January 2002

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Recommended Citation

Rodriguez-Navarro, Alejandro; Gaines, Karen F.; Romanek, Christopher S.; and Masson, G. R., "Mineralization of Clapper Rail Eggshell from a Contaminated Salt Marsh System" (2002). *Faculty Research & Creative Activity*. 49.
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Mineralization of Clapper Rail Eggshell from a Contaminated Salt Marsh System

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Received: 18 December 2001/Accepted: 25 March 2002

Abstract. The effect of contamination on eggshell mineralization has been studied for clapper rails (*Rallus longirostris*) inhabiting a contaminated salt marsh in coastal Georgia. To assess the impact of contaminants, the thickness, microstructure (crystal orientation), mineral composition, and chemistry of shell material were analyzed from a contaminated site and a nearby reference site using optical microscopy, X-ray diffraction, inductively coupled plasma mass spectrometry, and gas chromatography with electron capture detector. Eggshells from the contaminated site were generally thinner than those from the reference site. Also, eggshells from the contaminated site were abnormally brittle and contained anomalous microstructural attributes. The combination of reduced shell thickness and anomalous microstructure resulted in weaker eggshells, which in turn could pose a significant threat to the reproductive success of the affected population. PCB concentrations in eggshells were at background levels in both sites. Eggshells from the contaminated site had higher concentrations of heavy metals, specifically mercury, than the reference site. The structural changes observed in eggshells may be related to the concentration of specific metals (e.g., Mg, Cu, Zn, Pb, and Hg) in shell, however, statistical analyses indicated that metals only explained a small portion of the observed variation in properties (i.e., thickness, crystal orientation). Further analysis is required to better constrain the factors leading to unusually weak eggshells in the contaminated site.

and bacterial invasion; it also controls the exchange of water and gases through pores and it is the main calcium reservoir for skeletal formation during embryogenesis (Richards and Packard 1996; Nys *et al.* 1999). Thin-shelled eggs are accidentally crushed in the nest during parental incubation, and this has resulted in a population decline of many raptorial and fish-eating birds (e.g., bald eagle, *Haliaeetus leucocephalus*). Historically, the principal environmental contaminant affecting the eggshell was the metabolite of DDT and p,p'-DDE (Cooke 1973). Other organochlorides (e.g., polychlorinated biphenyls [PCBs]; Cooke 1973) as well as some heavy metals (Hg, Pb, and Al) can also induce this effect (Stoewsand *et al.* 1971; Lundholm and Mathson 1986).

During a population study of clapper rails (*Rallus longirostris*) inhabiting a highly contaminated marsh in coastal Georgia, it was noticed that many eggshells were abnormally brittle. Specifically, eggshells easily cracked when being marked with a pencil. The main contaminants in this marsh system were PCBs, Pb, and Hg. DDTs and other organochlorides (e.g., PCBs) can affect enzyme activity involved in calcium transportation and consequently affects eggshell thickness (Cooke 1973; Baird 1995). On the other hand, trace metal contaminants can influence the mineralization of eggshell in three fundamental ways.

1. They can reduce the availability of calcium in the diet. For instance, dietary calcium uptake is generally poorer in low-pH environments because calcium-rich foods, such as crayfish, snails, and mussels, are scarce (Okland 1992; Nybo *et al.* 1997). These low-pH environments commonly contain other environmental contaminants, such as metals (e.g., Ni, Co, Cu, Al) due to the greater mobility of these elements in acidic environments (Adriano 1986; Baird 1995).
2. Contaminants can interfere with calcium metabolism. For instance, heavy metals (e.g., Pb) compete with Ca^{2+} and interfere with Ca^{2+} -mediated processes (Baird 1995; Scheuhammer 1987; Richards and Packard 1996; Schirmacher *et al.* 1998).
3. Contaminants can interfere with the mineralization process itself by affecting the precipitation rate, mineralogy, size,

Environmental contamination has an adverse impact on avian reproduction. One of the most evident effects of environmental pollutants on birds is eggshell thinning (Ratcliffe 1970; Cooke 1973; Fry 1995). The eggshell functions as a protective housing for the developing chick embryo against mechanical impact

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and morphology of crystals that make up the eggshell (Cooke 1973). For instance, it is well known that Mg^{2+} inhibits the precipitation of calcite relative to the $CaCO_3$ polymorph aragonite in marine environments (Folk 1974). Several other divalent cations are known to inhibit the precipitation of calcite in a similar way, including Cu, Zn, Pb, and Fe (Wada *et al.* 1995; Parsiegla and Katz 1999a).

Eggshell is a bioceramic composite consisting mainly (> 95%) of the mineral calcite ($CaCO_3$) and a pervading organic matrix (1–3.5%), resulting in a structure that has excellent mechanical properties (Simkiss and Wilbur 1989; Fink *et al.* 1992; Nys 2001). The most important parameter for structural strength of the egg is shell thickness (Tyler 1961). However, considering that the eggshell is essentially a ceramic material, its mechanical properties (breaking strength) should be also determined by shell microstructure. The key microstructural parameters that control the mechanical properties of ceramic materials are crystal size and orientation (Davidge 1979). The inherent strength of a ceramic increases with decreasing crystal size and orientation of crystals. For instance, Rodriguez-Navarro *et al.* (2002) demonstrated a strong inverse correlation between the preferential orientation of crystals in an eggshell and the inherent breaking strength of the shell. Their results demonstrated that for eggshells of equal thickness, about 40% of the variance in shell strength can be explained by differences in the degree of orientation of crystals. Eggshells showing a lower degree of crystal orientation were mechanically stronger than those with a higher degree of crystal orientation.

A study has been conducted to compare eggshell structure in a contaminated marsh site to a nearby unimpacted reference marsh. The microstructure (*i.e.*, thickness, crystal orientation) and chemistry (metals, PCBs) of eggshells collected from each site were characterized to understand whether contaminants influenced the eggshell structure and its mechanical strength. In particular, we were interested in determining if contaminants (*e.g.*, metals) directly affect the formation process of eggshell forming crystals. A nonspecific inhibition of their growth will result in a decreased eggshell thickness, whereas a more specific inhibition of specific crystal direction will result in a change in crystal morphology and eggshell microstructure.

Clapper rail eggs were studied because this species has been the focal point for a variety of environmental toxicology studies (Vanvelzen and Kreitzer 1975; Lonzarich *et al.* 1992; Jarman *et al.* 1993; Kannan *et al.* 1998; Schwarzbach *et al.* 2001) due to their well-known foraging habits and strong site fidelity (especially in the southeastern United States). The clapper rail's diet in the southeastern United States consists primarily of crabs, crayfish, mollusks, worms, and other marine organisms. Small fish, aquatic insects, and amphibians are consumed to a lesser degree (Terres 1991; Eddleman *et al.* 1998). Feeding on benthic organisms increases the likelihood of individuals accumulating significant amounts of contaminants associated with coastal sediments.

Materials and Methods

Study Area

Contaminated Marsh—LCP Site: Clapper rail eggs were collected from a salt marsh contaminated with mercury, other heavy metals, and

the PCB Aroclor 1268 at the high priority Superfund site (Linden Chemicals and Plastics; LCP) in Brunswick, GA. At this site, both mercury and PCBs are present in elevated levels in the sediments and resident fauna (fiddler crabs, fish, etc.; Sprenger *et al.* 1997; Kannan *et al.* 1998). The site is located on the western shore of the Brunswick peninsula.

Reference Marsh—Blythe Island: Eggs were also collected at a nearby reference marsh at Blythe Island, located approximately 5 km from the LCP site. This site was chosen as a close analog to the LCP site based on similar vegetation, tidal influence, tidal creek diversity, and water chemistry.

Nest Searches

Nest searches were performed during low and high tide at both the LCP site and Blythe Island locations during the clapper rail nesting season in March–June 2000. Once nests with eggs were found, all eggs were collected and marked. Eggs were brought back to the Savannah River Ecology Laboratory on the same day as collection and were incubated at 99°F and 87°F relative humidity in an incubator (Humid-aire incubator separate hatcher). They were rotated automatically every 12 h for 21 to 23 days, until hatching. After hatching, eggshells were collected and put into individual plastic bags and stored in a freezer for later analysis. Eggs that did not hatch after 30 days of incubation were collected and stored in a freezer. Most eggshells broke in two main pieces on hatching, and this permitted the sampling of shell pieces from a known location on the original shell.

Eggshell Thickness

Eggshell thickness was measured with a point micrometer (Mitutoyo), to a precision of 1 μm . Thickness was measured at 90° intervals along the egg waist (transverse section through the egg having the greatest diameter, equator) and was reported as an average value ($n = 4$).

X-ray Diffraction

The mineral composition of eggshell was determined using a powder X-ray diffractometer (Scintag X1). To determine the orientation of crystals in the eggshells, 1-cm² pieces of shell were excised from the waist. These pieces were mounted onto stubs with the outer surface facing upward and were flattened by hand pressure. X-ray diffraction patterns were then registered for each sample.

A diffractogram was collected from a sample of ground shell for comparison. The ground eggshell diffractogram was used as a reference pattern for crystals having a completely random orientation. In Figure 1a, the X-ray diffraction patterns for a ground eggshell and a sample (intact) eggshell are shown. Note that the relative intensities of the hkl peaks vary for these two diffractograms. The intensity of each hkl peak is proportional among other factors, to the number of irradiated crystals having (hkl) crystal planes in Bragg orientation (Cullity 1977). Due to the absorption of X-rays, only the crystals at the outer surface of each sample contribute to the diffraction pattern. The ratio between the integrated peak areas of hkl peaks from the intact and ground shell provides a measure of the relative orientation of crystals in the intact shell. In the case of eggshells, generally the area ratio for the 006 peak is the highest, indicating that crystals are preferentially oriented with their (001) planes parallel (c -axis perpendicular) to the shell surface. Finally, the distribution of the orientation of crystals can be calculated by plotting the area ratio of each hkl peak (I/I_o or $area_{hkl(sa)}/area_{hkl(std)}$, where sa = intact shell and std = ground shell) normalized to the highest peak area ratio (I/I_o for 006 peak) versus the

Fig. 1. (a) X-ray diffraction pattern of: (1) a randomly oriented sample prepared by grinding one of the eggshells before XRD analysis, and (2) the outer surface of an intact Clapper Rail eggshell. The relative intensities of the hkl peaks differ among samples, most notably the 006 peak, indicating that the crystals are oriented with their (001) planes parallel to the eggshell surface. (b) Calcite crystal showing the angular relationship between the (001) and (104) faces. (c) The area for each hkl peak from the intact shell (I) is divided by the area of the same peak in ground shell (I_o) and plotted against the angle between the normal of each (hkl) plane and a reference plane (001). The area ratios are all normalized to the maximum area ratio observed (in this case the 006 reflection). From this plot, the degree of orientation of crystals is measured as full width half maximum (FWHM), where FWHM equals two times the angular distance of the half peak at half maximum (*i.e.*, where $I/I_o = 0.5$)

angle between the associated (hkl) plane and a reference plane (in this case (001) plane, see Figure 1b, c; Sharp and Silyn-Roberts 1984). Figure 1b shows an example of the angular relationship between the reference plane (001) and the (104) plane for calcite. For a randomly oriented sample, all peak area ratios will plot along a horizontal line of $I/I_o = 1$; the breadth of this distribution is uniform. With preferred crystal orientations in the sample, the line will develop some structure (peaks and valleys). The structure can be characterized by measuring the width of peaks at half the maximum height (*e.g.*, at $I/I_o = 0.5$, called full width at half maximum or FWHM). FWHM is used as a gauge of crystal orientation in a composite structure. The smaller the number of peaks and the narrower the FWHM distribution, the higher the degree of crystal orientation in the shell. Because it is well documented that eggshell strength increases with thickness and FWHM (Tyler 1961; Rodriguez-Navarro *et al.* 2002), the variable *MULT*, defined as the product of thickness and FWHM, can be used as an estimate of eggshell strength.

Organic Matter Content

The content of organic matter in eggshells was estimated by measuring the % N using an elemental analyzer (NC 2500, CE instruments). Percentages of N were measured on a composite sample from each

nest. Seven milligrams of ground eggshells were packed in a tin cup. The error in the determination of the % N was 0.05%.

ICP Mass Spectrometry

The concentration of Mg, Al, P, K, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Pb, and Hg in the eggshell was measured by inductively coupled plasma mass spectrometry (ICP-MS; Perkin-Elmer Elan 6000). Concentrations were measured on a composite sample from each nest. Pieces of eggshells from each nest were collected and ground with an agate mortar and pestle; 0.1 g of the ground material was digested in 10 ml of a 10% solution of trace metal-grade nitric acid. The lower detection limit for trace metal analysis was 1 ppb.

PCB Analysis

Two 5.0 g combined samples of eggshell were collected for PCB analysis; one from all nests of the contaminated site and other from all nests of the reference site. The methodology for PCB extraction and analyses used in this study is described elsewhere in detail (Mora *et al.* 1993). Composite samples were dissolved in a 10 ml solution of 10% nitric acid. Fifty milliliters of hexane were added to the solution and PCBs were extracted in the nonpolar solvent for gas chromatography analysis using an electron capture detector (GC ECD).

Thin Section Preparation and Optical Microscopy

Eggshell samples from nests with the highest and lowest concentration of Hg, Pb, Cu, Fe, and Al were selected for further examination using an optical microscope. The thinnest and thickest shells and shells with the highest and lowest degree of crystal orientation were also selected for the comparative study of microstructure. Thin sections ($\sim 30 \mu\text{m}$ thick) were prepared from radial sections cut from the waist of the eggshells for optical microscopy. The size, morphology, and orientation of crystals were observed using cross-polarized light.

Statistical Analysis

First, the distribution of all data variables was studied using Shapiro-Wilkes statistics (PROC UNIVARIATE, v. 6.12; SAS Institute 1998). Tests of hypotheses that these data were random samples from normal distributions were all rejected (p 's < 0.05 , except for Cu), and stem-and-leaf plots suggested that a log-transformation of these data was necessary. To determine if egg structure differed by site all data values were used. General linear model procedures (PROC GLM) were used to determine if thickness and MULT differed between sites. A Kruskal-Wallis ANOVA (PROC NPARIWAY) was used to determine if FWHM differed between sites because the overall distribution of this variable was not normal for raw or transformed data.

Because metal concentrations were not measured in individual eggshells but rather in nests, other eggshell properties (*e.g.*, thickness, crystal orientation) were averaged per nest to facilitate a meaningful comparison. Pearson's correlations were used to determine the relationships between metal concentration, eggshell thickness, and crystal orientation (FWHM). The relationship between eggshell structure and metal concentration was determined by performing linear regressions using all metals as separate dependent variables. A principal component analysis (PCA; PROC PRINCOMP) was used to explore the underlying covariation structure of the metal data. A covariance matrix was used in the PCA to preserve the scale relationship of the variance differences among metals. Because values for Mg were so much higher than other trace elements, this variable was not included in the PCA. Three principal components (PC 1–3) were identified and used along with Mg and location in general linear model analyses (PROC GLM) to explore how these variables were related to eggshell structure (thickness, FWHM, MULT).

Results

Samples

Eggs were collected from 13 nests in the reference site (Blythe Island) and 21 nests in the contaminated site (LCP). The number of eggs collected per nest in both sites varied from 2 to 10, with an average of 4 eggs per nest, though nests may not have a complete clutch.

Eggshell Thickness

Eggshell thickness was uniform along the waist of an individual shell based on the measurements recorded at 90° intervals ($\pm 3 \mu\text{m}$, 1σ , $n = 4$) from X shells. Also, eggshell thickness among shells of a nest was uniform with variability typically of $\pm 2 \mu\text{m}$ (1σ ; see Appendix 1 for details). Eggshell thickness, averaged by nest, ranged from 150 to $190 \mu\text{m}$ in the

contaminated site and from 160 to $220 \mu\text{m}$ in the reference site (Table 1; Appendix 1). Eggshells were 7% thinner in the contaminated site ($\bar{x} = 171 \pm 10 \mu\text{m}$, $n = 21$) compared to the reference site ($\bar{x} = 183 \pm 15 \mu\text{m}$, $n = 13$). In some nests from the contaminated site, eggshells (averaged per nest) were up to 17% thinner than the reference site. A comparison of all eggshells from the two sites revealed a significant difference in thickness ($p < 0.0001$, $n = 23$).

Microstructure of Eggshells

Figure 2 shows the shell microstructure in a cross-section of an eggshell from the reference site as viewed with an optical microscope. Under cross-polarized light (Figure 2b), the boundaries between columnar calcite crystals were clearly visible by the slightly different degree of extinction of the crystals. This property indicated that crystallographic orientations differed slightly among grains. The lateral width of crystals increased from about $25 \mu\text{m}$ at the mammillary layer to $50 \mu\text{m}$ at the top of the palisade layer. The palisade layer had a relatively high concentration of organic matrix compared to the outermost shell and the mammillary layer, as seen in plane-polarized light (Figure 2c). In many shells, there was a layer of red pigmentation that lay just below the cuticle that cross-cut part of the palisade layer (Figure 2d).

Eggshell Thickness and Crystal Orientation

The crystal orientation, measured as $\text{FWHM}/2$ (averaged by nest) ranged from 16° to 41° in the contaminated site and from 12° to 32° in the reference site (Table 1; Appendix 1). The variability in the degree of crystal orientation of eggshells from a single nest was larger in the contaminated site ($\pm 7^\circ$, 1σ , $n = 21$) than in the reference site ($\pm 5^\circ$, 1σ , $n = 13$). A comparison of all eggshells ($n = 146$) from the two sites revealed a significant difference in FWHM ($p = 0.0003$, $n = 131$). Furthermore, the product of FWHM and thickness (MULT) was significantly different between sites ($p = 0.0003$, $n = 131$).

To determine if any pattern could be found for the relationship of crystal orientation and thickness between the two sites, the data were smoothed by evaluating the mean of thickness and FWHM for each nest. Eggshell thickness and crystal orientation were highly correlated ($r = -0.66$, $p < 0.0001$, $n = 34$). Figure 3 shows the angular relationship between crystals of the outer shell surface (measured as FWHM) and eggshell thickness. The data are plotted as averages by nest in a ln-ln plot. The value of FWHM decreased with shell thickness. This trend indicated that as the thickness of the eggshell increased, calcite crystals at the outer surface of the eggshell were more uniformly oriented. For the reference site, the log-transformed data were best described by a polynomial function $\{\ln(\text{FWHM}) = 319 - 118 * \ln(\text{Thickness}) + 11 * [\ln(\text{Thickness})]^2$; $r^2 = 0.83$; $n = 13$).

Based on this relationship, it is possible to evaluate the data of the contaminated site within the context of a reference model (the eggshells from the reference site were assumed to be free from the effects of contamination). The number of points from the contaminated site lying under the curve is 15, compared to

Table 1. Eggshell thickness and crystal orientation, by nest, of clapper rails (from the LCP site and Blythe Island; variance is reported as standard deviation, 1 σ)

	Thickness (mm)	FWHM (Degrees)	% N
LCP—contaminated site			
Max	190	41	0.33
Min	150	16	1.13
Mean	171 $\pm$ 10	24 $\pm$ 7	0.53 $\pm$ 0.20
Blythe Island—reference site			
Max	220	32	0.27
Min	160	12	2.43
Mean	183 $\pm$ 15	19 $\pm$ 5	0.78 $\pm$ 0.70

only 6 above the curve. This is anomalous given the fact that eggshells from the reference site are equally distributed above and below the curve (by definition). This observation indicates that eggshells from the contaminated site have a higher crystal orientation than eggshells with the same thickness from the reference site. However, because the data were not normally distributed and the relationship is among averaged data, more meaningful relationships could not be defined.

PCBs and Metals in the Eggshells

The concentration of PCBs was at background levels for a composite sample of eggshell from each site. Summary statistics for the concentration of metals in eggshells (averaged by nest) from the two sites are given in Table 2. Eggshells from nests from the contaminated site (LCP) have a higher concentration of Mg, P, Mn, Cu, Zn, Se, Pb, and Hg than those from the reference site (Blythe Island). However, when using a significance level of $p < 0.05$, only the elevated concentration of mercury was statistically significant ($p = 0.00001$). The concentration of mercury in eggshells from the LCP site ranged from 0.095 to 0.766 ppm ($\bar{x} = 0.368 \pm 0.193$ ppm, $n = 21$), while from the reference site (Blythe Island), the values ranged between 0.039 and 0.241 ppm ($\bar{x} = 0.105 \pm 0.066$ ppm, $n = 13$).

There was a strong positive correlation between the concentration of several metals in the eggshell (see correlation matrix; Table 3, top). The concentrations of Al, Fe, and Mn were most strongly correlated ($p \leq 0.0002$ for all data, $n = 34$). Also, there was a significant positive correlation between the concentration P and Zn ($p = 0.0003$), with several other metals being correlated at $p < 0.01$ (e.g., P and Mg, Pb and Al, Zn and Hg, and Cu and Mg). In the reference site, the positive correlations of Fe with Al and Mn, Pb with Fe and Mn, and Mn and Al were significant ($p < 0.0001$), whereas Pb and Al was significant at $p \leq 0.0002$ (Table 3, middle). No other association were significant, except Cu and Hg ($p = 0.05$) and Cu and Mg ($p = 0.04$). In the contaminated LCP site, the concentrations of P and Mg, P and Zn, Fe and Al, and Fe and Mn were positively correlated and significant ($p < 0.001$). Pb was negatively correlated with Zn, and Hg was positively correlated with Zn ($p < 0.01$), while six other metal pairs displayed significant positive or or negative correlations ($p < 0.05$; Table 3, bottom).

For the first principal component (PC1), Al, Fe, and Pb loaded heavily in a positive direction, and Hg and Zn heavily loaded in the opposite (negative) direction. Hg, Al, Mn, Zn, and Fe heavily loaded in a positive direction for PC2, and no metals loaded in the opposite direction. Last, PC3 loaded heavily for Al, Mn, Fe, and Zn, while Pb and Hg loaded heavily in the opposite direction (Table 4).

Organic Matter Content in Eggshells

The content of organic matter in eggshells estimated as the % N was higher in the reference site compared to the contaminated site. The % N in eggshells ranged from 0.33 to 1.13% in the contaminated site and from 0.27 to 2.43% in the reference site (Table 1). Eggshells have a higher % N in the contaminated site ($\bar{x} = 0.53 \pm 0.20$ %, $n = 21$) compared to the reference site ($\bar{x} = 0.78 \pm 0.70$ %, $n = 13$). However, the comparison of eggshells from the two sites reveals no significant difference in the % N ($p = 0.13$, $n = 34$).

Comparison of Eggshell Microstructure Between Sites

It was not possible to distinguish eggshells among sites based on optical properties alone.

Effect of Metals on Eggshell Microstructure

Correlation analysis showed that the thickness of the eggshell was negatively correlated with the concentration of certain metals (Table 5). When analyzing all data from both sites and using a level of significance of $p < 0.05$, eggshell thickness decreased significantly only with increasing concentration of Hg ($r = -0.36$, $n = 34$, $p = 0.04$), and marginally with Mg ($r = -0.34$, $n = 34$, $p = 0.05$; Table 5, top). However, when the data were analyzed by site (Tables 5, middle, bottom), there was no correlation of thickness with Hg ($p > 0.75$ for both sites). Interestingly, Cu was highly correlated with thickness at the contaminated site ($r = -0.64$, $p = 0.002$; Table 5, bottom) but showed little correlation at the reference site ($r = 0.32$, $p = 0.29$; Table 5, middle).

Likewise, the correlation analysis showed that LFWHM was related to the concentration of certain metals. When analyzing all data from both sites and using a level of significance of $p < 0.05$, LFWHM increased significantly with increasing concentration of magnesium ($r = 0.42$, $n = 34$, $p = 0.01$) and zinc ($r = 0.44$, $n = 34$, $p = 0.01$; Table 5, top). When the data were analyzed by site (Table 5, middle, bottom), the only metal that correlated with LFWHM at the reference site was Zn ($r = 0.56$, $p < 0.05$) while at LCP, LFWHM was correlated with Mg ($r = 0.55$, $p = 0.01$) and P ($r = 0.46$, $p = 0.03$). Interestingly, the correlation of LFWHM with Mg and P at the contaminated site ($p \leq 0.03$ for both metals; Table 5, bottom), was not reproduced at the reference site ($p > 0.4$ for both metals; Table 5, middle).

LNMULT (thickness* FWHM) showed a significant correlation with Mg ($r = 0.36$, $p = 0.04$), Zn ($r = 0.41$, $p = 0.02$),

Fig. 2. (a) Sketch showing the microstructure of an eggshell. Arrows represent the orientation of *c*-axis of calcite crystals. (b–d) Photomicrographs of cross-sections of clapper rail eggshells from Blythe Island, as observed with an optical microscope. Scale bars = 100 μm . (b) Cross-polarized light. Calcite crystals show different degrees of extinction, making the boundaries between crystals clearly visible. (c) Plane-polarized light. The palisade layer (dark) has a relatively high concentration of organic matter. (d) Plane-polarized light. Some eggshells show a red pigmented layer just below the cuticle

and Hg ($r = 0.35$, $p = 0.04$). When analyzing the data by site, LNMULT was correlated only with Zn at the reference site ($r = 0.56$, $p = 0.05$) and Mg ($r = 0.51$, $p = 0.02$) at the LCP site.

Because metals individually showed only relatively weak correlations to microstructural parameters, multiple regression analyses between metals and microstructural parameters were performed to test for any synergetic cooperations. These analyses indicated there was no relationship between eggshell thickness and all metals ($p = 0.3123$, $F = 1.25$, Adj. $r^2 = 0.06$, $n = 34$). There was no relationship between the LFWHM and all metals ($p = 0.1857$, $F = 1.55$, Adj. $r^2 = 0.13$, $n = 34$) and between LNMULT (the product of FWHM and thickness) and all metals ($p = 0.1636$, $F = 1.63$, Adj. $r^2 = 0.15$, $n = 34$). PCA analyses (Table 4) revealed that 86% of the variation in the metal data could be explained in the first three PCs (41%, 31%, and 14%, respectively). Due to low eigenvalues (≤ 0.15) and variance proportions ($\leq 0.06\%$), only the first three PCs were used in GLM procedures. There was no relationship between eggshell thickness, crystal orientation (FWHM), or the product of thickness and orientation (MULT) with PC 1 through 3, Mg, or location.

Fig. 3. Relationship between the degree of orientation of crystals (FWHM) and the thickness of the eggshell (by clutch) for the two populations of eggshells studied (Blythe Island = closed squares, LCP = open circles). The line describes the least squares fit for the reference site data. Data from the contaminated site generally fall below this line

Table 2. Concentrations (ppm) of different metals in eggshells of clapper rails from the LCP site and Blythe Island

	Mg	Al	P	Mn	Fe	Cu	Zn	As	Se	Pb	Hg
LCP—contaminated site											
Max	2,146	186	5,938	13	286	3.30	19.37	0.42	1.58	2.62	0.766
Min	1,212	18	2,597	3	77	0.81	2.15	0.03	0.48	0.08	0.095
Mean	1,617	79	3,617	7	147	1.71	6.33	0.21	0.90	0.37	0.368
SD	204	42	804	3	60	0.58	4.19	0.09	0.26	0.52	0.193
Blythe Island—reference site											
Max	1,934	207	4,469	11	330	2.59	11.69	0.43	1.39	0.46	0.241
Min	1,037	39	2,601	2	89	0.31	2.19	0.11	0.40	0.10	0.039
Mean	1,513	88	3,473	5	159	1.37	4.80	0.21	0.71	0.23	0.105
SD	237	48	555	2	72	0.64	2.36	0.09	0.28	0.10	0.066

Table 3. Top: Correlation matrix for metals in eggshell for clapper rail eggs from both the contaminated (LCP site) and unimpacted marsh (Blythe Island). Middle: Correlation matrix for metals in eggshell for clapper rail eggs from the unimpacted marsh (Blythe Island). Bottom: Correlation matrix for metals in eggshell for clapper rail eggs from the contaminated site (LCP)

	Mg	Al	P	Mn	Fe	Cu	Zn	Pb	Hg
Pearson correlation coefficients, n = 34 Prob > r under H0: Rho = 0									
Mg		−0.09493	0.49504	0.09914	−0.06793	0.4485	0.34856	−0.05464	0.19521
Al	0.5933		−0.37187	0.59683	0.91731	−0.16538	−0.13303	0.50729	−0.23794
P	0.0029	0.0303		−0.05556	−0.33967	0.19473	0.57972	−0.38419	0.24746
Mn	0.577	0.0002	0.755		0.71635	−0.00512	0.20165	0.22662	0.35584
Fe	0.7027	<0.0001	0.0494	<0.0001		−0.14081	−0.06129	0.4328	−0.16707
Cu	0.0078	0.3499	0.2698	0.9771	0.427		0.05419	−0.07833	0.35701
Zn	0.0434	0.4532	0.0003	0.2528	0.7306	0.7608		−0.35994	0.47459
Pb	0.7589	0.0022	0.0249	0.1975	0.0106	0.6597	0.0365		−0.11285
Hg	0.2686	0.1754	0.1582	0.0389	0.345	0.0382	0.0046	0.5251	
Pearson correlation coefficients, n = 13 Prob > r under H0: Rho = 0									
Mg		0.26877	0.10699	0.30826	0.28563	0.58686	0.06729	0.22407	0.34339
Al	0.3746		−0.15466	0.89571	0.97345	−0.12689	0.27152	0.8573	−0.1569
P	0.7279	0.6139		−0.02015	−0.17992	0.43359	−0.20868	−0.09453	0.20269
Mn	0.3055	<0.0001	0.9479		0.91538	0.00618	0.42795	0.9282	0.11077
Fe	0.3442	<0.0001	0.5564	<0.0001		−0.12082	0.2197	0.8756	−0.10025
Cu	0.035	0.6795	0.1388	0.984	0.6942		0.06261	0.01864	0.72104
Zn	0.8271	0.3695	0.4938	0.1446	0.4708	0.839		0.47258	0.36091
Pb	0.4618	0.0002	0.7587	<0.0001	<0.0001	0.9518	0.1029		0.12923
Hg	0.2507	0.6087	0.5066	0.7187	0.7445	0.0054	0.2257	0.6739	
Pearson correlation coefficients, n = 21 Prob > r under H0: Rho = 0									
Mg		−0.31722	0.68702	−0.18011	−0.40194	0.33105	0.45144	−0.20975	−0.01208
Al	0.1612		−0.45918	0.53842	0.88544	−0.09313	−0.21558	0.401	−0.05032
P	0.0006	0.0363		−0.1472	−0.46533	0.06494	0.73599	−0.48616	0.28131
Mn	0.4346	0.0118	0.5243		0.67673	−0.18063	0.06719	−0.21907	0.36746
Fe	0.0709	<0.0001	0.0335	0.0008		−0.06764	−0.14324	0.2225	0.0521
Cu	0.1427	0.688	0.7797	0.4333	0.7708		−0.02224	−0.15416	−0.12294
Zn	0.04	0.348	0.0001	0.7723	0.5357	0.9238		−0.56926	0.62539
Pb	0.3615	0.0716	0.0254	0.34	0.3323	0.5046	0.0071		−0.4691
Hg	0.9586	0.8285	0.2167	0.1013	0.8225	0.5955	0.0024	0.0319	

All data except Mg were log-transformed to meet normality assumptions. The upper matrix represents the *r*-values of the correlation; the bottom matrix shows the associated *p*-values

Discussion

There were significant differences in eggshell structure between the two sites, suggesting that some attributes of the LCP site adversely affected eggshell mineralization. Eggshells from the contaminated site were substantially thinner (7%), while for shells of the same thickness, those from the contaminated site had a relatively higher crystal orientation than those from the reference site. Specifically, eggshells from nest HH from the

contaminated site are the thinnest shells and have an abnormally high orientation given their thickness. In addition, shells from the contaminated site had higher concentrations of most heavy metals. Specifically, mercury concentrations in eggshells from the contaminated site (up to 0.766 ppm) are the highest reported in the literature. However, there are only a few studies for comparison (Gonzalez and Hiraldo 1988; Burger 1994; Morera *et al.* 1997) in which concentrations of heavy metals were measured in the eggshell.

Table 4. Principal component analyses (PCA) to determine the relationship between metal concentrations in eggshells from the contaminated site (LCP site) and the unimpacted site (Blythe Island). (A covariance matrix was used in the PCA to preserve the scale relationship of the variance differences among the metals)

Covariance Matrix	Al	P	Mn	Fe	Cu	Zn	Pb	Hg
Al	0.43	-0.05	0.21	0.31	-0.04	-0.04	0.24	-0.13
P	-0.05	0.04	-0.01	-0.03	0.02	0.06	-0.05	0.04
Mn	0.21	-0.01	0.29	0.19	0.01	0.05	0.09	0.16
Fe	0.31	-0.03	0.19	0.25	-0.03	-0.02	0.15	-0.07
Cu	-0.04	0.02	0.01	-0.03	0.15	0.01	-0.01	0.13
Zn	-0.04	0.06	0.05	-0.02	0.01	0.25	-0.13	0.20
Pb	0.24	-0.05	0.09	0.15	-0.01	-0.13	0.50	-0.07
Hg	-0.13	0.04	0.16	-0.07	0.13	0.20	-0.07	0.68

Eigenvalues of the Covariance Matrix								
	Eigenvalue		Difference		Proportion		Cumulative	
1	1.06		0.26		0.41		0.41	
2	0.80		0.43		0.31		0.72	
3	0.37		0.21		0.14		0.86	
4	0.15		0.04		0.06		0.92	
5	0.11		0.05		0.04		0.96	
6	0.06		0.04		0.02		0.99	
7	0.02		0.01		0.01		0.99	
8	0.01				0.01		1.00	

Eigenvectors	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8
Al	0.57	0.23	0.27	-0.05	0.15	-0.55	0.44	-0.17
P	-0.09	0.02	0.07	0.13	0.21	0.23	0.58	0.73
Mn	0.21	0.47	0.25	-0.20	-0.24	0.70	0.16	-0.26
Fe	0.40	0.22	0.27	-0.09	0.05	-0.03	-0.64	0.54
Cu	-0.11	0.14	-0.17	-0.49	0.82	0.11	-0.07	-0.13
Zn	-0.21	0.26	0.35	0.74	0.39	0.05	-0.15	-0.21
Pb	0.49	0.14	-0.75	0.38	0.09	0.17	-0.02	0.00
Hg	-0.41	0.76	-0.27	-0.08	-0.22	-0.34	0.00	0.13

Laboratory and field studies suggest that metals like mercury, lead, copper, and aluminum impair eggshell formation and cause eggshell thinning (Stoewsand *et al.* 1971; Lundholm and Mathson 1986; Lundholm 1991; Eeva and Lehtikoinen 1995). The observed reduction of eggshell thickness at the contaminated site (up to 17% in some nests) is similar to that described for other areas contaminated with high concentrations of heavy metals in Finland (Eeva and Lehtikoinen 1995) and acidified areas in Norway (Nybo *et al.* 1997).

Also, metals can directly inhibit the growth of calcium carbonate crystals, which constitute the eggshell (Bischoff 1968; Wada *et al.* 1995). In fact, small concentrations of certain metals can have a dramatic effect on the growth rate, mineral type, size, and morphology of crystals (Parsiegla and Katz 1999a, 1999b). It is well known that Mg inhibits calcite nucleation, favoring the precipitation of aragonite (Bischoff 1968; Morse 1985) in marine settings. In fact, Mg^{2+} controls the polymorphic form of calcium carbonate in many environments (Bischoff 1968). Wada *et al.* (1995) showed that Fe^{2+} , Ni^{2+} , and Zn^{2+} favor the precipitation of aragonite over calcite. Parsiegla and Katz (1999a, 1999b) showed that Cu strongly reduced the precipitation rate of calcite. Also, phosphate and orthophosphate ions are strong inhibitors of calcite crystal growth (Mucci 1986).

It has been tested by statistical analysis if any of the metals

or a combination were responsible for the alterations in eggshell properties (described at the beginning of this discussion). The observed reduction of eggshell thickness could be explained in part by the inhibitory effect of these elements on calcite growth. However, the statistical analysis indicated that the variation in eggshell properties (*i.e.*, thickness, crystal orientation) explained by metals is relatively small. This suggests that other pollutants may be responsible for the observed changes in shell attributes or that the matrix of interactions between the studied variables is more complex than can be resolved by the experimental and statistical protocols used here. For instance, a major problem for the interpretation of the effect of individual metals is their association. The association of different metals found in the eggshells, *e.g.*, Al, Fe, and Mn, can be explained by specific metalloproteins that bind these metals and carry them in the bloodstream to the shell gland where they may be incorporated in organic matrix. For instance, vitellogenin binds Fe and Mn and conalbumin binds Al and Fe (Richards and Packard 1996). Also, the association of metals could be related to the precipitation of other phases known to exist in eggshells at low concentration. For example, phosphate minerals, which commonly occur in mineralized tissues, are known to bind a variety of metals (*e.g.*, Zn) in their structure.

Other major contaminants in the LCP site were PCBs. They

Table 5. Top: Correlation matrix for eggshell structure and metals for clapper rail eggs from both the contaminated (LCP site) and unimpacted marsh (Blythe Island). Middle: Correlation matrix for eggshell structure and metals for clapper rail eggs from the unimpacted marsh (Blythe Island). Bottom: Correlation matrix for eggshell structure and metals for clapper rail eggs from the contaminated (LCP site)

	LNMULT	LFWHM	LTHICK
Pearson correlation coefficients, n = 34 Prob > r under H0: Rho = 0			
Mg	0.36 <i>0.04</i>	0.42 <i>0.01</i>	-0.34 <i>0.05</i>
Al	-0.13 <i>0.45</i>	-0.05 <i>0.77</i>	0.15 <i>0.41</i>
P	0.24 <i>0.17</i>	0.30 <i>0.09</i>	-0.11 <i>0.54</i>
Mn	0.14 <i>0.42</i>	0.20 <i>0.26</i>	-0.18 <i>0.31</i>
Fe	-0.18 <i>0.30</i>	-0.09 <i>0.62</i>	0.13 <i>0.45</i>
Cu	0.18 <i>0.32</i>	0.16 <i>0.36</i>	-0.31 <i>0.08</i>
Zn	0.41 <i>0.02</i>	0.44 <i>0.01</i>	-0.25 <i>0.16</i>
Pb	-0.15 <i>0.41</i>	-0.15 <i>0.39</i>	0.14 <i>0.45</i>
Hg	0.35 <i>0.04</i>	0.30 <i>0.09</i>	-0.36 <i>0.04</i>
Pearson correlation coefficients, n = 13 Prob > r under H0: Rho = 0			
Mg	0.09 <i>0.78</i>	0.22 <i>0.47</i>	-0.25 <i>0.40</i>
Al	0.03 <i>0.91</i>	0.16 <i>0.60</i>	-0.14 <i>0.64</i>
P	-0.23 <i>0.45</i>	-0.05 <i>0.86</i>	0.30 <i>0.33</i>
Mn	0.12 <i>0.69</i>	0.25 <i>0.41</i>	-0.21 <i>0.48</i>
Fe	0.05 <i>0.88</i>	0.17 <i>0.59</i>	-0.12 <i>0.70</i>
Cu	-0.29 <i>0.34</i>	-0.15 <i>0.63</i>	0.32 <i>0.29</i>
Zn	0.56 <i>0.05</i>	0.56 <i>0.05</i>	-0.42 <i>0.16</i>
Pb	0.03 <i>0.91</i>	0.16 <i>0.61</i>	-0.06 <i>0.85</i>
Hg	0.24 <i>0.43</i>	0.29 <i>0.34</i>	-0.10 <i>0.76</i>
Pearson correlation coefficients, n = 21 Prob > r under H0: Rho = 0			
Mg	0.51 <i>0.02</i>	0.55 <i>0.01</i>	-0.37 <i>0.10</i>
Al	-0.12 <i>0.60</i>	-0.14 <i>0.55</i>	0.23 <i>0.31</i>
P	0.41 <i>0.06</i>	0.46 <i>0.03</i>	-0.28 <i>0.23</i>
Mn	-0.01 <i>0.96</i>	0.04 <i>0.87</i>	0.10 <i>0.66</i>
Fe	-0.31 <i>0.18</i>	-0.30 <i>0.19</i>	0.28 <i>0.21</i>
Cu	0.31 <i>0.17</i>	0.29 <i>0.19</i>	-0.64 <i>0.00</i>
Zn	0.33 <i>0.14</i>	0.40 <i>0.07</i>	-0.11 <i>0.62</i>
Pb	-0.28 <i>0.22</i>	-0.35 <i>0.12</i>	0.34 <i>0.13</i>
Hg	0.08 <i>0.73</i>	0.15 <i>0.51</i>	0.01 <i>0.98</i>

All data except Mg were log-transformed to meet normality assumptions. The first line of the matrix represents the *r*-values of the correlation; the second line (italics) shows the associated *p*-values.

are hormonal disrupters and can affect the enzyme activity involved in calcium transportation and consequently affect eggshell thickness (Cooke 1973; Baird 1995). However, this hypothesis could not be tested because PCBs were not measured above background concentration in eggshells from the contaminated LCP site. This is not surprising considering the hydrophobic character of PCBs. Because the eggshell is mostly calcite, an ionic solid, the absence of PCBs in the eggshells were expected even though the levels in the environment were high. For instance, it has been already shown that PCBs are bioavailable in the LCP system and have been found in elevated levels in chick embryos (Kannan *et al.* 1998). Therefore, the possibility still exists that PCBs were responsible for the eggshell thinning. The influence of PCBs on the eggshell mineralization has yet to be explored in sufficient detail to make a definitive interpretation of the results presented here.

Finally, the observed eggshell thinning (7%) in the contaminated site is below the suggested critical value of 15–20%, beyond which extensive eggshell breakage occurs in nests of native birds (Newton 1979). However, the combination of a reduction in eggshell thickness and a higher degree of orientation of crystals (lower values of FWHM) observed in eggshells from the contaminated site probably affected the mechanical properties of the eggshells, explaining their abnormal brittleness. Furthermore, based on the relationship found between crystal orientation and eggshell strength (maximum load) by Rodriguez-Navarro *et al.* (2002), the 20° decrease in FWHM observed for some eggshells from the contaminated site could result in a decrease in the strength of the eggshell of up to 30%, clearly above the 20% threshold identified by Newton (1979).

The possibility has been explored that metals could affect directly the mineralization of eggshells by inhibiting crystal growth. This may have affected the eggshell thickness as well as its microstructure. In future studies, this hypothesis will be explored in detail.

Acknowledgments. This study has been financed by the U.S. Fish and Wildlife Service and an award (DE-FC09-96-SR18546) from the U.S. Dept. of Energy to the University of Georgia Savannah River Ecology Laboratory. We thank Jim Novak for helpful discussions about the statistical analysis. Keith Hastie (USFWS) is thanked for useful comments and corrections to the manuscript. We thank Brian Jackson for the ICP-MS analysis of samples and Gary Mills and Dwella Moton for the analysis of PCBs.

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Appendix 1. Eggshell thickness and crystal orientation (FWHM) for LCP site and Blythe Island

Clutch	Egg ID #	Thickness (μm)	FWHM/2 (Degrees)	Clutch	Egg ID #	Thickness (μm)	FWHM/2 (Degrees)
LCP site				LCP site			
A	3	188	20	GG	1	157	25
A	4	194	24	GG	5	168	18
A	5	176	18	GG	6	164	18
A	6	212	16	GG	8	152	27
A	7	199	21	H	2	164	33
A	8	172	15	H	3	157	29
B	2	184	20	H	4	161	28
B	3	172	20	H	5		36
B	4	189	16	HH	1	143	25
B	6	178		HH	2		38
B	5		33	HH	3	153	
C	2	174	16	HH	4	146	30
C	5	178	16	HH	5	159	20
BB	1	169	16	HH	6	156	19
BB	5	180	16	HH	8	153	
BB	8	161	16	HH	9	142	35
CC	2	179	16	I	1		15
CC	3	164	15	I	2	179	27
CC	6	197	16	I	4	171	16
DD	10		21	I	5	182	22
DD	2	179	29	I	6	153	46
DD	4	165	26	I	7	178	17
DD	5	168	28	I	8	170	21
DD	7	168	23	II	2	180	19
DD	8	184	23	II	3	164	15
DD	9	178	21	II	4	182	17
E	1	176	21	J	1		19
E	2	192	25	J	3	175	28
E	4	189	20	J	5	164	27
E	5	160	23	J	6	173	23
E	7	185	33	J	7	158	28
E	8	175	20	K	1	169	22
EE	1	173	29	K	2	170	19
EE	2	145	31	K	4	170	14
EE	3	168	28	K	5	160	13
EE	4	181	21	K	6	173	38
EE	5	157	23	K	7	174	18
EE	7	152	31	K	8	178	15
EE	8	162	21	K	9	167	15
EE	9	167	24	L	8	190	19
F	1	167	48	L	#4/29		16
F	2	177	31	M	4	175	21
F	3	178	21	M	1	143	30
F	5	168	35	M	2	171	22
F	6	163		M	3	171	41
FF	2	172	18	N	1	167	20
FF	5	177	18	N	2	164	35
FF	6	165	20	N	3	174	22
FF	7	170	19	N	4	168	24
G	4	157	26				
G	6	156	47				
G	7	161	50				

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Appendix 1. Continued

Clutch	Egg ID #	Thickness (μm)	FWHM/2 (Degrees)
Blythe Island			
AA	4	173	19
AA	5	185	18
D	1		17
D	2	181	22
D	3	173	29
D	4	183	17
D	6	188	18
D	7	173	19
D	8		20
D	9	173	27
O	1	171	20
O	2	178	20
O	3	179	24
O	4	172	17
O	5	174	17
O	6	182	16
P	1	169	22
P	2	165	31
R	4	160	32
S	2	220	12
T	1	183	24
T	2	180	19
T	4	199	16
T	5	175	16
T	6	195	17
T	7	170	18
T	8	195	18
T	9		14
U	1	177	21
U	2	175	15
U	4	176	18
U	8	184	15
V	1	197	
V	5	202	18
V	6	208	15
W	1	185	20
W	2	184	16
W	3	193	19
W	4	184	17
W	7	192	18
X	2	181	17
X	4	173	19
Y	5	183	14
Z	1	182	23
Z	2	179	21

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Appendix 2. Concentrations of metals (ppm) and % N in eggshells per clutches for LCP site and Blythe Island

Clutch	Mg	Al	P	K	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Cd	Pb	Hg	% N
LCP site																
A	1212	102.7	2631	798	12.99	228.5	0.734	0.481	1.018	3.78	0.168	0.475	0.007	0.225	0.502	0.46
B	1472	32.4	4007	1617	4.36	97.0	0.865	0.821	1.400	12.54	0.203	0.854	0.006	0.081	0.766	0.35
BB	1313	127.4	3281	867	12.54	249.9	0.784	0.421	1.912	4.38	0.300	0.822	0.003	0.340	0.606	0.44
C	1567	48.1	3285	1018	5.96	111.6	0.907	0.655	1.294	5.84	0.207	0.505	0.005	0.302	0.675	1.13
CC	1701	186.4	3179	1288	8.79	286.4	0.766	0.491	1.464	4.13	0.399	0.858	0.004	0.367	0.207	0.59
DD	1611	78.5	2597	650	12.67	162.3	0.732	0.420	1.715	4.05	0.247	0.814	0.005	0.237	0.342	0.41
E	1488	73.3	4073	880	7.09	132.6	0.916	0.489	0.815	6.98	0.131	0.619	0.004	0.129	0.313	0.48
EE	1721	73.8	3139	1051	6.35	130.3	0.796	0.592	1.996	3.80	0.296	1.445	0.003	0.257	0.288	0.79
F	1716	106.5	3344	956	6.42	123.7	0.814	0.533	1.884	4.73	0.144	0.964	0.005	0.272	0.269	0.33
FF	1419	115.2	3004	606	7.30	242.6	0.794	0.418	1.618	3.36	0.236	0.923	0.002	0.299	0.265	0.33
G	1852	94.0	4358	1141	7.37	117.3	0.773	0.426	1.608	6.98	0.198	0.870	0.004	0.166	0.766	0.81
GG	1636	72.4	3652	940	6.41	127.7	0.769	0.407	1.976	2.87	0.192	1.125	0.003	0.307	0.138	0.36
H	2146	17.6	5938	1770	6.60	78.0	0.784	0.468	2.195	10.95	0.181	0.954	0.003	0.106	0.457	0.40
HH	1450	21.1	2951	668	3.17	76.7	0.789	0.485	2.207	3.51	0.029	0.798	0.002	0.106	0.234	0.53
I	1942	44.9	4734	790	4.96	115.4	0.840	0.420	1.494	19.37	0.156	1.118	0.004	0.173	0.468	0.60
II	1789	30.5	4102	576	3.40	82.7	0.806	0.543	2.347	3.60	0.176	0.743	0.003	0.151	0.214	0.53
J	1645	53.3	5186	1014	6.35	106.7	0.746	0.463	1.330	13.34	0.197	0.695	0.005	0.176	0.371	0.40
K	1547	63.3	3124	505	4.05	101.0	0.781	0.505	1.364	2.82	0.120	0.820	0.005	1.179	0.234	0.49
L	1543	54.7	2929	616	2.50	100.7	0.735	0.449	1.170	2.15	0.188	0.898	0.003	2.623	0.148	0.38
M	1563	152.6	3200	1096	4.70	209.6	0.685	0.427	2.766	7.52	0.420	1.299	0.005	0.425	0.404	0.64
N	1804	117.0	3689	657	6.85	198.3	0.692	0.430	1.866	9.16	0.262	0.977	0.005	0.300	0.312	0.62
Blythe Island																
AA	1813	433.5	2688	850	19.87	819.9	0.913	0.755	1.199	5.15	0.816	0.730	0.007	0.808	0.062	0.99
D	1655	64.7	3106	862	3.48	132.2	0.956	0.771	1.777	4.84	0.199	0.655	0.006	0.288	0.241	0.38
O	1685	97.1	3472	472	4.45	150.6	0.756	0.550	1.782	4.16	0.177	0.588	0.004	0.181	0.163	0.49
P	1464	65.1	3416	4028	4.12	154.0	0.724	0.458	0.922	4.43	0.427	1.388	0.001	0.161	0.110	0.27
R	1934	207.1	3766	865	11.25	329.8	0.804	0.735	1.660	6.18	0.349	0.773	0.009	0.461	0.153	2.43
S	1472	173.9	4468	782	7.83	303.4	0.824	0.566	1.867	3.53	0.333	0.468	0.004	0.434	0.108	2.16
T	1523	45.8	3162	629	4.24	88.9	0.768	0.533	1.793	6.79	0.204	0.609	0.004	0.234	0.197	0.49
U	1325	59.6	4134	699	4.59	110.0	0.742	0.387	1.199	3.81	0.170	0.418	0.002	0.190	0.072	0.41
V	1447	38.5	3259	998	1.83	89.1	0.769	0.408	1.688	3.38	0.186	0.769	0.001	0.114	0.096	0.50
W	1525	61.5	3626	566	2.97	111.5	0.908	0.619	0.974	4.01	0.147	0.402	0.003	0.214	0.046	0.39
X	1037	130.3	2601	320	3.86	197.3	0.659	0.457	0.309	4.25	0.150	0.448	0.007	0.234	0.036	0.44
Y	1912	57.8	3393	978	2.14	104.2	0.802	0.481	1.599	2.51	0.174	0.632	0.001	0.101	0.055	0.65
Z	1556	97.4	3459	642	3.52	132.6	0.765	0.566	1.506	6.84	0.200	0.666	0.004	0.194	0.066	0.48
Blank sample	2	-2.7	22	6	0.07	59.4	0.024	-0.009	0.006	0.19	-0.018	-0.053	-0.001	-0.002	-0.013	0.00

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