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RESEARCH ARTICLE

Fine-scale chemical fingerprinting of an open coast crustacean for the assessment of population connectivity

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Abstract Chemical fingerprinting techniques recently have been used to track larval dispersal of estuarine species that bear calcified structures, but the applicability of this important approach may be limited on the open coast where chemical signatures may be less distinctive and for the many species that do not retain calcified structures throughout development. Externally brooded embryos of the porcelain crab, *Petrolisthes cinctipes*, and inductively coupled plasma mass spectrometry were used to determine whether fine-scale variation in trace-elemental composition occurred along an open coast. Embryos were collected from 16 sites from 37.8° to 39.5° north latitude along the Pacific Coast of California, USA during late January and early February 2003. Discriminant function analysis revealed that collection sites, many separated by only a few kilometers along an open coast, could be differentiated with an overall accuracy of 73%, and combining the sites into three regions increased the accuracy to 88%. Thus, distinctive elemental signatures can be detected in open coast species even at a fine scale raising the possibility that larval tags can be developed for many more species than previously thought possible.

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Introduction

Dispersal plays a central role in the dynamics and structure of marine communities, speciation, fisheries management and conservation but has remained unknown for most marine animals that typically develop as microscopic larvae in the plankton (Levin 1990). The utility of artificial tagging is severely constrained by the considerable potential for advection and mortality, but recent progress has been made in developing natural, environmentally induced tags for measuring larval dispersal of estuarine fishes and molluscs (DiBacco and Levin 2000; Thorrold et al. 2002; Gillanders 2005). Distinctive chemical signatures from natural and anthropogenic inputs into estuaries generate "chemical fingerprints" in calcified structures, such as otoliths, shells or statoliths (Levin et al. 1993; DiBacco and Levin 2000; Becker et al. 2005; Gillanders 2005). Other studies have established the existence of chemical fingerprints in calcified structures of open coast species (Swearer et al. 1999; Zacherl et al. 2003; Zacherl 2005; Warner et al. 2005). The advantage of these natural tags is that every larva collected has been imprinted with the chemical signature of the site of origin. However, most species do not reside in estuaries nor do most of them retain calcified structures throughout development, potentially limiting the applicability of this promising approach to measuring dispersal.

We took an initial step in exploring the utility of chemical fingerprinting for species that live on open coasts and lack permanent calcified structures by determining whether distinctive chemical signatures were evident in externally brooded embryos of the porcelain crab, *Petrolisthes cinctipes*, along wave-swept rocky shores of northern California, USA. Dense aggregations of these filter-feeding adults live in cobble and mussel beds. Females brood fertilized eggs beneath the abdomen until hatching whereupon larvae molt

through two larval stages before settling as post-larvae in adult habitats (Morris et al. 1980). Embryos are brooded for about 7 weeks (S. G. Morgan, unpublished data) and molt through two larval stages in 32 days (Gonor 1970) and settle after spending as long as 22 days as postlarvae (Jensen 1991).

We determined whether geographical variation in the concentrations of 16 readily detectable elements in embryos was evident at 16 stations along 255 km of open coastline. Our goal was to determine whether sites had distinctive elemental signatures that could be used to establish the natal origins of settling larvae and connectivity of populations.

Materials and methods

Ovigerous female *P. cinctipes* were collected from beneath rocks at 16 sites along the coast of northern California (Fig. 1) during late January and early February low tides in 2003. Females were frozen for later analysis. In August 2005, 50–100 embryos were removed from 10 females per site, rinsed in ultra-pure water (resistivity $\geq$18 M Ω cm, Millipore, Billerica, Massachusetts) and deposited in 50-ml polypropylene vials. Before use, vials were acid-washed by leaching in 1N hydrochloric acid (Trace Metal Grade, Fisher Scientific, Waltham, Massachusetts) at sub-boiling temperatures for several hours followed by three rinses in ultra-pure water. Forceps and other equipment coming in contact with embryos were acid-leached at room temperature and ultra-pure water rinsed in between samples. Embryos were dissolved in 1 ml of nitric acid (Trace Metal Grade, Fisher Scientific) and the solution was diluted with 40 ml of ultra-pure water. The relatively large volume of water was necessary to reduce the acidity for analysis. Rinses of the forceps were saved and processed as samples to become method blanks.

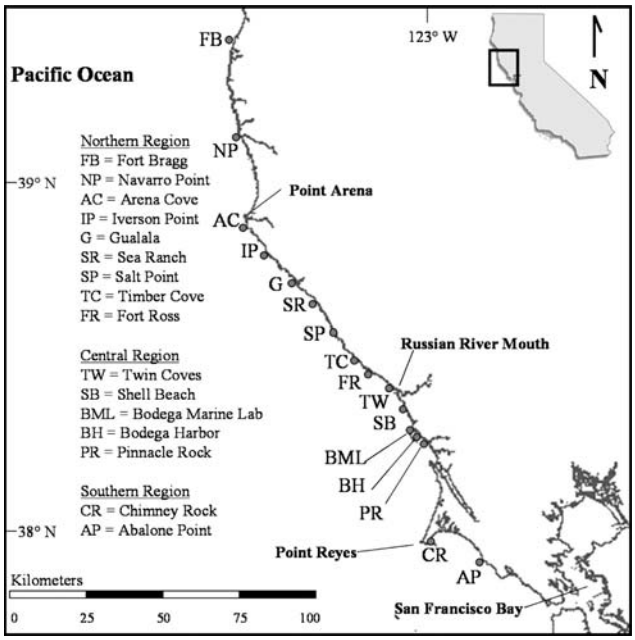

Fig. 1 Map of 16 sample locations along the coast of northern California, USA

The resulting 160 samples and blanks were analyzed using an Agilent (Palo Alto, California) 7500i inductively coupled plasma mass spectrometer (ICP-MS). The ICP-MS sampled at 0.4 ml min^{-1} by peristaltic pump through a Babbington-style nebulizer into a 2°C cooled double-pass spray chamber leading to a 1,300 W plasma. The number of doubly charged Cerium ions (Ce^{++}) from a tuning solution analyzed in the plasma was 1.5% that of singly-charged Cerium ions (Ce^{+}). To minimize polyatomic ions, the number of molecular oxides (CeO^{+}) was tuned to 0.3% of the total number of Cerium ions. External standards (0.01–100 ng ml^{-1}) were prepared from NIST-traceable stock multi-element standard 2A (Spex Certiprep, Methuen, New Jersey) plus six major elements Na, Mg, Al, K, Ca and Fe (also NIST-traceable, from Ultra Scientific, N. Kingstown, RI) from 200 to 100,000 ng ml^{-1} and verified against natural water standard references SLRS-4 and NASS-5 (NRC Canada, Ottawa, Ontario). Intersample rinses of the machine using dilute nitric acid in ultra-pure water were analyzed every 20 samples to quantify machine drift. Counts-per-second of 22 elements were detected for each sample. A linear fit to the standards for each element provided a counts-per-second to parts-per-billion conversion slope for use on the unknowns. Elements measured were Magnesium (Mg), Manganese (Mn), Copper (Cu), Calcium (Ca), Barium (Ba), Iron (Fe), Chromium (Cr), Vanadium (V), Potassium (K), Nickel (Ni), Rubidium (Rb), Cobalt (Co), Zinc (Zn), Arsenic (As), Strontium (Sr), Selenium (Se), Aluminium (Al), Cadmium (Cd), Lead (Pb), Cesium (Cs), Thallium (Tl), and Uranium (U). Instrument detection limits (three standard deviations, EPA 6020) ranged from $\sim$1 ng ml^{-1} for abundant elements, such as Mg, K, Ca and Fe, down to $\sim$0.01 ng ml^{-1} for Co, Rb, Sr and Ba (Fig. 2). Method detection limits for the 16 elements interpreted were lower than sample concentrations. Concentrations for each element were adjusted to a baseline zero point by subtracting the average background levels determined in all analyzed rinses throughout the sample run.

The goal of this research was to eventually compare the chemistry of embryos to that of the soft tissues of settling larvae. The larval exoskeleton should be excluded from analysis because elements from the surrounding water are incorporated just after molting thereby obscuring the natal signature when a larva molts repeatedly during development, as is the case for most crustacean larvae. Soft tissues of larvae must be dissolved from the exoskeleton making it

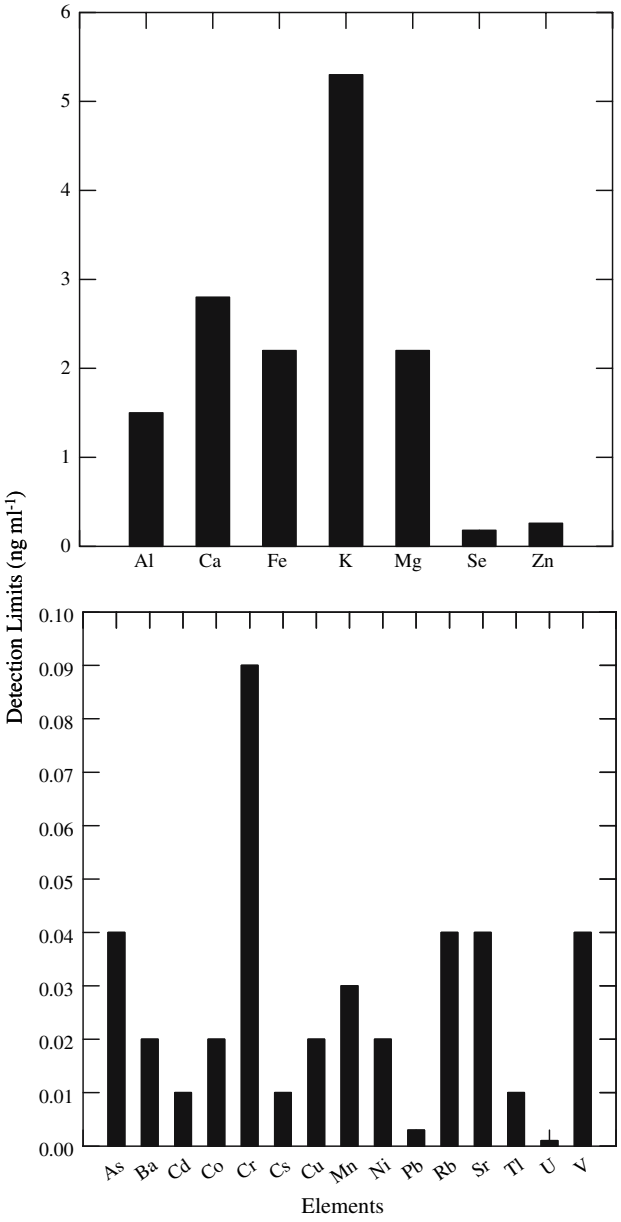

Fig. 2 Element-specific detection limits for the 22 elements analyzed using ICP-MS, expressed in ng ml^{-1}

difficult to dry and weigh them for standardizing their elemental concentrations to weight. Therefore, we did not dry and weigh embryos to avoid artifacts of different standardization techniques for embryos and larvae. Instead, differing amounts of material in each embryo sample were standardized by presenting the concentration for each element as a ratio to the amount of Carbon determined in that sample by ICP-MS analysis. The resulting ratios were compared among samples in a linear discriminant function analysis using Systat version 10.0. The model was complete in that all elements were available to discriminate among samples. Each embryo sample was assigned to the closest group of the 16 possible sites in multivariate space, while not using its own chemical information to define any of the groups (jackknifing). The accuracy of assignment of the embryos to their correct site was used to evaluate the existence of a site-specific chemical fingerprint.

The data in some cases did not conform to the assumptions of a parametric discriminant function analysis. In particular, the high variability in element ratios at some sites (see Fig. 3) made reaching homoscedasticity among all 16 sites difficult to achieve despite several transformation schemes. Fortunately, linear discriminant function analysis is relatively robust to departures from these assumptions (Lachenbruch 1975).

To validate our parametric analysis against these assumption violations, we also analyzed the data using a non-parametric "k-nearest-neighbor" discrimination method, which assigns a sample to the group in which the majority of its nearest neighbors reside. This approach is not restricted by the assumptions of the underlying distribution that constrain the parametric analyses (Cover and Hart 1967). There is no universally accepted method to decide the appropriate number of nearest neighbors (k) to use in defining a sample. We classified the samples eight separate times, using between three and ten nearest neighbors, resulting in classification accuracies between 76.8 and 82.5%. Since these analyses assigned embryos to their correct sites with better accuracy than the parametric results, we concluded that the assumption violations of our parametric analyses did not artificially inflate assignment accuracy. All results, tables and graphs were produced with the parametric analyses.

Chemical fingerprints were placed in a larger geographic context to improve the assignment accuracy by lumping nearby sites into groups and evaluating these regional groups in the discriminant function analysis in place of sites. The final groupings maximized the assignment accuracy among groups in the discriminant function analysis while remaining geographically contiguous.

Spatial trends were explored for each element separately by relating the site location along the coastline to the site-averaged ratio of that element in a linear regression. Values for the location variable were calculated using ArcExplorer GIS version 2.2, with zero being the southernmost site (Abalone Point) and the distance north following the coastline for each subsequent site.

In spring 2007, we retrospectively assessed whether embryo size or stage significantly affected the ratio of each element to Carbon. We thawed the ovigerous females and measured the long axis of five haphazardly selected embryos each from those remaining on the ten females using imaging software. These measurements were averaged across each site. We also classified the stage of embryonic development using microscopy. Late stage embryos were identified by fully formed eyes. We calculated the

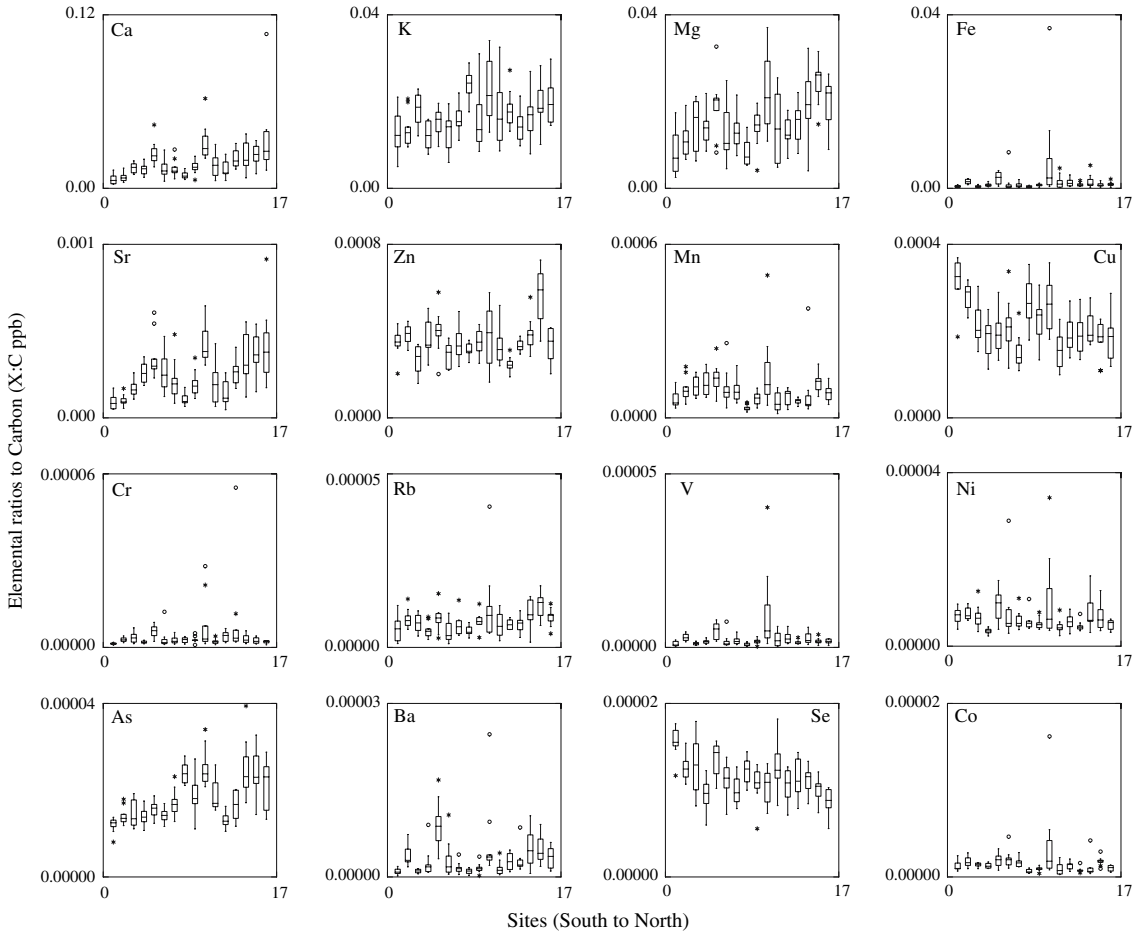

Fig. 3 Ratio of each of the 16 elements to the amount of Carbon detected. The *x*-axis shows the 16 collection sites, arranged from southernmost (1) to northernmost (16). *Center lines* of the *box plots* represent the median, *rectangles* encompass the second and third quartiles, whiskers extend to observed values within the inner fences, *asterisks* denote values outside the inner fences (outliers) and *open circles* denote values outside the outer fences (extreme outliers). The plots are arranged in descending order of *y*-axis scale, *left–right*, *top*–bottom

percentage of females with broods that were in the late stage of development at each site.

We tested the null hypothesis that embryos at all sites were the same size and in the same stage of development by conducting one-way ANOVAs followed by pairwise comparisons among sites using Tukey tests. Linear regression was used to detect spatial patterns in the size and stage of embryos among study sites. Lastly, we examined the relationship of the average size and stage to the ratios of each element to Carbon using linear regression.

Results

Three of the 22 elements were not distinguishable above background levels and therefore were excluded from the analysis (Cs, Tl, U). Most of the remaining elements occurred in concentrations of at least an order of magnitude above the method blanks. However, three elements were detected in significant amounts in the blanks most likely due to contamination, and were therefore excluded from the analysis (Al, Cd, Pb). The remaining 16 elements were included in the analysis (Mg, Mn, Cu, Ca, Ba, Fe, Cr, V, K, Ni, Rb, Co, Zn, As, Sr, Se), and the ratio of these elements to Carbon for each of the 16 sites is reported in Fig. 3.

Ratios of seven elements were related to the location of the collection site (Table 1). Five of these elements increased in proportion to Carbon moving north along the coastline (Ca, Mg, Sr, As and Rb), and two elements increased within the embryos at southern sites (Se and Cu).

All 16 elements were used in the discriminant function analysis to distinguish among the 16 sites with an overall 73% jackknifed accuracy. In four cases, nine out of ten embryo samples were correctly assigned to site: the southernmost location at Abalone Point, Chimney Rock at the tip of Point Reyes, and Gualala and Navarro Point on the northern coast (Table 2). The lowest assignment accuracy occurred at Iverson Point on the northern coast, where only

Table 1 Relationship between location along the coast and the ratio of seven elements in porcelain crab embryos to Carbon in a linear regression

Element	r^2	P	Greatest ratios
Calcium	0.521	0.002	Northern sites
Selenium	0.485	0.003	Southern sites
Magnesium	0.442	0.005	Northern sites
Strontium	0.421	0.007	Northern sites
Arsenic	0.372	0.012	Northern sites
Rubidium	0.316	0.023	Northern sites
Copper	0.312	0.024	Southern sites

Ratios of five of these elements increased at northern sites, while Se and Cu increased at southern sites. The other nine elements analyzed did not show a significant relationship to location

four out of ten embryo masses were correctly assigned. Of the 27% of embryo masses that were misplaced overall, one fifth were incorrectly assigned to one of the neighbors on either side of the actual collection site; 36% of misplaced embryo masses were assigned to one of the two sites immediately north or two sites immediately south of the correct site.

The overall assignment accuracy was maximized by lumping the 16 sites into 3 regions: a southern region composed of the two sites south of Point Reyes, a central region consisting of the five sites between Point Reyes and the mouth of the Russian River, and a northern region consisting of the nine sites ranging from the Russian River to Fort Bragg (Fig. 1). The analysis was able to discriminate among these regions with an overall 88% jackknifed accuracy (Fig. 4, Table 3).

Mean embryo size differed among sites ($df = 15$, $F = 8.208$, $P < 0.0001$), ranging from an average of 0.75 ± 0.03 mm SD at Bodega Harbor to 0.96 ± 0.18 mm SD at Shell Beach. Embryos from Chimney Rock, Bodega Harbor, Bodega Marine Laboratory, and Fort Bragg were smaller than average, and embryos from Shell Beach and Sea Ranch were larger than average. These differences, however, were not related to position along the coastline ($r^2 = 0.015$, $P = 0.654$) or any of the 16 element ratios used in the discriminant function analysis (regression results ranged from Cu, $r^2 = 0.05$, $P = 0.404$; to Sr, $r^2 < 0.001$, $P = 0.961$).

The number of late stage broods differed among sites ($df = 15$, $F = 1.918$, $P = 0.026$), and embryos in late stages of development were larger than those in middle or early stages of development ($df = 2$, $F = 19.3$, $P < 0.0001$). More females possessed late-stage embryos at northern than other sites ($r^2 = 0.283$, $P = 0.034$). Average embryo stage generally was not related to the ratio of each element to Carbon ($P > 0.05$ for all regressions), although three element ratios were weakly related to embryo stage. Rb ($r^2 = 0.193$, $P = 0.089$) trended toward high concentrations relative to Carbon in late-stage sites, while Se ($r^2 = 0.189$, $P = 0.093$) and Fe ($r^2 = 0.191$, $P = 0.091$) tended to have lower levels at sites with later stages.

Discussion and conclusions

Unique chemical fingerprints were detected within brooded crustacean embryos from the open coast, and these fingerprints define ecologically relevant spatial scales. Even though sites were separated by as little as a few kilometers, the overall accuracy of assignment is comparable to those detected in previous studies that included polluted estuarine or more distantly separated open coast sites (Becker et al. 2005, 2007; Warner et al. 2005; Zacherl 2005). The southern sites were most readily distinguished, with an average of 81% accuracy for the eight sites from Fort Ross south. Most of the sites that were located hundreds of kilometers to the north could also be satisfactorily distinguished. When the eight southern sites were excluded, the remaining eight sites were distinguished with 71% accuracy. The ability to combine sites into coherent regions that could be discriminated with increased accuracy (88%), and that over a third of the misclassifications occurred at nearby sites further demonstrated that these fingerprints are not simply site specific, but that they exist in a larger geographic context as well. Thus, this study is the first step in applying natural chemical fingerprints more broadly than to just estuarine species or those with permanent calcified structures.

Although the sites differed in average embryo size and stage, these differences did not have demonstrable effects on the chemistry of the embryos; size was not significantly related to any of the element ratios to Carbon in linear regressions. Even so, future researchers might avoid potential interference by ensuring that all analyzed embryos were in the same stage of development.

The physical and biological factors that maintain these unique signatures along the coast are not fully understood. Elements in seawater vary spatially for many reasons, such as anthropogenic sources (e.g., Pb, Cu and Zn Nriagu 1989), biological uptake of nutrients and removal of multivalent metals by precipitation (Faure 1998), riverine input (e.g., dissolved Ca and particulate Al; Faure 1998), differing freshwater source geology (e.g., elevated Cr and Ni from serpentine soils or Se from shales; Faure 1998), and upwelling (e.g., Cd, Mn, Zn, Pb, Ni, Cu and Cr; Sanudo-Wilhelmy and Flegal 1991, 1996; Lares et al. 2002; Chase et al. 2005). The two southernmost sites are influenced by outwelling of polluted waters from San Francisco Bay, whereas the northern region of the coastline does not contain major harbors, population centers or outfalls from major agricultural watersheds. Consequently, the relationship between increased heavy metals Se and Cu at southern

Table 2 Jackknifed assignment accuracy for porcelain crab embryos into one of sixteen sites produced by a linear discriminant function analysis of their chemistry

	Abalone point	Chimney rock	Pinnacle rock	Bodega harbor	BML	Shell beach	Twin coves	Fort ross	Timber cove	Salt point	Sea ranch	Gualala	Iverson point	Arena cove	Navarro point	Fort bragg	% Correct
Abalone point	9	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	90
Chimney rock	0	9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	90
Pinnacle rock	0	0	7	0	0	1	1	0	0	0	0	1	0	0	0	0	70
Bodega harbor	0	0	0	8	1	0	0	0	0	0	1	0	0	0	0	0	80
Bodega M.L.	0	0	0	1	8	0	0	0	0	0	1	0	0	0	0	0	80
Shell beach	0	0	0	0	0	8	1	1	0	0	0	0	0	0	0	0	80
Twin coves	0	0	0	0	0	1	8	0	0	0	1	0	0	0	0	0	80
Fort ross	0	0	0	0	0	0	0	8	1	0	1	0	0	0	0	0	80
Timber cove	0	0	0	0	0	0	0	1	7	0	0	0	1	0	0	1	70
Salt point	0	1	0	0	0	1	0	0	1	5	0	0	0	2	0	0	50
Sea ranch	0	0	0	0	0	0	0	1	1	0	7	0	0	0	0	1	70
Gualala	0	0	1	0	0	0	0	0	0	0	0	9	0	0	0	0	90
Iverson point	0	0	0	0	1	0	1	0	0	0	2	0	4	0	0	2	40
Arena cove	0	0	0	1	0	0	0	0	2	0	1	0	0	6	0	0	60
Navarro point	0	0	0	0	0	0	1	0	0	0	0	0	0	0	9	0	90
Fort bragg	0	0	0	0	0	0	0	0	2	0	1	0	1	1	0	5	50
Total	9	10	9	10	10	11	12	11	15	5	15	10	6	9	9	9	73

Sites are arranged from south (Abalone Point) to north (Fort Bragg)

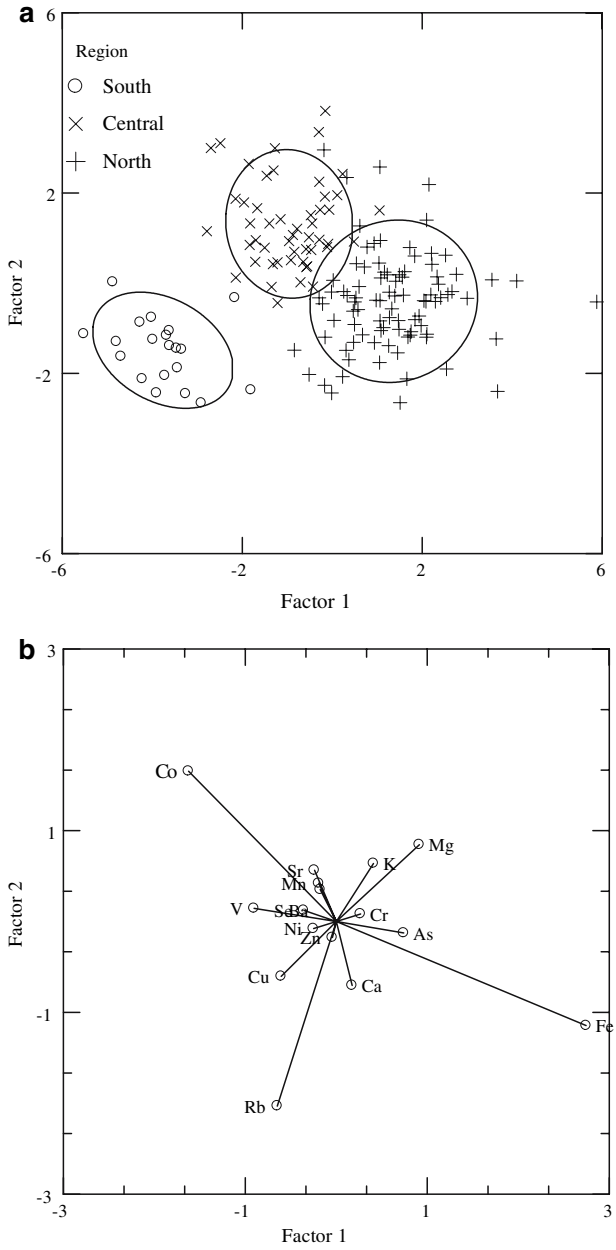

Fig. 4 Canonical factor one versus canonical factor two for the regional discriminant function analysis (**a**) showing chemical differences among the three regions in multivariate space, and (**b**) showing the relative importance of the 16 elements in discriminating among regions. Vector length is proportional to the absolute value of the canonical functions, standardized by within variances. Note that the scale of both axes in **b** is half that of **a** to show detail

sites may be due to the proximity of these sites to known sources of pollution (Flegal et al. 2005). However, this relationship remains intact when the two sites south of Point Reyes and closest to San Francisco Bay were excluded from the analysis. With all sites included, the r^2 values (49 and 31% variation explained) for these relationships suggest that the overall picture is more complex than the distance from a large source of heavy metals.

Table 3 Jackknifed assignment accuracy for porcelain crab embryos into one of three regions produced by a linear discriminant function analysis of their chemistry

Regions	South	Central	North	% Correct
Southern 2 sites	19	1	0	95
Central 5 sites	0	47	3	94
Northern 9 sites	3	13	74	82
Total	22	61	77	88

Several of the elements used in the analysis are known to be related to upwelling (Cd, Mn, Zn, Pb, Ni, Cu and Cr), which varies in intensity along the coast increasing near headlands (Huyer 1983; Sanudo-Wilhelmy and Flegal 1991, 1996; Lares et al. 2002; Chase et al. 2005). Local variability in the chemical composition of embryos also could be affected by the composition of the phytoplankton assemblage eaten by females and passed on to their broods.

The relationship between the chemistry of brooded embryos and the physical environment could be explored further by the collection of water samples or the monitoring of element-accumulating films. The goal of these "proxies" is not only to better understand the relationship between the environment and chemical fingerprints, but also to explore the possibility that larval tracking can take place without analyzing all possible source populations (Warner et al. 2005). So far these proxies have shown little promise in predicting the chemistry of natural fingerprints. With *P. cinctipes*, water samples and elemental accumulators are unlikely to predict the fingerprints, because the embryo chemistry depends on both the bioavailability of elements that are provided by the mother and those that are taken up directly from the environment (S.G. Morgan, unpublished data).

The geographical pattern in elemental concentrations in embryos cannot be counted on to remain static due to seasonal and interannual variability in rainfall, upwelling and anthropogenic inputs. We conducted our study during the season of peak reproduction by *P. cinctipes* and most other species on the West Coast, but many species have protracted reproductive seasons (Morris et al. 1980; Strathmann 1987; Shanks and Eckert 2005), necessitating that temporal variation in the signatures of embryos be taken into account. Attempts to reduce sampling effort by constructing a temporally stable atlas of chemical signatures across a geographic region have not yet been successful (Becker et al. 2005; Gillanders 2005; Warner et al. 2005).

Natural elemental tags have been used successfully to track the origins of crab larvae. First stage larvae of the lined shore crab, *Pachygrapsus crassipes*, could be distinguished as originating from San Diego Bay or the open

coast using trace element signatures (Ca, Mn, Zn, Cu, Sr, Mg, Al) in larval exoskeletons (DiBacco and Chadwick 2001). However, crab larvae shed their exoskeletons during molting, and therefore, a key component to the successful tracking of larval movement in the field is that the fingerprint is retained until the larvae can be captured farther along their journey or at their ultimate destination. Crab and other planktotrophic larvae grow rapidly during development, and there are little data available on the retention or regulation of specific elements in crustacean larvae. Elevated concentrations of Se have remained in tissues through planktonic development of crab larvae indicating that natural elemental tags in the soft tissues of larvae can be used to determine larval origins and pathways (Anastasia et al. 1998). Other elements that are not appreciably diminished during molting, such as Cu and Zn (Weeks et al. 1992), may be crucial to the development of a persistent natural chemical fingerprint.

One reason why we were better able to discriminate closely spaced sites along the open coast is that our analyses were based on far more elements than previous studies on exposed coasts (DiBacco and Levin 2000; Becker et al. 2005; Warner et al. 2005; Zacherl 2005). However, all of our 16 elements may not be retained in soft tissues throughout development, thereby potentially diminishing our ability to discriminate among closely spaced sites of natal origin. Had we only been able to utilize the seven elements used by DiBacco and Levin (2000) to discriminate among *P. crassipes* larvae (Al, Mg, Mn, Cu, Zn, Sr, Ca) our assignment accuracy among sites would drop from 73 to 52%. The ability to discriminate embryos by region, however, only drops from 88 to 82% accuracy with the reduced list of elements.

The next step in the development of this technique is to identify elements that are retained throughout development in laboratory assays. If distinguishable chemical fingerprints are indeed preserved in open-coast species that lack calcified structures, then larval tracking can be expanded to study many more species and in more places than previously thought possible. This will greatly improve our understanding of population connectivity in marine populations and aid in conservation efforts for a number of threatened or commercially valuable species.

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