Biomonitoring of trace metal bioavailability in the barnacle
Amphibalanus improvisus along the Iranian coast of the Caspian Sea

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Abstract
The fouling barnacle Amphibalanus improvisus is a cosmopolitan biomonitor of trace metal bioavailability in coastal waters of reduced salinity such as estuaries or the Baltic Sea. Bioavailability of Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V and Zn to A. improvisus was investigated in July 2014 at 15 sites along the Iranian coast of the understudied, yet biologically unique, Caspian Sea. In general, the bioavailability of these toxic metals in the water column, as biomonitored by the barnacle, was within the typical ranges expected from data available in the literature. Thus, there were no major environmental concerns raised. Nevertheless increased Cu and Zn were locally bioavailable at one site, perhaps associated with shrimp farming activities, and of Mn at another, probably from an industrial source. It is vital that the degree of contamination of the largest continental body of water in the world, particularly a water body with a unique biology is monitored.

Keywords: Caspian Sea, Trace metals, Biomonitoring, Barnacles, Amphibalanus improvisus

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Introduction
The Caspian Sea is the largest continental water body in the world. It is surrounded by five states – Azerbaijan, Iran, Turkmenistan, Kazakhstan and Russia, and any pollutants discharged into the Caspian Sea remain trapped within the basin of this non-tidal land-locked system (de Mora and Turner, 2004; de Mora et al., 2004). Major sources of pollutants into the Caspian Sea are considered to be associated with the oil production industry, for example via the Volga River in the north or along the coast of Azerbaijan (de Mora and Turner, 2004). Additional pollution threats are from organochlorinated compounds from industrial and agrochemical sources (de Mora and Turner, 2004), and from toxic trace metals in industrial and urban discharges (de Mora et al., 2004; Parizanganeh et al., 2007; Sohrabi et al., 2010; Saghali et al., 2014; Ghorbanzadeh Zaferani et al., 2016). For example, the Kura River, and its tributary the Aras River, drain mining and industrial regions of Georgia and Armenia before entering the Caspian Sea in Azerbaijan. The Kura River delivers high loads of trace metals such as copper into the Caspian Sea (Dumont, 1995; de Mora et al., 2004).

The Iranian coast stretches for nearly 700 km from Azerbaijan in the west to Turkmenistan in the east (Fig. 1).

Figure 1: Caspian Sea coastal waters showing the 15 sites from which barnacles (Amphibalanus improvisus) were collected. See Table 1 for details.

Sea currents transport entrapped pollutants, discharged locally or in adjacent Azerbaijan or even Russia, along the Iranian coast (Parizanganeh et al., 2007). Furthermore, more than 10 million people reside in the coastal
provinces of Iran that border the Caspian Sea, and Caspian Sea sturgeon and other fish are regularly consumed by this population (Parizanganeh et al., 2007). Trace metals accumulated by fish via local aquatic food chains have the potential to enter the human food chain (Luoma and Rainbow, 2008). Given the biological, conservational and commercial importance of members of the biological community of the Caspian Sea, it is vital to understand how any increased bioavailability of toxic contaminants such as trace metals might potentially affect the biota of the Caspian.

This study investigates the bioavailability of twelve trace metals in the coastal waters of the Iranian coast at the southern end of the Caspian Sea.

There have been previous studies of metal concentrations in local Caspian Sea sediments, but these are measures of either total metal concentrations (de Mora et al., 2004; Sohrabi et al., 2010), or metal concentrations after extraction with cold acetic acid (Parizanganeh et al., 2007). The latter extraction measures concentrations of relatively exchangeable metal in the sediments that might correlate with bioavailable metal concentrations, but only a bioaccumulated concentration can give a measure of a bioavailable concentration (Luoma and Rainbow, 2008). While it is of international importance to have knowledge of the bioavailability of trace metals in at least part of this important ecosystem that is the Caspian Sea, the data set collected will also add more data to the growing international database of accumulated trace metal concentrations in the bodies of Amphibalanus improvisus.

Materials and methods

Sample sites

Barnacles, Amphibalanus improvisus, were collected between 2 and 5 July 2014 from piers, pebbles and stones, rocks of artificial breakwaters or reeds along the shores of the 15 sites listed in
Table 1 (Fig. 1). The sites chosen cover a range of potential anthropogenic input of trace metals into the local Caspian Sea waters. They include sites in commercial ports (sites 4, 8), other ports and urban sources (sites 1, 12, 13), aquaculture facility outlets (sites 3, 14), river mouths (sites 3, 5, 6, 7, 10), wetland discharges (sites 4, 15), and open coast sites popular with tourists (site 9) (Table 1). Sites 1 and 15 would be most affected by any effluent discharge from sources in Azerbaijan and Turkmenistan respectively. At each site the salinity of the water was measured with a refractometer, and salinities are quoted using the Practical Salinity Scale without units (Table 1).

**Sample analysis**

Barnacles were removed from the substratum with a new stainless steel scraper, put in polythene bags for transport to the laboratory in a cool box. 10 (sites 1, 2, 3, 4, 10, 11, 14, 15) or 20 (sites 5, 6, 7, 8, 9, 12, 13) bodies were subsequently dissected out with new stainless steel instruments, each rinsed in distilled water, and pooled in each of 10 replicate samples from each site.

Table 1: The 15 sampling sites along the Iranian coast of the Caspian Sea, water salinities and dates of collection of barnacles.

<table>
<thead>
<tr>
<th>Site</th>
<th>Name</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Salinity</th>
<th>Date</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Astara</td>
<td>38°23'41.9&quot;N</td>
<td>48°52'00.1&quot;E</td>
<td>13</td>
<td>02.07.2014</td>
<td>Urban, tourism; most input from Azerbaijan</td>
</tr>
<tr>
<td>2</td>
<td>Haviq</td>
<td>38°09'05.8&quot;N</td>
<td>48°54'47.7&quot;E</td>
<td>12.5</td>
<td>02.07.2014</td>
<td>Small town; no specific source of pollution</td>
</tr>
<tr>
<td>3</td>
<td>Hashtpar</td>
<td>37°49'53.9&quot;N</td>
<td>48°58'43.6&quot;E</td>
<td>13.5</td>
<td>02.07.2014</td>
<td>Discharge from Kargan-Roud river and sturgeon farms</td>
</tr>
<tr>
<td>4</td>
<td>Anzali port</td>
<td>37°28'45.9&quot;N</td>
<td>49°27'29&quot;E</td>
<td>10</td>
<td>02.07.2014</td>
<td>International commercial port, urban, discharge from Anzali wetland</td>
</tr>
<tr>
<td>5</td>
<td>Kiashahr</td>
<td>37°26'31.9&quot;N</td>
<td>49°57'59&quot;E</td>
<td>11</td>
<td>03.07.2014</td>
<td>Discharge from a small river, very small barnacles on Phragmites, 5 km from Sefid-Roud river mouth</td>
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<tr>
<td>6</td>
<td>Chamkholeh</td>
<td>37°12'57.4&quot;N</td>
<td>50°16'29.03&quot;E</td>
<td>11</td>
<td>03.07.2014</td>
<td>Discharge from Chamkholeh river, draining an industrialised region with a refinery, machinery production, and textile and soap processing industries</td>
</tr>
<tr>
<td>7</td>
<td>Chaboksar</td>
<td>36°59'07.5&quot;N</td>
<td>50°33'53.1&quot;E</td>
<td>12</td>
<td>03.07.2014</td>
<td>Small river discharge</td>
</tr>
<tr>
<td>8</td>
<td>Noshrar port</td>
<td>36°39'30.5&quot;N</td>
<td>50°16'29.03&quot;E</td>
<td>12.5</td>
<td>03.07.2014</td>
<td>International commercial port, urban</td>
</tr>
<tr>
<td>9</td>
<td>Sisangan</td>
<td>36°35'02.8&quot;N</td>
<td>51°48'18.5&quot;E</td>
<td>12.5</td>
<td>04.07.2014</td>
<td>Open tourist coast; no specific source of pollution</td>
</tr>
<tr>
<td>10</td>
<td>Fereidunkenar</td>
<td>36°41'19&quot;N</td>
<td>52°30'12.04&quot;E</td>
<td>12</td>
<td>04.07.2014</td>
<td>Near Fereidunkenar river mouth</td>
</tr>
<tr>
<td>11</td>
<td>Khazarabad</td>
<td>36°47'28.6&quot;N</td>
<td>53°02'10.1&quot;E</td>
<td>12</td>
<td>04.07.2014</td>
<td>Small river discharge within 6 km</td>
</tr>
<tr>
<td>12</td>
<td>Amir Abad port</td>
<td>36°51'36.5&quot;N</td>
<td>53°22'30.01&quot;E</td>
<td>12</td>
<td>04.07.2014</td>
<td>Fisheries port; 8-10 km from Neka power plant, industrial area, oil and gas factories</td>
</tr>
<tr>
<td>13</td>
<td>Torkman port</td>
<td>36°53'46.7&quot;N</td>
<td>54°02'32.2&quot;E</td>
<td>12.5</td>
<td>05.07.2014</td>
<td>Small open port</td>
</tr>
<tr>
<td>14</td>
<td>Gomishan shrimp farm outlet</td>
<td>37°13'37.3&quot;N</td>
<td>54°01'13.6&quot;E</td>
<td>55</td>
<td>05.07.2014</td>
<td>Very big barnacles, very high salinity, high phytoplankton density</td>
</tr>
<tr>
<td>15</td>
<td>Gomishan shrimp farm inlet</td>
<td>37°16'45.4&quot;N</td>
<td>54°01'54.3&quot;E</td>
<td>30</td>
<td>05.07.2014</td>
<td>Very big barnacles, high salinity, high phytoplankton density</td>
</tr>
</tbody>
</table>
The body of a barnacle typically consists of the thorax with six pairs of filtering thoracic limbs (cirri), the reduced abdomen and part of the head (the oral cone). Bodies are easily recognised and removed, leaving behind the rest of the head forming the mantle, base, shell plates and opercular plates. Any egg masses in the mantle cavity were ignored. Given their ease of recognition, removal and therefore reproducibility, it is the bodies of barnacles that are usually analysed in biomonitoring studies (e.g. Rainbow et al., 2002, 2004a; Nasrolahi et al., 2014). Barnacles were not maintained in the laboratory in any effort to depurate the gut contents, in order to avoid laboratory contamination. All the barnacle bodies, therefore, contain gut contents but these are considered to represent only a small proportion of total body metal content, given the characteristics of barnacles as particularly strong trace metal accumulators (Rainbow, 1987, 2007).

The pooled bodies were dried in acid-washed pre-weighed plastic vials to constant weight at 60°C. The pooled samples were then each acid-digested in acid-washed test tubes at 100°C (glass-stoppered reflux) in Aristar grade (Merck) concentrated nitric acid. Each digest was made up to 10 mL with double-distilled water and analysed for up to 12 trace metals, Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V and Zn, on a Vista-Pro CCD Simultaneous ICP-OES. The limit of detection in the digests was typically 0.01 µg mL⁻¹ for each metal, equivalent to 0.1 µg in each 10 mL digest. Across the range of weights analysed, these translate into concentration detection limits between 0.5 and 10 µg g⁻¹ dry weight, according to the weight of tissue available. Simultaneous comparative analyses of trace metals were made in blanks (every 10 samples) and digests of two Standard Reference Materials, Mussel Tissue ERM-CE278 (Institute for Reference Materials and Measurements (2440 Geel, Belgium) and Oyster Tissue 1566b (US Department of Commerce, Technology Administration, National Institute of Standards and Technology, Gaithersburg, MD, USA). The precision of the analyses are expressed in terms of the 95% confidence limits of the measured mean concentrations (Table 2). Agreement with the certified concentrations of the reference material, where available, is considered satisfactory (Supplementary Table 1). All metal concentrations are quoted in terms of µg g⁻¹ dry weight.

Statistical analyses
It is important to consider any potential effect of size on body metal concentrations before comparing bioaccumulated concentrations of metals in invertebrates from different locations. Size will inevitably be correlated with age, but in the absence of an independent measure of age (but see Marsden et al., 2013), it is typically size that is used to allow for age differences.
Table 2: Comparisons of mean measured trace metal concentrations (µg g⁻¹ dry weight) against certified concentrations in Standard Reference Materials - Mussel Tissue ERM-CE278 (Institute for Reference Materials and Measurements (2440 Geel, Belgium) and Oyster Tissue 1566b (US Department of Commerce, Technology Administration, National Institute of Standards and Technology, Gaithersburg, MD, USA). (± 95% Confidence Limits, n = 4 (mussel) or 3 (oyster)).

<table>
<thead>
<tr>
<th></th>
<th>Measured Concentration</th>
<th>Certified Concentration</th>
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<tbody>
<tr>
<td><strong>Mussel Tissue</strong></td>
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<tr>
<td>As</td>
<td>5.17 ± 1.34</td>
<td>6.07 ± 0.13</td>
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<tr>
<td>Cd</td>
<td>0.27 ± 0.05</td>
<td>0.35 ± 0.01</td>
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<tr>
<td>Cr</td>
<td>0.73 ± 0.20</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td>Cu</td>
<td>8.45 ± 0.85</td>
<td>9.45 ± 0.13</td>
</tr>
<tr>
<td>Mn</td>
<td>6.56 ± 0.69</td>
<td>7.69 ± 0.23</td>
</tr>
<tr>
<td>Pb</td>
<td>1.85 ± 0.45</td>
<td>2.00 ± 0.04</td>
</tr>
<tr>
<td>Zn</td>
<td>71.5 ± 12.3</td>
<td>83.1 ± 1.7</td>
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<tr>
<td><strong>Oyster Tissue</strong></td>
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<tr>
<td>Ag</td>
<td>0.67 ± 0.48</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>Cd</td>
<td>1.99 ± 0.28</td>
<td>2.48 ± 0.08</td>
</tr>
<tr>
<td>Co</td>
<td>0.29 ± 0.03</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Cu</td>
<td>66.9 ± 2.2</td>
<td>71.6 ± 1.6</td>
</tr>
<tr>
<td>Fe</td>
<td>201 ± 9</td>
<td>206 ± 7</td>
</tr>
<tr>
<td>Mn</td>
<td>16.6 ± 0.6</td>
<td>18.5 ± 0.2</td>
</tr>
<tr>
<td>Ni</td>
<td>1.98 ± 1.15</td>
<td>1.04 ± 0.09</td>
</tr>
<tr>
<td>Pb</td>
<td>0.42 ± 0.23</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>V</td>
<td>0.54 ± 0.05</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>Zn</td>
<td>1,214 ± 27</td>
<td>1,424 ± 46</td>
</tr>
</tbody>
</table>

The power function \( y = ax^b \), where \( y \) is the metal concentration (µg g⁻¹), \( x \) is body dry weight (g), and \( a \) and \( b \) are constants, is a widely used model for the relationship between accumulated trace metal concentrations and individual body weights in barnacles (Rainbow et al., 2002, 2004a; Nasrolahi et al., 2014). \( x \) is the mean body dry weight of the 10 or 20 pooled bodies in each sample. All data were transformed logarithmically (to the base ten) to render the variance homogeneous and to normalise the data, creating an additive data set with skews removed, before the application of parametric statistics.

Data were first analysed for significant regression coefficients (slopes) in the regressions of log individual body weight (\( \log x \)) against log metal concentration (\( \log y \)) in the data set for each metal at each site, and in the whole data set for each metal. For each of the 12 metals analysed, there was in fact a significant effect of size...
(body weight) on accumulated body metal concentration in the whole data set and/or in at least one of the data sets for an individual site. It is, therefore, not meaningful to quote means or use Analysis of Variance to make comparisons between barnacle accumulated metal concentrations at different sites. Analysis of Covariance (ANCOVA) was therefore carried out in order to allow for the size effect. The data for each metal were first analysed to check if the data set for any site showed a regression coefficient significantly different from those of the other sites. When this occurred, that data set was removed from any further statistical comparison against the remaining sites. Analysis of the data for those sites for which the slopes of best-fit regressions did not differ significantly, was continued to identify any differences in elevation of metal concentration / body dry weight regressions (double log) between sites. For As, Cu and Mn, sites fell into two groups in which regression coefficients did not differ significantly between sites within each group, but did differ significantly between sites in the two different groups. Separate ANCOVA analyses were carried out for each of the two groups for each of these three trace metals. For every trace metal there was a significant a priori difference in accumulated metal concentration across the sites (up to 15) included in the ANCOVA comparison. Subsequently Tukey’s Honest Significant Difference (HSD) test was applied a posteriori, to identify which sites differed significantly from each other in accumulated metal concentrations in the barnacle bodies. Regression analyses and ANCOVA were carried out using STATISTICA (Statsoft).

Because mean accumulated metal concentrations in the bodies of barnacles from different sites have no comparative meaning in the presence of size effects, even in just a subset of data sets for a metal, comparative accumulated metal concentrations are quoted as the estimated accumulated concentrations (with 95% confidence limits) in barnacle bodies of a standard dry weight, as calculated from each double log regression. The mean dry weight of all barnacle bodies analysed was 0.0033g. This average dry weight, however, was highly affected by the extraordinarily large barnacles collected at sites 14 and 15 (Table 1). The mean dry weight of the barnacle bodies at the other 13 sites was 0.0023g. This is considered to be a more representative individual body dry weight across the collection. After further simplification, the standard individual body dry weight chosen for the expression of data was, therefore, 0.002g dry weight. The ordination technique Principal Components Analysis (PCA) was used finally to compare the similarity between sites, with respect to trace metal profiles for the 12 metals using estimated metal concentrations for barnacle bodies of 0.002g dry weight where available. CAP5 (Community Analysis Package, Pisces Conservation
Ltd, Lymington SO41 8GN, UK) was used for ordination analyses (Henderson and Seaby, 2008).

**Results**

Supplementary Table 2 summarises the metal concentration data for 12 trace metals accumulated in the bodies of *A. improvisus* from 15 sites along the Iranian coast of the Caspian Sea in July 2014. The accumulated concentrations of Ag, As, Cd, Co, Cr and Pb were often below detection limits at particular sites, and the concentrations reported summarise only the measured concentrations (Table 3).

While Supplementary Table 2 shows clear differences in accumulated metal concentrations between barnacles from different sites, these concentrations cannot be compared statistically between sites without allowing for any effects of differences in barnacle body weight between the samples from different sites. For every metal there was a significant effect of size on accumulated body metal concentration in the whole data set and/or in at least one of the data sets for an individual site. For each metal this size effect was, therefore, allowed for by ANCOVA of best-fit double log regressions of individual body dry weight against accumulated metal concentrations for each site (Table 4). For every metal there was a significant *a priori* difference in metal concentrations across the sites compared, a prerequisite of this concentration comparison being a lack of significant difference across the regression coefficients of the regressions under comparison. Concentration data for all 15 sites could be compared by ANCOVA for Cu (in two groups), Fe, Ni and V, but reduced numbers of sites were compared for As (6 sites in 2 groups), Co (2 sites), Cr (14 sites), Mn (14 sites in 2 groups), Pb (10 sites) and Zn (14 sites) as a result of elimination of data sets with no or insufficient concentration data (Table 3), or because of significantly different regression coefficients (Table 4). The data sets for each of As, Cu and Mn could be divided into two groups for comparison by ANCOVA (Table 4). Each group contained data sets with no significant differences between regression coefficients, although the regression coefficients in each group differed significantly from those of sites in the other group for that metal (Table 4). The need to separate the As, Cu and Mn data each into two sets for ANCOVA analysis on the basis of differences in regression coefficients is probably not of biological significance, but simply reflects the width of the range of best-fit regression coefficients across the whole data set for these metals. Individual site data sets with fewer than 3 replicates were excluded from ANCOVA analyses (Table 4), but accumulated concentrations are presented in Supplementary Table 2.
Table 3: Minimum and maximum concentrations (µg g⁻¹ dry weight) of trace metals in bodies (mean individual dry weight (g)) of up to 10 replicate samples (n), each of 10 or 20 pooled bodies, collected from 15 sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Mn</th>
<th>Cu</th>
<th>Ni</th>
<th>Pb</th>
<th>Zn</th>
<th>Fe</th>
<th>Cd</th>
<th>Hg</th>
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<tbody>
<tr>
<td></td>
<td>max</td>
<td>min</td>
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<td>1</td>
<td>80.1</td>
<td>194</td>
<td>137</td>
<td>302</td>
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<td>1040</td>
<td>750</td>
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<td>9</td>
<td>1.05</td>
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Table 4: *Amphibalanus improvisus*: Comparison by ANCOVA of accumulated concentrations of Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V and Zn in the bodies of barnacles from the 15 sites listed in Table 1.

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<th>ANCOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td></td>
<td></td>
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<tr>
<td>12</td>
<td>10</td>
<td>2,800</td>
<td>2,530</td>
<td>3,110</td>
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<td>14</td>
<td>9</td>
<td>16,800</td>
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<td>108,000</td>
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<td>1,660</td>
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<td>1</td>
<td>10</td>
<td>969</td>
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<td>511</td>
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<td>10</td>
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<td>675</td>
<td>1,850</td>
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</table>

Trace metal concentrations [M] are estimated concentrations (µg g⁻¹ dry wt) with 95% confidence limits (CL) in a barnacle body of 0.002 g dry weight [W] from each site, using the best-fit regression equation \( \log [M] = \log a + b \log [W] \). Barnacles from any sites sharing a common letter in the ANCOVA column for a metal do not differ significantly in body concentrations of that metal. Sites with fewer than 3 replicate samples for an individual metal (see Table 2) were excluded from the ANCOVA analysis. In the cases of Mn and Zn, the asterisk indicates a site that could not be included in the ANCOVA analysis for that metal because of a significant difference in regression coefficient (b) in comparison with best-fit regressions from other sites. In the cases of As, Cu and Mn, sites fell into two groups of sites in which regression coefficients did not differ significantly between sites within each group, but did differ significantly between sites in different groups. Separate ANCOVA analyses were carried out in each group. n is the number of replicate samples for each metal at each site.

Table 2 presents a summary of the ANCOVA comparisons made for each metal, comparative accumulated metal concentrations being quoted as the estimated concentrations (with 95% confidence limits) in barnacle bodies of a standard 0.002g dry weight. Site data for Cd, Mn and Zn excluded from ANCOVA comparisons are included for completeness (Table 2). Barnacles from any sites sharing a common letter in the ANCOVA column for a metal do not differ significantly \( a \ posteriori \) in body concentrations of that metal. In Table 2, sites have been ordered firstly in terms of the \( a \ posteriori \) ANCOVA groups to which they belong (as denoted by a single letter), and secondly within those groups in terms of the estimated body metal concentration of a body of 0.002g dry weight. It needs to be remembered, however, that it is the best-fit regression lines that are being compared. These lines will cross (even in the absence of significant differences between slopes), and estimated metal concentrations for different body weights would probably give different site orders. Secondly, according to how the chosen standardised weight falls in (or indeed outside) the real data set of body weights analysed for a given site, the 95% confidence limits of the estimated
concentrations might be narrow or broad. For these two reasons, a posteriori ANCOVA site groupings will not always follow the strict rank order of the weight standardised metal concentrations quoted (Table 4). Barnacles at site 5 were much smaller (mean individual body weight 0.00061g) than at other sites (Table 3), and confidence limits for estimated metal concentrations in a body of 0.002g dry weight are correspondingly very large, as exemplified in the cases of Cu, Fe, Mn, Ni and V at this site (Table 4). By the same argument confidence limits about estimated metal concentrations for a 0.002g dry weight body are also wide for the very large barnacles at sites 14 and 15 (e.g. for As, Cr, Fe, Mn, Pb, V and Zn at site 14 – Table 4). Furthermore, when the number of measurable replicates is small, confidence limits about estimated concentrations in a standard sized barnacle body are large (Table 4). As is clear from Table 4, there were significant differences in accumulated concentrations of each metal across the sites compared, with a gradient of metal concentrations in the bodies of barnacles across the sites.

Only the barnacles from site 14 had accumulated measurable concentrations of silver (Table 4).

In the case of arsenic, significantly increased accumulated concentrations were present in the barnacles from sites 14 and 15, higher again at site 14 (Table 4). Only four other sites had enough measurable accumulated As concentrations for ANCOVA analysis, and these followed a gradient down from site 4, through sites 13 and 12, to site 9 (Table 4).

Cadmium concentrations accumulated by the barnacles were only measurable at site 14 (again) (Table 2).

Similarly accumulated cobalt concentrations were measurable only in the barnacles collected from sites 14 and 15, with higher concentrations at site 14.

There was a more complete data set available for chromium accumulated by the barnacles, with the sole exception of site 5 (Table 3, Table 4). There was a clear gradient of accumulated chromium concentrations in the barnacles at the other 14 sites, headed by sites 11 and 1, then sites 7 and 10 (Table 4). In contrast to the preceding metals, sites 14 and 15 were at the lowest end of the accumulation gradient for chromium (Table 4).

In the case of copper, site 14 was back at the top of the list of metal concentrations accumulated by the barnacles in a comparison of 13 of the 15 sites, with site 15 at the bottom of this list (Table 4). Accumulated copper concentrations were also significantly increased at sites 2 and 1, with a gradient of decreasing copper concentrations thereafter, down to site 15 (Table 4). Sites 11 and 5, with significantly different regression coefficients from the majority, were excluded from this larger ANCOVA comparison, but their accumulated...
copper concentrations were not atypically high (Table 4).

Iron concentrations in the barnacle bodies varied significantly across all sites, with the highest concentrations at sites 11, 10, 13 and 1, and the lowest at sites 4 and 6 (Table 4). Sites 14 and 15 were in the bottom half of the accumulated concentration list (Table 4).

Much of the highest accumulated Mn concentrations were found in barnacles at site 6, although these could not be compared statistically against the other accumulated Mn concentrations because of a significant difference in regression coefficients (Table 4). In fact accumulated Mn concentrations in the barnacles from the remaining 14 sites could only be compared by ANCOVA in two groups. Accumulated Mn concentrations were significantly increased in barnacles from sites 5 and 3 in the first group, and site 4 in the second group (Table 4). Sites 15 and 14 respectively appeared at the bottom of the two lists of accumulated Mn concentrations in the barnacles (Table 4).

Lead concentrations in the barnacle bodies showed relatively little (but still significant) variation across the 10 sites comparable by ANCOVA, with concentration data for 7 of these sites showing no significant difference (ANCOVA group B, Table 4). There were insufficient measureable Pb concentration data for site 15, but site 14 was at the bottom of the list of the 10 accumulated lead concentrations compared by ANCOVA (Table 4).

There were significant variations in vanadium concentrations accumulated by the barnacles across all 15 sites (Table 4). Sites 11 and 1 were at the top of the list, sites 14 and 15 at the bottom (Table 4).

Zinc concentrations in the barnacle bodies could be compared across 14 of the 15 sites (Table 4. Barnacles from the missing site 10 did not, however, appear to have an atypically high or low accumulated zinc concentration (Table 4). Of the 14 sites compared by ANCOVA, sites 12 and 14 were at the top of the list of accumulated zinc concentrations, while sites 4, 13, 3 and 5 were at the bottom (Table 4).

Fig. 2a shows the PCA plot using the six metals for which data sets of estimated accumulated concentrations in barnacle bodies were available for all 12 sites. Site 14 is well separated from the remainder with eigenvectors for Cu and Zn acting as the important drivers (Fig. 2a). Site 5 was also separated from the others with the eigenvectors for Fe and V being the drivers, and the smaller separation of site 6 was driven by the Mn eigenvector (Fig. 2a). The two Principal Axes shown accounted together for 68% of the variance in the data. When the incomplete data sets for the other six metals were added in to the PCA, site 14 remained very distinct from the other sites (Fig. 2b). Eigenvectors for Cd and As were now added to those of Cu and Zn as drivers of this separation (Fig. 2b).
Figure 2: Principal Components Analysis of site profiles for the metals accumulated by *Amphibalanus improvisus*. Fig 2a concerns only the 6 trace metals (Cu, Fe, Mn, Ni, V and Mn) for which bioaccumulation data are available at all 12 sites. Fig 2b concerns all 12 trace metals, the additional 6 metals (Ag, As, Cd, Co, Cr and Pb) presenting incomplete data sets across the 12 sites. The numbering of sites is as in Table 1.
Site 5 again appears distinct, still under the (reduced) influence of the eigenvectors for Fe and V, but also now of the Mn eigenvector (Fig. 2b). Site 6 is now back in the pack, and site 15 is more removed from the others in Fig 2b than in Fig 2a. Eigenvectors for Cr and Ni are close together, and have pulled out the pack of sites in their direction (Fig. 2b). The two new Principal Axes accounted for 62% of the variance in the data.

**Discussion**

Biomonitors provide direct information on differences in the local bioavailability of contaminants between locations, or at the same location over time (Phillips and Rainbow, 1994; Luoma and Rainbow, 2008). A biomonitor is defined here as an organism which accumulates chemical contaminants in its tissues, the accumulated concentration of which provides a relative measure of the total amount of contaminant taken up by all routes by that organism, integrated over a preceding time (Luoma and Rainbow, 2008). The accumulated concentration, in this case of a trace metal, is, therefore, an integrated measure of the total amount of the metal in bioavailable form to which that organism has been exposed over a period that can be defined for that organism (Luoma and Rainbow, 2008). Only bioavailable forms of a contaminant are taken up by an organism, and so have the potential to be of ecotoxicological significance.

The accumulated concentration of a trace metal in the body of a barnacle is an integrated measure of the total metal taken up by that barnacle from solution and diet over a preceding period. It is appropriate, therefore, to refer to high accumulated body concentrations of a trace metal as indicative of high ambient bioavailability (summed from both sources) to that barnacle over that period.

Barnacles take up trace metals from solution through the large permeable surface area of their bodies with their extensive cirri, and from the suspended food filtered by these cirri. In the case of balanid barnacles, including *Amphibalanus improvisus*, the filtered food includes fine detritus particles and phytoplankton, as well as larger zooplankton (Anderson, 1994). These small suspended particles adsorb and absorb high concentrations of trace metals, representing a considerable trophic input of trace metals to barnacles which have high filtering rates. Although barnacles have high uptake rates of trace metals from solution in comparison to other crustaceans (Luoma and Rainbow, 2008), the high trophic input of metals, coupled with high assimilation efficiencies, mean that barnacles take up most of their accumulated metals from the diet (Wang *et al.*, 1999a,b; Rainbow and Wang, 2001; Rainbow *et al.*, 2003; Rainbow 2007). Barnacles also have extremely low excretion rates of accumulated metals, which they store in the body in detoxified forms.
(Rainbow, 2007; Rainbow and Luoma, 2011), leading to accumulated concentrations of trace metals that are notoriously high amongst crustaceans (Rainbow, 2007; Luoma and Rainbow, 2008). The low excretion rates also mean that the period of metal exposure represented by an accumulated concentration in the body of a barnacle is very long, of the order of months or even more than a year (Rainbow et al., 2003, 2004b; Rainbow and Luoma, 2011).

Table 5 summarises literature data on accumulated concentrations of trace metals in the bodies of *A. improvisus* from other coastal sites, specifically the Thames Estuary, UK (Rainbow, 1987; Rainbow et al., 2002) and the Gulf of Gdansk at the southern end of the Baltic Sea (Rainbow et al., 2004a). Comparative international data are lacking as yet for As, Co, Cr and V. Typical accumulated trace metal concentrations range from body concentrations found in presumed uncontaminated conditions to those concentrations more usually measured, given the presence of some trace metal contamination in most coastal waters analysed (Luoma and Rainbow, 2008). The high bioaccumulated concentrations listed in Table 3 are found less often, and are considered to indicate atypically high local bioavailability of the trace metals concerned.

The divisions made here into typical and high concentrations (Table 3) are inevitably arbitrary, and should be modified as more data become available (Luoma and Rainbow, 2008). Included in Table 3 for ease of comparison are the ranges across the 15 Caspian Sea sites of the estimated metal concentrations in 0.002g dry weight barnacle bodies. A similar, but more comprehensive, comparative data base is available for the related but more widespread fouling barnacle *Amphibalanus amphitrite* (Luoma and Rainbow, 2008; Nasrolahi et al., 2014). While the arbitrary designation of typical and high concentrations has resulted in similar concentration ranges chosen for some metals for the two barnacles (see Table 5 and Nasrolahi et al., 2014), interspecific differences are inevitable, given the interspecific differences in growth rates (causing differential growth dilution of accumulated concentrations), in physiological parameters of metal accumulation, and subtle differences in diet such as size of particle filtered (Luoma and Rainbow, 2008).
Table 5: Amphibalanus improvisus: Accumulated concentrations of trace metals (µg g⁻¹ dry weight), arbitrarily categorised as ‘typical’ or ‘high’ when possible, in the bodies of the barnacle A. improvisus. The low end of the typical range is indicative of uncontaminated conditions; the high end is indicative of concentrations representative of moderate contamination on a regional scale. The ‘high’ concentrations are indicative of atypically raised bioavailability of that metal in the local habitat. (After Rainbow, 1987; Rainbow et al., 2002; 2004a). Also shown for comparison are the ranges across the Caspian Sea sites of estimated metal concentrations in 0.002 g dry weight barnacle bodies, summarised from Table 2.

<table>
<thead>
<tr>
<th>Amphibalanus improvisus</th>
<th>Ag</th>
<th>As</th>
<th>Cd</th>
<th>Co</th>
<th>Cr</th>
<th>Cu</th>
<th>Fe</th>
<th>Mn</th>
<th>Ni</th>
<th>Pb</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Woolwich, Thames 1985</td>
<td>700</td>
<td>38</td>
<td>2,000</td>
<td>250</td>
<td>814</td>
<td>964</td>
<td>3,300</td>
<td></td>
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<td></td>
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<td>Thames Estuary, UK</td>
<td>7.9</td>
<td>7.4</td>
<td>11.2</td>
<td>9.1</td>
<td>23.6</td>
<td>815</td>
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<td>15.5</td>
<td>3,300</td>
<td>7,400</td>
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<tr>
<td>Gulf of Gdansk, Poland</td>
<td>7.7</td>
<td>9.3</td>
<td>13.2</td>
<td>39.3</td>
<td>70.8</td>
<td>1,720</td>
<td>41.4</td>
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<td>Caspian Sea</td>
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<td></td>
<td></td>
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<tr>
<td>Range of weight-</td>
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<td>4.0</td>
<td>2.8</td>
<td>3.3</td>
<td>2.5</td>
<td>19.1</td>
<td>230</td>
<td>12.4</td>
<td>3.7</td>
<td>2.4</td>
<td>0.8</td>
<td>150</td>
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<tr>
<td>corrected metal</td>
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<td>49.4</td>
<td>8.8</td>
<td>16.6</td>
<td>143</td>
<td>1,000</td>
<td>2,500</td>
<td>67.2</td>
<td>7.2</td>
<td>53.3</td>
<td>16,800</td>
<td></td>
</tr>
</tbody>
</table>

Of particular significance in any comparison between A. improvisus and A. amphitrite is the preferred lower salinity regime of A. improvisus. Decreased salinity causes the decreased inorganic complexation of certain dissolved trace metals, including Ag, Cd, Ni and Zn (Luoma and Rainbow, 2008). This decreased inorganic complexation increases the availability of the uncomplexed free metal ion, to which increased uptake rates from solution are correlated (Campbell, 1995; Luoma and Rainbow, 2008). The use of an intraspecific comparative database for A. improvisus would allow for any effect of low salinity to increase the uptake of dissolved metals from solution, all the barnacles in the data base occupying a similar low salinity range.

The few measurable accumulated concentrations of Ag and Cd in the Caspian Sea barnacles do not appear to indicate the presence of any increased bioavailability of either metal along the Iranian coast (Tables 4, 5).

There are no comparable data on the accumulated concentrations of As in A. improvisus (Table 5). At the risk of reading too much into an interspecific comparison, the range of accumulated As concentrations in the Caspian Sea A. improvisus (Table 5) falls well below what would be considered a high concentration (200 µg As g⁻¹) in the closely related species A. amphitrite (Nasrolahi et al., 2014). It is unlikely therefore that As bioavailability in barnacles at the sites in the southern Caspian Sea are very high on an international scale, although they are
increased at site 14 (especially) and site 15 (Table 4).

In the absence of any comparative data for accumulated concentrations of cobalt in bodies of *A. improvisus*, or indeed of any barnacle (Rainbow, 1987; Reis, 2011), it is impossible to draw any conclusions on whether bioavailability of cobalt is increased in the Iranian coastal waters of the Caspian Sea.

The situation for chromium is similar to that for arsenic, comparative accumulation data only being available for *A. amphitrite* (Table 5). As for arsenic, the accumulated chromium concentrations in *A. improvisus* (Table 5) were below the threshold concentration (20 µg Cr g⁻¹) considered high for *A. amphitrite* (Nasrolahi et al., 2014). Again, therefore, it is unlikely that Cr bioavailability to barnacles is high at any of the Caspian Sea sites investigated.

In the case of copper, we came across the first likelihood of an increased bioavailability at a site in the southern Caspian. The concentration of 143 µg Cu g⁻¹ estimated for the barnacles at site 14 (Table 2) just falls within the high range identified in Table 3. All other sites have accumulated copper concentrations (Table 4), that can be considered typical (Table 5). Thus we conclude that there was a high local bioavailability of copper to barnacles at site 14, and by implication to other aquatic biota there.

Iron and manganese are rarely considered to be of ecotoxicological significance in oxygenated surface waters of marine or estuarine systems (Luoma and Rainbow, 2008). Their high accumulated concentrations in deposit or suspension feeding detritivorous invertebrates often together indicate the presence of sediment in the gut of the invertebrate analysed. Interestingly, in the PCA of these samples, Fe and Mn eigenvectors did fall together as expected when all 12 metals were included in the analysis (Fig. 2b), but were distinct when only 6 metals were used (Fig. 2a). In the case of iron, barnacles at several of the sites (11, 10, 13 and 1) had estimated accumulated iron concentrations above a threshold of 2,000 µg Fe g⁻¹ (Tables 4, 5), indicating a high local bioavailability of iron. Using a threshold of 300 µg Mn g⁻¹ as a threshold for a high concentration of Mn (Table 5), sites 3, 5 and especially 6 had barnacles with higher concentrations (Table 4). Thus it is concluded that sites 3 and 5 showed high Mn bioavailability, and site 6 a very greatly increased Mn bioavailability. The very high bioavailability of Mn at site 6 is absolutely not associated with that of iron (site 6 is at the bottom of the iron list in Table 5), and so is probably not related to the presence of suspended sediment particles. It is probably biologically relevant that the regression coefficient of the Mn data at site 6 is significantly different from those of the best-fit regressions at all other sites. This difference may represent the
presence of a unique source of high bioavailability Mn at site 6. Accumulated nickel concentrations in A. improvisus range from the bottom uncontaminated end of the typical range, just into the bottom end of what might be considered high at site 1 (Tables 4, 5).

Lead concentrations in the barnacles are all firmly in the uncontaminated end of the typical range of accumulated lead concentrations in A. improvisus (Table 3), indicating that no site showed any evidence of an increased lead bioavailability.

Comparative bioaccumulation data are lacking for vanadium in A. improvisus, but Nasrolahi et al. (2014) did measure V concentrations accumulated by A. amphitrite in the Persian Gulf. These ranged from 1.4 to 24.0 µg V g⁻¹ across 19 sites, and the authors concluded that the latter value should be considered high (Nasrolahi et al., 2014). In the case of A. improvisus in the southern Caspian, all but one of estimated bioaccumulated V concentrations at each site fell below 19 µg V g⁻¹ (Table 4). The estimated concentration at the remaining site (53.3 µg V g⁻¹, Table 4) was derived for the very small barnacles at site 5, with correspondingly very wide confidence limits (Table 4). This is not strong evidence for a very high V bioavailability at site 5. What can be concluded, though, is that V bioavailability to the barnacles did vary significantly across the 15 Caspian Sea sites (Table 4). Some sites (headed by sites 11 and 1) showed the highest V bioavailability, and this bioavailability can be considered as significantly raised, in comparison to example to V bioavailability at sites 14 and 15 (Table 4).

There was a significant gradient in zinc concentrations accumulated by the barnacles across the sites (Table 4). At first sight, the bioaccumulated concentration of Zn at site 14 seems high, given a threshold concentration of 10,000 µg Zn g⁻¹ to define a high concentration (Tables 4 and 5). Because of the atypically large size of the barnacles here, however, there are wide confidence limits on the value of 16,800 µg Zn g⁻¹. It is probable, nevertheless, that the zinc bioavailability at site 14 is high, while zinc bioavailability at the remaining 14 sites can be considered typical, and in many cases low (Tables 4, 5).

How can trace metal bioavailability be summarised across sites? Site 14 is clearly characterised by raised bioavailability of As and Zn, but particularly Cu. Site 14 is located near the outlet of a shrimp farm (Table 1), and it is possible that metal-containing products are in use there, such as, for example, trace metal food additives (particularly zinc), antioxidants and copper-based fungicides (Alvarado, 1997; Yeh et al., 2004; Lin et al., 2013). Silva et al. (2003) similarly found raised bioavailability of Cu and Zn (in this case to an oyster) downstream of the effluent outlet of a large shrimp farming enterprise in the
Curimatau estuary, Rio Grande do Norte, Brazil. The other site showing evidence of a trace metal bioavailability of international significance was site 6 in the case of manganese. Site 6 receives discharge from the Chamkhaleh River, draining an industrialised region with a refinery, machinery production, and textile and soap processing industries (Sohrabi et al., 2010) (Table 1). There was evidence of raised bioavailability of manganese also at sites 3 and 5, and of iron at sites 11, 10, 13 and 1.

The major pollution problems of the Caspian Sea are often considered to be associated with the production of oil (de Mora and Turner, 2004). As far as the Caspian Sea is involved, oil production is predominant in the Volga region of Russia and in Azerbaijan. Does oil pollution spread south to Iran? Two trace metals commonly associated with oil are vanadium and nickel (Pearson and Green, 1993), and increased bioavailability of either of these two metals indicates the presence of spilled oil or refined fuel in a coastal system. In fact there was evidence of increased bioavailability of both V and Ni at site 1, the site nearest the Azerbaijan border (Fig. 1).

None of the other trace metals investigated showed evidence of high bioavailability at any sites.

Previously measured concentrations of trace metals in Caspian Sea sediments in the region (de Mora et al., 2004; Parizanganeh et al., 2007; Sohrabi et al., 2010) can be compared against these metal bioavailability results. On the wide scale of the whole Caspian Sea, de Mora et al. (2004) reported increased sediment concentrations of As, Cu, Cr, Ni and V on average in coastal sediments of Azerbaijan and Iran in comparison to the rest of the Caspian. The increased Cu and Zn sediment concentrations in these two countries were attributed to mining activities (de Mora et al., 2004). By comparison to marine sediment quality guidelines, sediment concentrations of As, Cr, Cu, Ni and Zn off Azerbaijan and Iran were considered to be of potential ecotoxicological significance (de Mora et al., 2004). While this potential ecotoxicity may apply to infauna of the sediments, particularly deposit feeding infauna, there was no evidence from this study that trace metals were being supplied from the sediments to the overlying water column in sufficient quantities to be of ecotoxicological significance.

Parizanganeh et al. (2007) measured acetic acid-extractable concentrations of trace metals in sediments along the Iranian coast of the Caspian. There was evidence of increased extractable concentrations of Cu, Ni, Pb and Zn in sediments closest to Azerbaijan, but even higher sediment concentrations of these metals at sites in the east near Turkmenistan (Parizanganeh et al., 2007). Sohrabi et al. (2010) also found increased total concentrations of Cu, Ni and Zn in sediments at the eastern end of the Iranian coast, although the
highest sediment concentration of Cu was at the other end next to Azerbaijan. Again, therefore, these sediment concentrations do not correlate well with our results on local variations in trace metal bioavailability in the water column (dissolved and in suspended matter) to barnacles. Similarly it can be concluded that sediment metals are not being released to provide high bioavailability in the water column, even when present in high concentrations in those sediments. A logical follow up to this study would be to investigate the bioavailability of such sediment metals to an infaunal deposit feeding polychaete (Luoma and Rainbow, 2008).

This paper defines the present situation as regards the bioavailability of toxic metals in the water column along the Iranian coast of the Caspian. It can be concluded in general that there are no major environmental concerns raised by the data collected, although the locally increased bioavailabilities of Cu and Zn at site 14, and of Mn at site 6, may warrant further environmental management attention. It needs to be stressed that such results are not just of local significance. It is vital that the degree of contamination of the largest continental body of water in the world, particularly a water body with a unique biology, is monitored so that any necessary action can be taken. Furthermore the Caspian Sea data add to a growing A. improvisus database, increasing its future value in assessing the ecotoxicologically significant trace metal contamination of low salinity coastal waters across the world.

References


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previous field exposure history on the uptake of trace metals from water and food by the barnacle *Balanus amphitrite*. *Marine Ecology Progress Series*, 259, 201-213.


