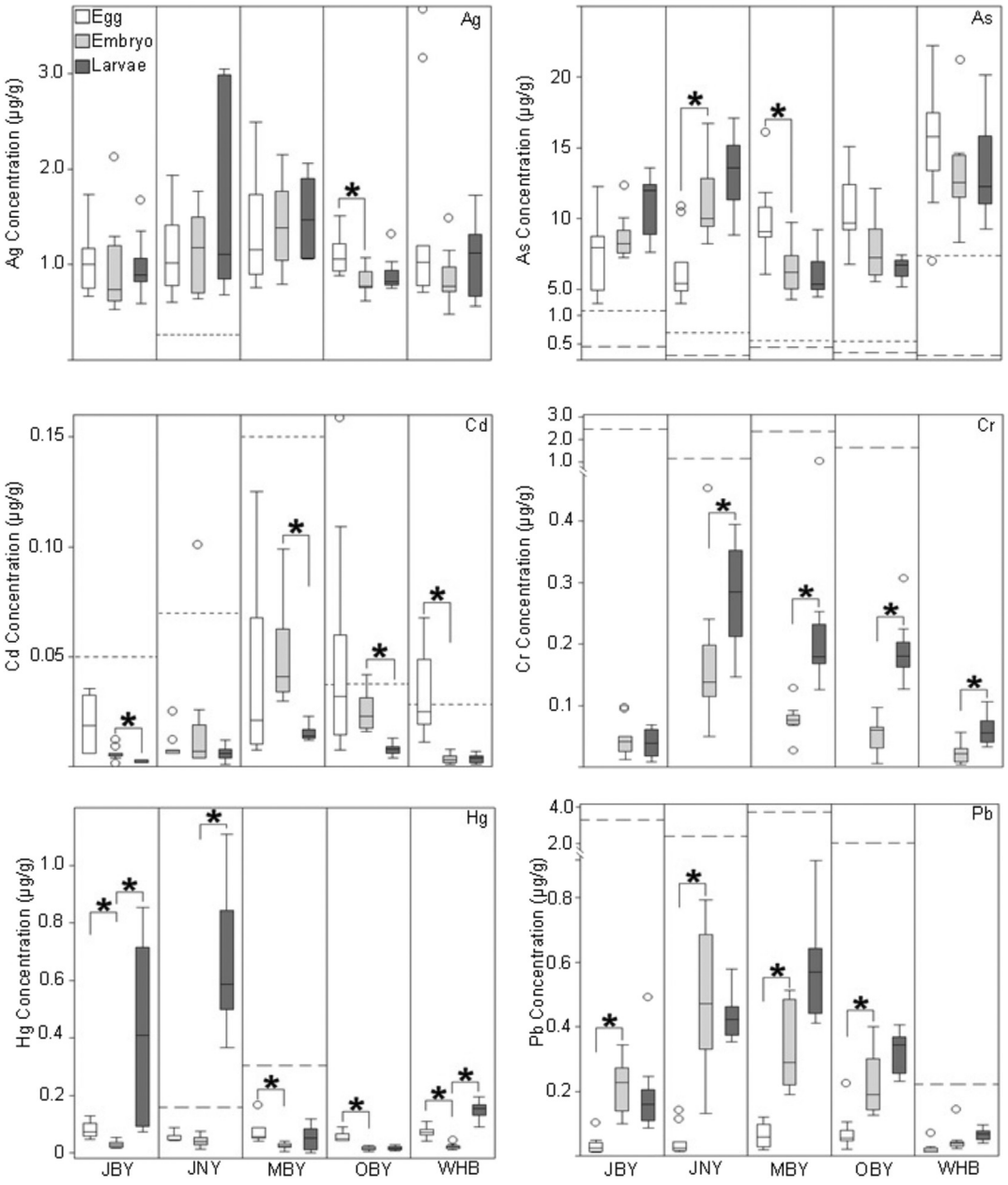

Fig. 2. Concentration (µg/g dry weight) of Ag, As, Cd, Cr, Hg, and Pb in Atlantic horseshoe crab (*Limulus polyphemus*) eggs (white), embryos (grey), and larvae (black). Box and whisker plot represents median (hash line within box), first and third quartiles. Whiskers indicate range between ±1.58 IQR $n^{-1/2}$ (IQR = interquartile range; n = number of points in the distribution). Circles indicate data outside this range (outliers). Significant comparisons between stages within a site for each element are indicated; * = $p < 0.05$. Large dashed lines represent average sediment concentrations and small dashed lines represent average pore water concentrations (pore water was converted from µg/l to µg/g for same unit comparison). Missing lines or boxes indicate BDL (below detection limit). Site abbreviations are as follows: JBY - Jamaica Bay; WJB - West Jones Beach; MBY - Manhasset Bay; OBY - Oyster Bay; and WHB - Westhampton Bay.

1.38 and 1.61 respectively. No BCF was calculated for Cr or Hg given that all pore water values were BDL.

Similar to the BCF trends, As had the highest BSAF at all sites, all with ratios > 1. Chromium and Pb both had fairly low BSAFs with ratios < 1 at all sites where they were detected in the sediment. Mercury was only present at two sites, one of which (West Jones Beach) had a ratio above 1 at 2.33, and the other (Manhasset Bay) had a ratio < 1 at 0.16. No BSAF was calculated for Ag or Cd as these were both BDL in sediment collected from all sites.

4. Discussion

Prior studies have investigated the concentration of nonessential trace elements in horseshoe crab eggs through field studies (Burger, 1997; Burger et al., 2002; Burger and Tsipoura, 2014) and exposure effects on developing embryos and larvae using laboratory-based experiments (Itow et al., 1998a, 1998b; Botton, 2000). However, to our knowledge no prior studies have assessed the accumulation of nonessential trace elements in early life stages in local habitats while addressing the "starting points" of these concentrations due to maternal transfer. Some elements, like Cr and Pb, showed an increases in concentration between egg and embryo across many of the sites indicating consistent uptake from the habitat (i.e. pore water). Others, like Cd, showed a decrease between stages (egg to embryo at one site and embryo to larvae at three sites), which could be a result of reduced uptake with increasing life stage, an increase in the rate of excretion, or growth dilution. Silver, As, and Hg showed a variable pattern of increase, decrease, or no change with life stage across sites. Overall, the

Table 3
Nonparametric statistical comparisons and correlations for eggs, embryos, and larvae. Kruskal Wallis statistic with *p* value in parentheses. Significant overall Kruskal Wallis tests were followed up with a Dunn post hoc comparisons of each stage combination. Significant *p* values indicated by *. (Chromium was BDL for eggs at all sites and therefore not included in table; see Materials and Methods for all statistical details).

	Ag	As	Cd	Hg	Pb
Jamaica Bay (JBY)					
Kruskal Wallis	1.10 (0.58)	8.19 (0.02)*	23.31 (<0.01)*	22.13 (<0.01)*	19.17 (<0.01)*
Egg versus Embryo		1.00	0.11	0.02*	<0.01*
Embryo versus Larvae		0.09	0.02*	< 0.01*	1.00
West Jones Beach (WJB)					
Kruskal Wallis	0.78 (0.68)	16.08 (<0.01)*	0.93 (0.63)	20.12 (<0.01)*	19.16 (<0.01)*
Egg versus Embryo		0.04*		1.00	<0.01*
Embryo versus Larvae		0.44		<0.01*	1.00
Manhasset Bay (MBY)					
Kruskal Wallis	1.30 (0.52)	12.08 (<0.01)*	10.56 (<0.01)*	9.79 (<0.01)*	21.82 (<0.01)*
Egg versus Embryo		0.024*	0.15	<0.01*	0.03*
Embryo versus Larvae		1.00	<0.01*	0.24	0.22
Oyster Bay (OBY)					
Kruskal Wallis	8.02 (0.02)*	10.31 (<0.01)*	12.06 (<0.01)*	16.92 (<0.01)*	16.14 (<0.01)*
Egg versus Embryo	0.03*	0.14	1.00	<0.01*	0.03*
Embryo versus Larvae	1.00	0.92	0.02*	1.00	0.82
Westhampton Bay (WHB)					
Kruskal Wallis	3.16 (0.21)	2.76 (0.25)	19.56 (<0.01)*	25.31 (<0.01)*	16.44 (<0.01)*
Egg versus Embryo			<0.01*	0.03*	0.10
Embryo versus Larvae			1.00	1.00	0.16

concentrations found in specific life stages could be related to the length of time each stage is exposed to these trace elements due to local habitat concentrations in the sediment and pore water. This also could indicate that there is not only variability in habitat factors influencing uptake or elimination of certain nonessential trace elements, but also stage differences, since embryos will be exposed through aqueous uptake only, whereas larvae will be exposed via dietary and aqueous exposure pathways.

Concentrations of As was both the highest in both sediment and pore water of all elements tested, as well as was the highest concentration in all horseshoe crab early life stages. Thus, it is not surprising that As also showed the highest BFC and BSAF for all sites and stages (except at West Jones Beach). Because the BFC and BSAF is a simple ratio, it essentially normalizes an element's accumulation based on environmental concentrations. Thus, despite the high levels of As in the environment, horseshoe crab embryos and larvae accumulate As more readily than the other nonessential elements. Arsenic concentration patterns showed a sequential increase from egg to larvae at Jamaica Bay and West Jones Beach but a decrease for Manhasset Bay and Oyster Bay, despite the four sites having comparable sediment, pore water, and overlying water values. Westhampton Bay was the only site that showed no change, which might be related to the high pore water concentration (the concentration here was seven times higher than at all other sites). The factors that influence bioavailability for As uptake are complex as there are multiple pathways by which As can be released into solution. For example, As can be released into pore water during redox potential changes from the dissolution of Fe and Mn oxides (Neff, 1997). Interestingly, when we compared the As data in this study to the Fe and Mn concentrations from the same sites in our previous study (Bakker et al., 2016), As had an indirect relationship with each element. In addition, these redox reactions for As may be influenced by changes in temperature; for example, As accumulated at a higher rate in the sea snail (*Littorina littoralis*) when the temperature was 26 °C, but only when salinity the was at 36‰ (Klumpp, 1980). In the current study, only Westhampton Bay had similar temperature (25.9 °C) and salinity (34.2‰) values and indeed there was a high concentration of As

Table 4
Bioconcentration Factor (BCF) and Biota-Sediment Accumulation Factor (BSAF) for embryos are larvae.

	BCF				BSAF			
	Ag	As	Cd	Pb	As	Cr	Hg	Pb
Jamaica Bay (JBY)								
Embryo		2.61	0.60					
Larvae		3.38	0.12		13.26	0.02		0.06
West Jones Beach (WJB)								
Embryo	6.79	6.09	0.10	1.38				
Larvae	9.95	7.23	0.28	1.61	34.44	0.13	2.33	0.21
Manhasset Bay (MBY)								
Embryo		5.12	0.33					
Larvae		4.74	0.33		7.59	0.12	0.16	0.16
Oyster Bay (OBY)								
Embryo		6.61	0.75					
Larvae		5.39	0.25		13.77	0.11		0.15
Westhampton Bay (WHB)								
Embryo		1.74	0.13					
Larvae		0.22	0.13		4.43			0.29

here; Therefore, both salinity and temperature could be contributing factors at this site. Lastly, studies using phytoplankton and fish have shown that arsenate [As(V)], the dominant As species in oxygenated marine waters (Neff, 1997), shares the same uptake pathway as phosphate (Sanders and Windom, 1980; Beene et al., 2011), an element thought to be required for exoskeleton formation in horseshoe crabs (Raabe et al., 2005), into cells. Arsenic was not speciated in this study, so the species of As that was maternally transferred (i.e., arsenate, arsenite [As(III)] or organoarsenic compounds) is not known, nor can we conclude what species of As accumulated in the embryos and larvae from the surrounding environment.

Despite the low BSAF for Cr relative to the other tested elements, it appears that this accumulation is significant. Specifically, Cr was the only element to be undetectable in pre-spawned eggs but was detected in embryos from all sites, with four of the five sites showing an additional significant increase from embryos to larvae. While the accumulation of Cr is clear, the pathway of accumulation is less apparent. Of the four sites where Cr was observed to increase between stages, only three of these sites had Cr detected in the sediment (West Jones Beach, Manhasset Bay, and Oyster Bay). Chromium was detected in Jamaica Bay sediment, but there was no significant increase in Cr from embryo to larvae (though embryos contained Cr while pre-spawned eggs from that site did not). Conversely, we did not detect Cr in the sediment at Westhampton Bay while the larvae there did show significant increases in Cr levels from embryos. Chromium was not detected in pore water at any site and was only detected in overlying water at two sites (Jamaica Bay and Oyster Bay). The cause for the difference in observed patterns is not apparent to us. In addition, Cr was not speciated into trichromate [Cr(III)] and hexavalent chromate [Cr(VI)], each of which has been shown to have different uptake routes in marine mussels, with Cr(VI) accumulating predominantly from the aqueous phase and Cr(III) through dietary exposure (Wang et al., 1997). Thus, the initial uptake might occur as Cr(VI), the dominant form of Cr in marine waters, through aqueous exposure for embryos and first instar larvae, and then as Cr(III) through the diet once larvae develop into the second instar stage.

Mercury showed consistent uptake in larval stages at each site while showing lower levels in embryonic stages compared to eggs. This pattern could play an important ecological role. For example, three sites (Jamaica Bay, West Jones Beach, and Westhampton Bay) showed a successive increase in Hg from embryo to larvae while four sites (Jamaica Bay, Manhasset Bay, Oyster Bay, and Westhampton Bay) showed a significant decrease from egg to embryo. Mercury is maternally transferred to horseshoe crab eggs (Bakker et al., 2017) so the reduction in body burden that occurs during embryo development could be important in reducing overall levels as larval stages accumulate more Hg. The accumulation pathways for Hg, however, are unclear. Sediment concentrations were only detected at two sites, West Jones Beach and Manhasset Bay, and of these two sites only West Jones Beach showed a significant increase from embryo to larvae. This is explained by the fourteen fold higher BSAF at West Jones Beach than Manhasset Bay. However, the vast majority of sediment across sites were BDL for Hg, which can be attributed to the majority of the sediment (>97%) being comprised of sand and granule sized particles. While no relationship could be determined between early horseshoe crab stages and habitat, uptake predominantly occurred the most in the larval stage. This could be due to low levels of Hg in the pore water and overlying water, as well as Hg speciation. Methylmercury (MeHg) is the most bioavailable species of Hg, but MeHg only accounts for 3% of the total Hg in seawater (Hammerschmidt and Fitzgerald, 2006). Although we did not speciate in this study, MeHg predominantly accumulates in marine organisms (Laporte et al., 1997; Hammerschmidt and Fitzgerald, 2006) and therefore low levels in the water may account for the little uptake that occurred in embryos. Alternatively, the increase in concentrations in larvae for select sites might be due to accumulation from particulates or contaminated food sources. In freshwater phytoplankton, Hg(II) has been found to attach to cell walls and MeHg to cross into cytoplasm (Pickhardt and Fisher, 2007), where it will be more bioavailable to zooplankton. Although there are no detailed studies on the diet of horseshoe crab larvae, one study suggests that second instar larvae assimilate nutrients from organisms whose own diet includes phytoplankton and macroalgal (Gaines et al., 2002).

Cadmium showed very low BCFs and no BSAF could be calculated due to sediment being BDL. Lead on the other hand had very low BSAF values, with BCF values around 1 at the one site where it was present in pore water. Both Cd and Pb have been documented to have similar uptake routes as Ca^{2+} in crustaceans (Torreblanca et al., 1987). Calcium is thought to be a required micronutrient for the calcification of the exoskeleton in all crustacea (Greenway, 1985); therefore, it is possible Cd and Pb were accumulated through this pathway. However, in the current study, different accumulation patterns were observed between the two elements. Cadmium significantly decreased from egg to larvae at four of the five sites (Jamaica Bay, Manhasset Bay, Oyster Bay, and Westhampton Bay). This decrease occurred from embryo to larvae for three of these sites while the decrease was from egg to embryo at the other site. Lead on the other hand significantly increased from egg to embryo for four sites (Jamaica Bay, West Jones Beach, Manhasset Bay, and Oyster Bay) with no change from embryo to larvae at any site. For Cd, a decrease in salinity causes an increase of free Cd^{2+} ions in the water thus making it available for uptake (Mantoura et al., 1978; Rainbow et al., 1993) as seen in a variety of crustacean taxa (O'Hara, 1973; Wright, 1977; Rainbow et al., 1993). Based on the lack of Cd accumulation from eggs to larvae for all of our sites and that Cd concentrations were similar in pore water between sites despite variable salinity parameters, we believe salinity in and of itself cannot explain these results. Rather, similar to Hg, Cd is known to chloro-complex in seawater at salinities ≥ 10 ppt. Thus the available Cd^{2+} could be bound to chloride, decreasing the bioavailability of Cd, as demonstrated in killifish (Dutton and Fisher, 2011).

Silver was the only element to have no clear accumulation pattern through sequential stages for most sites. Further complicating the story for Ag is its absence in the sediment across sites (and therefore no BSAF for comparison), and only being present in pore water at one site, West Jones Beach (though here it showed the highest BCF of any element). Other studies using American oysters (*Crassostrea virginica*) and grass shrimp (*Palaemonetes pugio*) have observed that Ag is readily accumulated via the dissolved phase, but when ingested via food or particulates, it is rapidly excreted (Abbe and Sanders, 1990; Connell et al., 1991). While these studies may present a model for Ag uptake, in the current study four of the sites did not detect Ag in the habitat which likely accounts for the lack of increase between life stages. Silver was only detected in the pore water at West Jones Beach and the larvae at this site had the greatest variability in Ag concentration. However, further studies on the uptake route of Ag are needed in order to understand the potential significance of this result.

While accumulation did occur in embryos and larvae for most elements, it is important to take into consideration the organism's life stage and how sensitive they are to a given element. During horseshoe crab embryo development, nonessential elements that associate with an embryo but are not incorporated into developing embryonic tissue may either attach to the outer layer of the chorion or pass across the chorion and accumulate in the perivitelline fluid. In either case, excretion of these elements would occur upon hatching since both components are lost at that time. Patterns of significant loss seen after hatching seem to fit this process for Cd and Hg, though it might be more common for essential elements including Co, Mn, and Ni (Bakker et al., 2016). Additionally, the partitioning of nonessential trace elements within larvae may be more complex as they may internalize or absorb elements into their carapace as has been shown for Cd and Pb in other crustacea taxa (Canli and Furness, 1993; Zanders and Rojas, 1996). One study has shown that trace elements can be absorbed into the carapace of horseshoe crabs (Kannan et al., 1995)

suggesting that these elements would be lost during ecdysis. This could explain some variability with the larvae in the present study as the amount of molting that occurred for each individual prior to the time of sampling is unknown. Such a mechanism would passively act as a form of protection, particularly in contaminated environments.

The present study indicates that nonessential elements bioaccumulate during early stages of development in horseshoe crabs. When concentrations from our current study are compared to the values of laboratory studies which showed a negative effect of Cd, Cr, Hg, Pb on early stage horseshoe crabs (Itow et al., 1998a, 1998b; Botton, 2000), the field values in this study are 100 to 1000 times lower. However, As and Ag were not investigated in prior studies and considering they were the two elements found at the highest concentration in each life stage, our study indicates there is a need for laboratory-based experiments to examine whether As and Ag pose a threat to the health of horseshoe crab early life stages. In addition, uptake routes may be element, speciation, and life stage specific; future laboratory-based studies are required to investigate the influence of abiotic factors including salinity, temperature and pH, as well as diet, on the accumulation of nonessential trace elements in early life stages of the Atlantic horseshoe crab.

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