Final Report: St. Lucie Oyster Biomarker Study

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Introduction

The presence of metals and other contaminants in the environment does not automatically translate into adverse effects since bioaccumulation reflects the net effects of various physico-chemical factors that affect bioavailability. Moreover, bioaccumulation does not automatically translate into toxicity as organisms may respond by sequestering or detoxifying the chemicals. Therefore, sensitive reliable biomarkers can be used to determine if there is evidence of significant contaminant exposures that have exceeded detoxification or compensatory mechanisms and are resulting in adverse effects on physiological and biochemical responses. Cellular biomarkers represent the most sensitive indicators of toxicity. Lysosomal destabilization, glutathione concentrations (GSH), and lipid peroxidation levels (LPx) have been investigated in a variety of bivalve species and are recognized as valuable indicators of sublethal toxicity. Eventually these cellular stress effects would be reflected in more general physiological effects such as decreased growth and reproduction, and ultimately cause significant effects on the sustainability of oyster populations.

Lysosomes are organelles that are involved in numerous functions, including cellular defense, tissue repair and turnover, and nutrition. There is a substantial body of literature validating that environmental pollutants cause destabilization of lysosomes, and that lysosomal function assays of hepatic or blood cells are regarded as valuable indicators of pollutant-induced injury (Auffret, 1988; Adema et al., 1991; Moore, 1994; Ringwood et al., 1999a). Neutral red techniques for assessing lysosomal destabilization have been used successfully in fish and invertebrate taxa (Lowe et al., 1995; Moore et al., 2004; Ringwood et al., 2005), and are being incorporated into major European programs (BIOMAR, Black Sea Mussel Watch). We have used these techniques with oysters since 1997 and some of our important early findings included: oysters (native animals as well as deployed juveniles) from polluted sites had increased destabilization of lysosomes relative to oysters collected from uncontaminated sites; lysosomal responses were significantly correlated with sediment and tissue contaminants; and laboratory studies indicated that lysosomal responses were not affected by salinity regimes, but were very sensitive to copper in a dose-dependent manner (Ringwood et al., 1998a; 1999a, 1999b).

Lipid peroxidation reflects oxidative damage to lipid-rich components such as cell membranes that occurs as a result of increased OH• radicals, especially in the presence of elevated levels of redox reactive metals such as copper (Cu). Moreover, the free radical induced damage propagates additional cytotoxic products that can damage DNA and enzymes (Kehrer, 1993; Yu, 1994). Increased lipid peroxidation has been demonstrated in response to contaminant exposures in fish and bivalves (Di Giulio et al. 1989; Viarengo et al., 1990; Ringwood et al., 1998b; Livingstone, 2001).

Glutathione (GSH) is an abundant tripeptide that is regarded as one of the most important "first-line" defense mechanisms of cells. Glutathione is the most abundant intracellular thiol and an important antioxidant detoxification mechanism. Potential oxidative damage associated with Cu exposures can be ameliorated when GSH binds Cu and effectively sequester it from interaction with other cellular components, thereby potentially reducing adverse effects. Animal cells may respond to contaminants by increasing GSH levels as well as other detoxification mechanisms such as metallothioneins in an effort to reduce adverse effects. However, if the detoxification mechanisms are overwhelmed, the GSH production and recycling may be impaired, leading to decreased or depleted GSH levels. Therefore elevated GSH levels can indicate that organisms are exposed to a pollutant, but ultimately adverse effects are associated with GSH depletion as the detoxification mechanisms are overwhelmed. Glutathione depletion has been observed in mammalian systems as well as marine organisms, and it has been hypothesized that GSH depletion is both a signal of stress and a predisposing factor for increased adverse effects (Meister and Anderson, 1983; Viarengo et al., 1990; Regoli and Principato, 1995). Our recent data support this hypothesis. Laboratory studies indicated that GSH depleted oysters were more susceptible to metal stressors (Conners and Ringwood, 2000; Ringwood and Conners, 2000).

The overall purpose of these studies was to use oysters, *Crassostrea virginca*, to assess the potential biological and ecological impacts of Cu concentrations in the St. Lucie, Fl system. For this assessment, oysters were placed in cages from January to May, 2004 at various sites in the St. Lucie in order to evaluate the effects on the accumulation of Cu, cellular biomarker responses, and overall physiological condition.

Methods

Hatchery-reared oysters (*Crassostrea virginca*) were deployed *in situ* at 5 sites in the St. Lucie system in January (Figure 1). Approximately 4 months later, the oysters were retrieved and subsamples of oysters were sent overnight to A. Ringwood's laboratory, and the cellular biomarker assays (lysosomal destabilization, GSH, lipid peroxidation) were conducted on hepatopancreas tissues of individual oysters (n=10 oysters per site). Another subset of oysters from each site (n=25) were used to determine the over condition index and to determine tissue metal levels. The dates of deployment and salinity regimes for each site are summarized in Table 1.

A neutral red assay was used to evaluate lysosomal stability in live cells derived from hepatopancreas tissues of oysters (Ringwood et al., 1998a; Ringwood et al., 2005). Cell preparations from hepatopancreas tissues of individual oysters were prepared using Ca^{++}-Mg^{++}-free saline (CMFS) and trypsin as illustrated in Figure 2. Cell preparations were incubated in CMFS containing neutral red for a fixed time period (60 minutes), and the numbers of cells with neutral red retained in the lysosomes (e.g. stable lysosomes) or with neutral red leaking into the cytoplasm (destabilized lysosomes) were determined with a compound microscope (40x magnification); examples of stable and destabilized lysosomes are presented in Figure 3. A minimum of 50 cells was scored for each preparation, and the results were expressed as percent destabilized lysosomes.

Figure 1. Oyster deployment sites in the St. Lucie, FL system. See Table 1 for explanation of abbreviations

Table 1. List of site locations and dates of deployment. Information on salinity regimes (means and standard deviations) is also listed (based on data collected by M. Kandrashoff at 8 different intervals during the deployment period)

Site Abbreviation	Site Description	Latitude	Longitude	Salinity Mean (Stdev)	Deployment Date	Retrieval Date
NSR	East of Roosevelt Bridge Northern Shoreline	27 12.686	80 14.534	23.6 (4.4)	1/27/2004	5/19/2004
SOR	East of the Roosevelt Bridge Southern Shoreline	27 12.198	80 15.315	21.5 (3.5)	1/27/2004	5/19/2004
NOB	North of Bessie Creek North Fork of River	27 12.956	80 17.216	18.9 (3.5)	1/27/2004	5/18/2004
EAB	East of Bessie Creek North Fork of River	27 12.36	80 16.396	18.6 (3.9)	1/27/2004	5/18/2004
PAL	Palm City South Fork of River	27 11.11	80 15.992	15.1 (5.0)	1/27/2004	5/18/2004

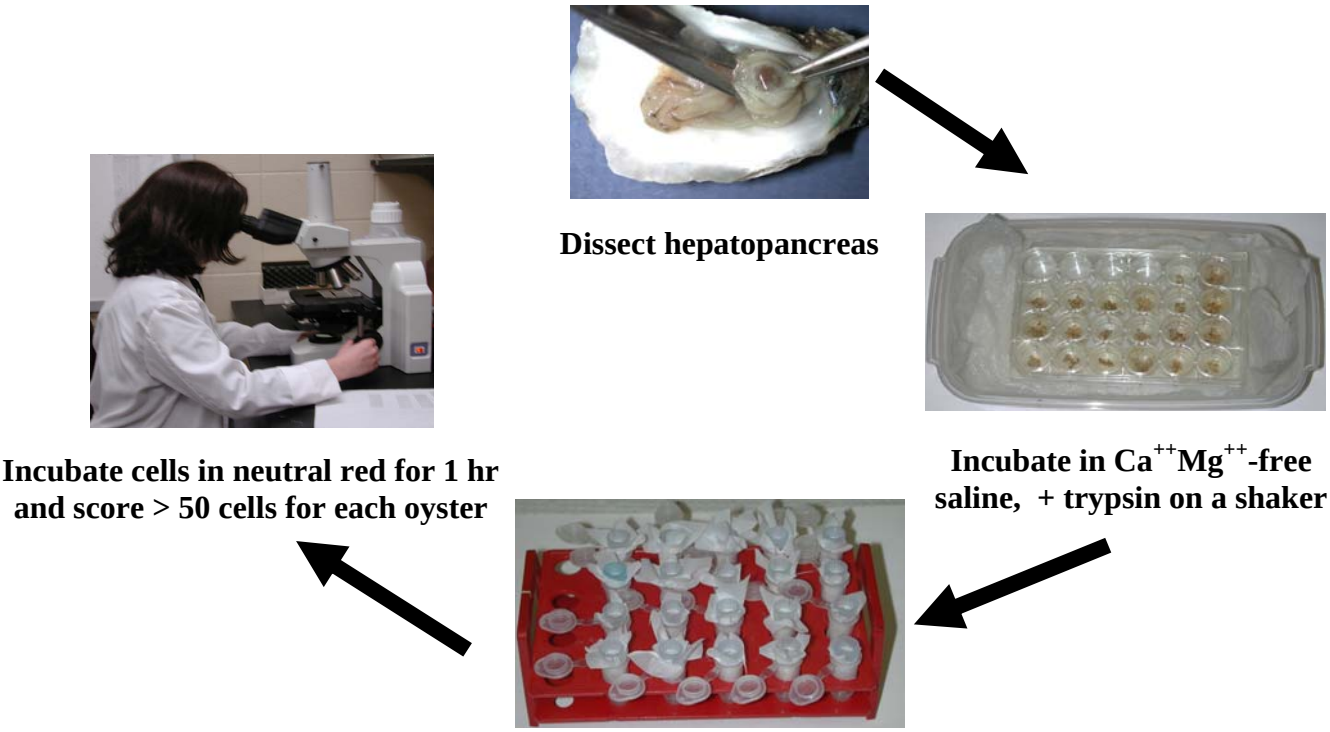
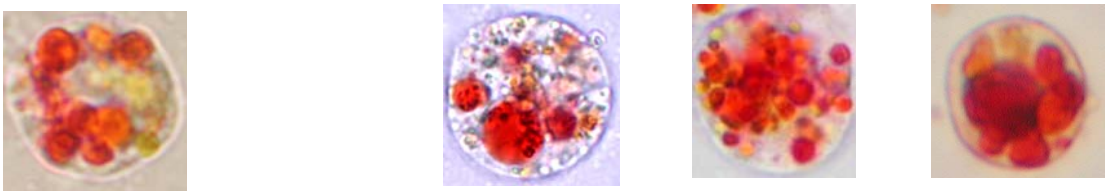


Figure 2. Summary diagram of the techniques use for the lysosomal destabilization assay



a. Stable Lysosomes **b. Destabilized Lysosomes**

Figure 3. Examples of oyster hepatopancreas cells with (a)stable lysosomes or (b) destabilized lysosomes using the lysosomal destabilization assay. In cells with stable lysosomes, the neutral red is retained inside the lysosomes. In cells with destabilized lysosomes, the neutral red is observed in the cytoplasm, which reflects the leaking of lysosomal enzymes into the cytoplasm, eventually leading to cell death.

Lipid peroxidation levels of oyster hepatopancreas tissues were measured using the thiobarbituric acid (TBA) reaction for the detection of malondialdehyde (MDA), a lipid peroxidation product (Gutteridge and Halliwell, 1990; Ringwood et al., 1998b and 1999a). Frozen tissues were homogenized in potassium phosphate buffer, and reacted with TBA and butylated hydroxytoluene, and the MDA derivatives were then detected with a spectrophotometer at 532 nm. Lipid peroxidation was estimated from a calibration curve with malondialdehyde tetraethylacetal (MDTA) as the standard, and the data were expressed as umol MDA/g tissue wet weight.

Glutathione (GSH) concentrations of hepatopancreas tissues were measured by the enzymatic recycling assay (Anderson, 1985; Ringwood et al., 1998b and 1999a). Total GSH was extracted from frozen tissues in 5% sulfosalicylic acid, and GSH concentrations, based on the rate of production of 2-nitro-5-thiobenzoic acid (detected at 412 nm with a spectrophotometer, measured over a period of 2 minutes), were estimated from a standard curve derived from purified GSH. The data were expressed as umol GSH/g tissue wet weight.

Condition index (CI) was determined on individual oysters from each site ($n = 22 – 25$). Oysters were dissected, and the shells and tissues were dried (tissues were lyophilized; shells were dried in an oven), and weighed. The condition index was calculated as tissue weight / shell weight x 100.

Metal analyses of the tissues were conducted using the same tissues that were used for the condition index. Three to five individuals were pooled for each replicate so that there were 5 replicate samples for the metal analyses from each site. The pooled lyophilized tissues for each replicate were ground to a powder using a mortar and pestle and mixed. A subsample of approximately 0.1 g was removed and placed into a Teflon digestion vessel. The tissues were digested with ultrapure nitric acid in a microwave, and diluted with deionized water for analysis by atomic absorption spectroscopy (Perkin Elmer AAnalyst 800). Copper concentrations were determined using the graphite furnace, equipped with Zeeman background correction; Zn concentrations were determined using the flame mode. Standard reference tissues (NIST oyster tissue 1566b) were processed and analyzed with each batch. The percent recoveries over all batches ranged between 90-105%. The data were expressed as ug metal / g tissue dry weight.

The data were summarized (means and standard deviations), and all data were analyzed using Sigma Stat. Significant differences were identified using ANOVA (significant when $p < 0.05$), and checks were conducted to ensure that the data fulfilled the requirements of normality and equal variance. Student-Newman-Keuls *a posterior* tests were used to identify site specific differences.

Results and Discussion

The primary impetus for these studies was a concern about potentially elevated Cu concentrations in the St. Lucie system. The results of these studies provide an assessment of tissue metal levels as well as biomarker responses to facilitate an appreciation of the significance of the metal concentrations. The results for all parameters, metals and biomarkers, are summarized in Table 2). The concentrations of Cu in oyster tissues over all sites ranged from 372 to 505 ug/g. The annual geometric mean concentrations reported in oysters from 1986-1993) as part of the NOAA mussel watch program ranged from 110 – 150 ug/g (O'Conner, 1996; summary table of these data also provided in Fisher, 2004). Similarly, in deployed oyster studies conducted in South Carolina from 1998 – 2000, the Cu levels of oysters from reference sites typically ranged from approximately 50 – 140 ug/g (Ringwood et al., 2003). Therefore the levels measured in the oysters deployed in the St. Lucie are around three times the levels reported in either of these assessments, and similar to levels observed in polluted systems such as Chesapeake Bay, etc. (Fisher, 2004). In this study, the highest tissue Cu levels were observed in the Bessie Creek areas (NOB and EAB) and the Northern Shore Roosevelt Bridge site (NSR), e.g. all approximately 500 ug/g; somewhat lower, but still relatively high, levels were observed at the Palm City (PAL) site and Southern Shore of the Roosevelt Bridge Site (SOR), e.g. approximately 400 ug/g. The sites with the highest Cu levels (NOB, EAB, and NSR) were all significantly higher than those measured in oysters from PAL and SOR. Therefore these data support the contention that there are high levels of Cu in the system that are bioavailable to oysters. Zinc levels on the other hand were similar to those reported by O'Conner (1996) and Ringwood et al. (2003), e.g. in the range of 2000 ug/g, indicating that while elevated Cu levels are present in the St. Lucie, Zn levels do not appear to be significantly elevated.

Table 2. Summary of biomarkers and tissue metal concentrations measured in oysters deployed in the St. Lucie.

Site	% Destabilized Lysosomes		Glutathione (umol /g wet wt)		Lipid Peroxidation (umol / g wet wt)		Condition Index		Tissue Cu (ug/g dry wt)		Tissue Zn (ug/g dry wt)	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
NSR	51.49	6.37	1007.1	285.4	182.0	83.5	3.06	0.79	505.4	43.9	2262.4	304.3
SOR	56.33	5.93	1248.1	383.5	203.5	100.2	2.54	0.68	418.8	72.1	2359.1	464.0
NOB	53.11	4.93	1021.6	375.6	474.1	203.7	3.31	0.99	495.6	160.8	2502.8	390.8
EAB	53.55	3.71	1076.3	154.6	216.0	146.0	2.85	0.68	485.6	145.7	2177.8	672.1
PAL	43.43	4.56	1003.7	185.5	192.7	86.5	3.90	1.03	372.3	85.0	2083.5	406.4

Lysosomal destabilization rates were also relatively high at all sites. The lowest levels were observed with oysters from the Palm City site, and the levels at NOB, EAB, NSR, and SOR

were all significantly higher than those of PAL. These results are consistent with those of laboratory studies in which oysters were exposed to Cu, which indicated that Cu-exposed oysters had significantly higher rates of lysosomal destabilization (Ringwood et al., 1998a). Generally lysosomal destabilization rates under control conditions (laboratory or field are less than 35%), so the levels measured in the St. Lucie oysters do indicate stress. Moreover, levels > 40% are typically linked to poor gamete viability. As shown in Figure 4, gametes from parents collected from field sites over a 2 year period with lysosomal destabilization rates >40% tended to have very low rates of embryo development (Ringwood et al., 2004).

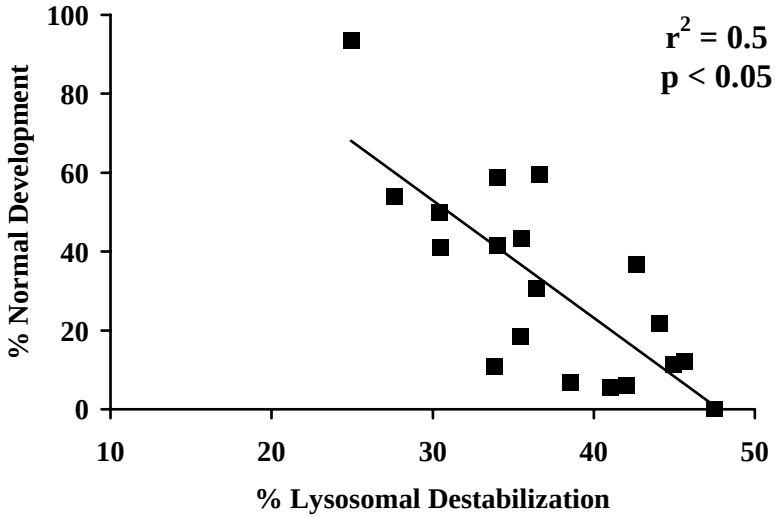


Figure 4. Relationship between lysosomal destabilization rates and embryo development. Oysters were collected from various field sites from 2000-2001 during their peak reproductive season (May). The lysosomal destabilization assay was conducted with the parents and their gametes were used for 48 hr embryo development assays, conducted with clean reference seawater (4 replicate tubes, each with 200 embryos per site using pooled gametes).

Only one site showed evidence of significantly higher lipid peroxidation levels, e.g. NOB. All other sites were low, and typical of what we have measured as being representative of normal levels (approx 150 umol/g). There were no significant differences in GSH levels in oysters from the different sites, and these are levels that are typical of those that we have consistently measured in oysters. Oysters from the Palm City site had a significantly higher condition index than the other four sites.

Copper is of course an essential element and is believed to play important roles in immune responses associated with hemocyte functions (Fisher, 2004) as well as other normal cellular processes. Oysters are renown for accumulating high levels of Cu in their tissues in response to elevated environmental levels. In Fisher's publication, he presents arguments that high levels of Cu that oysters accumulate are actually beneficial and important to robust immune

responses of hemocytes, endowing the ability to protect against invading pathogens and parasites. However, these issues were discussed in terms of mortality as an endpoint of toxicity, and he further acknowledges that he did not include publications or discussions of the extensive literature on sublethal effects. One of the points that is made is that very few laboratory studies demonstrated effects on survivorship, even though high levels of Cu were accumulated in the tissues. This is typical of metal related impacts. Acute toxicity is rarely observed quickly or over short time periods (weeks) that are typical of laboratory exposures. Metal toxicity is more chronic in nature, so sublethal effects can be observed over shorter time periods that will eventually result in mortality or significant impairment of physiological functions such as growth and reproduction. Therefore, while Cu (and Zn) are essential for robust immune responses and other essential cellular processes, levels such as those observed in oysters in the St. Lucie are likely to be related to significant adverse effects.

In summary, the Palm City site appeared to be the least impacted site. Oysters from this site had the lowest Cu (and Zn) concentrations, had the highest condition index, and had the lowest rates of lysosomal destabilization. However, the data suggests that even these oysters were suffering at least some level of stress that could affect normal physiological functions such as reproduction. At all of the other four sites, oysters had lysosomal destabilization rates > 50% and significantly higher tissue Cu concentrations, and would likely be even more stressed than those at the Palm City site. Overall these results suggest that elevated Cu levels in the St. Lucie are sufficient to contribute to chronic sublethal stress in oysters or other benthic organisms.

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