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## Comparative Analysis of Lead in *Crassostrea angulata* and *Cerastoderma edule* from Cádiz, Spain

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### Acknowledgements

I would like to sincerely thank Dr. Kyle Grice, Mary Jones, Dr. James Montgomery, Dr. Jason Bysrtiansky, and the Departments of Chemistry, Environmental Science and Studies, and Biology for facilitating this collaborative project. Shell samples were collected by Dr. Bystrianksy, Mary Jones, and students in the DePaul study abroad program "From Atoms to Ecology, Science is Global."

## Comparative Analysis of Lead in Clam and Oyster Shells from Cádiz, Spain

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**ABSTRACT** In order to study the capacity of bivalve shells as bioindicators in estuarine ecosystems, a comparative analysis of lead concentration in oyster (*C. angulata*) and clam (*C. edule*) shells collected near the coast of Cádiz, Spain was performed. Infrared spectroscopy was used to assess calcite and aragonite compositional differences in the shells. An atomic absorption spectrometer was employed for lead concentration method development and analysis. Infrared spectroscopy results found that *C. edule* shells may contain more aragonite than *C. angulata* shells. The average lead concentration in oyster shells was calculated to be  $44 \pm 24$  ppm from one site  $17 \pm 8$  ppm from a second site. Clam shells collected from a third site were found to have concentrations of  $43 \pm 10$  ppm. Further work is needed to determine relationships between lead accumulation in various species and anthropogenic pollution.

### INTRODUCTION

Anthropogenic influence on global climate and local environments has been occurring since antiquity (Cariou et al. 2017). Following the Industrial Revolution, human influence on different environments has grown exponentially, with wide-ranging effects from land degradation and mineral exploitation to the consumption of fossil fuels and plastic pollution. Carbon dioxide released from human activity like oil, gas, and coal production has led to the highest atmospheric concentrations of CO<sub>2</sub> in the last 420,000 years (Royal Society 2005). These anthropogenic ef-

fects continue to threaten all areas of the environment, and marine ecosystems are especially susceptible to environmental degradation and ecological collapse due to the carbon sequestration provided by the oceans and the near constant flux of mining effluents entering marine environments.

Currently, near 50% of all CO<sub>2</sub> from fossil fuel emissions in the last two centuries is sequestered into the ocean system (Royal Society 2005). Other anthropogenic effects damage ocean environments such as excessive nutrient runoff from

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agricultural activities and heavy metal pollution. In particular, lead (Pb) pollution dramatically increased during and after the Industrial Revolution (Cariou et al. 2017).

Benthic bivalve mollusks, a class of marine organisms, have an evolutionary adaptation in which large concentrations of trace essential and nonessential metals can be adsorbed into their calcium carbonate-based shells, including lead. Wang et al. (2018) conducted a literature review indicating that higher concentrations of lead in bivalve organisms lead to DNA breakage in oyster embryos, increased levels of proteins associated with protein misfolding repair, endoplasmic reticulum stress, inhibition of immune recognition processes, and accumulation of lead in lysosomes. Almeida et al. (1998) also indicated genomic mutations from increased concentrations of lead in *Crassostrea gigas*, an oyster from the same genus as the species observed in this study.

*Crassostrea virginica*, the eastern oyster, is a commonly consumed species of oyster found along the eastern coast of North America in the Atlantic Ocean (Kennedy 1996). Given the history of changes in the population ecology and local niche of *C. virginica* (Rothschild et al. 1994), this species marks an interesting case where both environmental degradation and ocean acidification have the capability to significantly influence the species. Huanxin and Presley (2000) found no significant, linear relationship between heavy metal concentrations in the Gulf of Mexico and in *C. virginica* oysters, but Guo et al. (2001) were able to identify an increase in metal uptake with an increase in dissolved organic carbon concentrations. The oyster species studied in this work was *Crassostrea angulata*, a relative of *Crassostrea gigas* and *Crassostrea virginica*.

As a fast-growing clam species found across the western coast of North Atlantic, the southern coast of the Mediterranean, and the Black Sea, *Cerastoderma edule* is also an ideal species to conduct trace metal pollution analysis (Tyler-Walters 2007). The wide geographic range the species is found in along with its sensitivity to a variety of environmental constraints produce a

strong case for *C. edule* to be used as a bioindicator of heavy metal contaminants.

This study sought to analyze benthic bivalve shells in order to establish lead content in different species collected from the same overall area in Spain. Additionally, the shell composition was assessed to determine any potential relationships between species, shell composition, and lead content. The results of this initial study are reported below.

## METHODS

### Sample Collection

*Crassostrea angulata* samples were collected in July 2019 off the coast of Spain from two different sites. One site was located at 36.2803 N, 6.1453 W, and was adjacent to an industrial warehouse with garbage along the coastline with a high flux of human interaction. The other site, 36.2923 N, 6.1556 W, was located on a nature preserve relatively isolated to the public. Comparatively, *Cerastoderma edule* were collected in July 2017 on the coast of the Río de San Pedro in Cádiz, Spain. Samples were located at 36.527841 N, 6.217789 W on a shoreline on a National Park site accessible to the public. All three sites were within approximately 15 miles of each other on the southern coast of Spain on the Atlantic Ocean.

### Infrared Analysis

Infrared spectroscopy is an incredibly useful tool for identifying molecular compounds and their concentrations in solid, liquid, or gaseous phases. Shell samples were ground in a mortar and pestle, and the resulting powders were analyzed for carbonate ratios qualitatively with an ABB FTLA 2000 Fourier-transformation infrared spectrometer. A PIKE Technologies Inc. MIRacle attenuated total reflectance (ATR) sample holder was used. Eight scans were collected from 2250-550  $\text{cm}^{-1}$  and averaged after subtracting the background spectrum (air). The wavenumber ( $\text{cm}^{-1}$ ) range was selected because it included the theoretical wavenumbers of calcite, aragonite, and lead, the species of interest in this study. These vibrations relate to the molecular structure of the carbonate anions packed in the solid.

### Sample Preparation and Lead Analysis

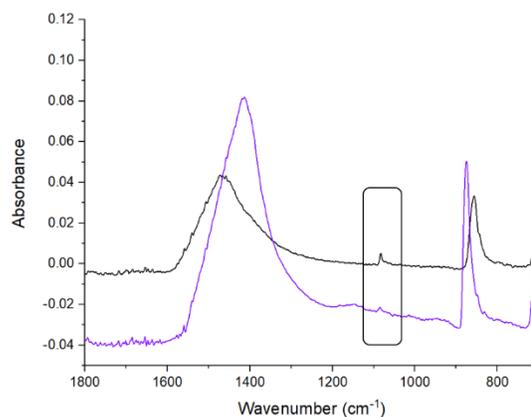
Pulverized shell samples were dissolved in excess 16 N nitric acid (Caution! Concentrated nitric acid is very caustic and releases noxious fumes during the dissolution of shells. Therefore, all reactions were carried out in a fume hood). Insoluble biopolymers were released from shells during dissolution, and the samples were filtered with 0.2  $\mu\text{m}$  syringe filters to remove particulates. Clam shell samples then were diluted to  $50 \pm 0.05$  mL while oyster shells were diluted to  $100 \pm 0.08$  mL with deionized water. Because of the mass difference between oysters and clams, different dilutions were necessary in order to dissolve the entire shell and keep the acidity of the solution at a level that would not disrupt the results from the instrument used. Then, samples were analyzed for lead concentration using a Varian AA240FS, flame atomic absorption spectroscopy (FLAA). The 217.0 nm Pb wavelength was used for analysis. The FLAA generates a calibration curve by diluting a stock solution of Pb and fitting it using the New Rational method. The New Rational method is one of many calibration methods available in the FLAA, but the New Rational method is most often the default. In addition, an independent calibration curve was generated using a linear regression based on Beer's Law and used in data analysis.

Beer's Law is a powerful tool in spectroscopic analysis. It states that there is a linear relationship between the light absorbed by a sample and the concentration of the chemical species of interest in solution. The equation is  $A = abc$ , where  $A$  is absorbance (unitless),  $a$  is molar absorptivity (in  $\text{M}^{-1}\text{cm}^{-1}$ ),  $b$  is the cell pathlength (cm), and  $c$  is concentration (M). Using a linear regression based on a calibration curve with known concentrations, the concentrations of species can be interpolated based on absorbance values. Absorbance holds no units because it represents a ratio of light that was able to pass through solution versus the amount of light that was absorbed. The wavelength of light that is used is crucial because different species will absorb different amounts of light at different wavelengths.

## RESULTS & DISCUSSION

### Infrared Spectroscopy

Bivalve shells are primarily composed of calcium carbonate ( $\text{CaCO}_3$ ) along with a small amount of other materials and biopolymers. There are multiple mineral forms of calcium carbonate, and the most common in bivalve shells are calcite and aragonite. These compounds can be characterized with infrared (IR) spectroscopy. The infrared spectra of both *C. angulata* and *C. edule* were taken from a variety of samples. Figure 1 presents a representative example of these different shell structures, where the black line represents the *C. edule* sample and the purple line represents the *C. angulata* sample. The large, broad peaks around  $1400\text{--}1450\text{ cm}^{-1}$  represent carbonate. The shape of the carbonate peak differs slightly between aragonite and calcite structures. The calcite peak appears to tilt to the right compared to the clear, Gaussian peak aragonite exhibits (Jovanovski et al. 1999) around the  $750\text{ cm}^{-1}$ . However, a clear key difference between aragonite and calcite is the peak at around  $1100\text{ cm}^{-1}$  that is characteristic of aragonite. Lastly, aragonite exhibits a split peak. Figure 1 reiterates that the *C. edule* shells contain significantly more aragonite than the *C. angulata* shells, though *C. angulata* most likely contained some aragonite due to the small peak at  $1080\text{ cm}^{-1}$ .



**Figure 1.** The infrared spectra of a clam shell (black) and an oyster shell (blue), with the characteristic signal for aragonite highlighted in the box.

### Trace Lead Concentration

In order to determine the trace lead concentration in *C. angulata* and *C. edule* shells, a calibration curve independent of the built-in software used by the Varian Atomic Absorption spectrometer was calculated through a linear regression. Through the calculated calibration curve, standard error was experimentally derived. Experimental error was found through the repeated testing of the same standard ten times within the same data set. The error was determined to be 4%, and Table 1 outlines the average concentration values found in the three different sample sets. For the *C. angulata* samples from a presumed uncontaminated site,  $44 \pm 24$  ppm of lead was found in the shells on average (ppm is equivalent to mg Pb per kg of shell). Located in what was considered a contaminated site, the lead concentration in samples was averaged to be  $17 \pm 8$  ppm. There was less lead found in samples from the “contaminated” site, indicating that what is deemed visible evidence of human influence and may not correlate with actual levels of heavy metal contamination. *C. edule*, calculated at  $43 \pm 10$  ppm, also had very high levels of lead.

Shell ID	Pb (mg/kg)	Pb error
<i>C. angulata</i> “Contaminated”	17	8
<i>C. angulata</i> “Uncontaminated”	44	24
<i>C. edule</i>	43	10

**Table 1.** The average concentration of lead found in benthic bivalves off the coast of Spain based on linear calibration curves generated from absorbance values.

Due to the high sensitivity of the FLAA, the instrument provides robust data on trace element concentration in dissolved bivalve samples. However, the machine has constraints in terms of the ability to develop independent calibration curves. Often throughout sampling or if ever paused, the FLAA recalibrates. The recalibration can pose an issue in data analysis because the realibration changes the baseline from which the samples are

being compared. Additionally, it is difficult to compare data sets between days due to the nature of instrument preparation. The FLAA has an optimization process in which the seat of the flame, the location of the beam, and the intensity of the beam are adjusted to optimize the robustness of the instrument per sample analyzed. Therefore, best practices ought to require the analysis of larger sample pools in one data set. We highly recommend running independent calibration curves before each sample set and examining the slope of the data. On the other hand, the FLAA is known to occasionally have values drift, which is why it is also necessary to perform quality control intermittently throughout the data set using pre-made standards. Additionally, redundancy in samples is crucial to ensure accurate results.

Table 1 highlights the resulting trace lead concentration found in *C. angulata* and *C. edule* shells. The results, though comparable to biomonitoring data found along the Bay of Cádiz, was much higher than expected. T.J.J. Hanebuth et al. (2018) found in modern day sites along the Bay of Cádiz, Pb in sediment ranged from 35-60 ppm. Usero et al. (2016) noted that bioaccumulation in benthic invertebrates occurs through both estuarine water and ingested sediment, which reflects a potential correlation between lead concentrations found in benthic invertebrates and localized sediment. Usero had also found that samples found south of the bridge José León de Carranza, along the Inner Bay, were in an estuary that favors pollution accumulation versus those collected along the Outer Bay along the Atlantic Ocean. Seeing as the *C. edule* samples were found near the opening, the higher concentration of lead found in those samples compared to oyster samples found along the Atlantic Ocean is reasonable.

The oyster data, additionally, suggests that even though a location may seem secluded from the public and protected by policy, environmental contamination can still occur. Overall, benthic invertebrates, and other forms of invertebrates, are great tools in environmental monitoring because of their dependence on environmental conditions to determine their growth patterns. Their ability to filter pollutants makes these creatures indicator species in their estuarine community ecosystems,

and valuable remediators, removing heavy metals from their environment and sequestering them in their shells.

The use of solid-state infrared spectra to determine relative abundance of carbonate structures in conjunction with trace lead concentration in shells collected from the same sites provided a

unique insight to the way that contamination is perceived. It was clear that heavy metal contamination was relatively less in areas with greater human activity, indicating that other factors like currents and topography have a greater influence on the flow of pollutants than the location of activity. There was no clear relationship between bivalve species and lead, but further testing is needed.

**ACKNOWLEDGEMENTS.** I would like to sincerely thank Dr. Kyle Grice, Mary Jones, Dr. James Montgomery, Dr. Jason Bysrtiansky, and the Departments of Chemistry, Environmental Science and Studies, and Biology for facilitating this collaborative project. Shell samples were collected by Dr. Bysrtiansky, Mary Jones, and students in the DePaul study abroad program "From Atoms to Ecology, Science is Global."

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