Accumulation and elimination of copper by the Flat-tree oyster *Isognomon alatus*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In

MARINE SCIENCE

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2007

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ABSTRACT

A baseline study of copper concentrations in *I. alatus* from Bioluminescent Bay during May 2004 to June 2005 was conducted. In general, average copper levels were (7.64-17.1 μg/g dry wt), which are lower than those previously reported for *I. alatus*. The accumulation dynamics of copper by *Isognomon alatus* was examined in the laboratory. Accumulation of Cu was observed at concentration ≥0.1 mg Cu/L. Survival was 100% in oysters exposed to concentrations of 0.1-1.0 mg Cu/L. High mortality was observed at concentrations from 1.5 to 3.0 Cu mg/L after 3-5 days of exposure while the average oyster´s Cu tissue content ranged 115-362 μg/g dry wt. At exposure of 3.87 mg Cu/L the oysters reached Cu concentrations of 1022 and 1259 μg/g dry wt, and 100% of mortality was observed. Furthermore, oysters exposed to 0.5 mg Cu/L for 5 days were transplanted to the field during a 14 days depuration/elimination study to determine depuration rates and biological half-lives ($B_{½}$) of Cu. In average, these oysters reached a maximum of 72 μg/g dry wt within the first day of incubation and 95% of the copper accumulated was eliminated after 11 days of depuration/elimination, reaching final concentration similar to the control (13 μg/g dry wt). An average biological half-live of 6.0 days was observed thus indicating an efficient depuration capacity by *I. alatus*.

RESUMEN

Se realizó un estudio de trasfondo de las concentraciones de Cu en *I. alatus* de la Bahía Bioluminiscente durante mayo 2004 a junio 2005. En general, los niveles de cobre promedio fueron (7.64-17.1 μg/g peso seco), los cuales son más bajos que los reportados en estudios previos. La dinámica de acumulación de cobre en *Isognomon alatus* fue examinada en el laboratorio. La acumulación de Cu fue observada a concentración ≥0.1 mg Cu/L. Los ostiones expuestos a concentraciones de 0.1-1.0 mg Cu/L presentaron 100% de sobrevivencia. Alta mortandad fue observada en ostiones expuestos a 1.5 a 3.0 Cu mg/L después de 3-5 días de exposición, mientras que el contenido de Cu promedio en el tejido de los ostiones fue de 115-362 μg/g peso seco. A exposición de 3.87 mg Cu/L los ostiones alcanzaron concentraciones de Cu de 1022 y 1259 μg/g peso seco y 100% de mortalidad fue observada. Además, ostiones expuestos a 0.5 mg Cu/L por 5 días fueron transplantados al campo durante un estudio de depuración/eliminación de 14 días para determinar las tasas de depuración y las medias vidas biológicas ($B_{½}$) de Cu. En promedio, esos ostiones alcanzaron un máximo de 72 μg/g peso seco en el primer día de incubación y 95% del cobre acumulado fue eliminado después de 11 días de depuración/eliminación, alcanzando una concentración final similar al control (13 μg/g peso seco). Se observó una media vida biológica promedio de 6 días, lo cual indica que *I. alatus* tiene una capacidad de depuración eficiente.

To my family . . .

ACKNOWLEDGEMENTS

Quiero agradecer a Dios, mi familia (en especial a mi "partner" del alma Josean y a mis padres) y amigos por su amor y darme el balance necesario durante esta larga……jornada. Le agradezco grandemente al Dr. Ernesto Otero por todos sus consejos y su tiempo para ayudarme en la realización de este trabajo. También le doy gracias al Dr. Arturo Massol por su gran apoyo y por adoptarme en su laboratorio. Agradezco también al Dr. José M. López por su ayuda con los materiales y recomendaciones. Le doy las gracias en especial a la Sra. Elba Díaz quien me ayudó y enseñó a trabajar en el laboratorio para analizar las muestras y por su apoyo y defensa de mis ostiones.

Agradezco a todos mis compañeros de CIMA por su compañía en los viajes de campo, en especial a Verónica Acevedo, Elizabeth Mclean, Brenda Soler, Luisa Ramírez, Tony y otros. Muchas gracias al Dr. Kubaryk por las facilidades de laboratorio y al Dr. González por los materiales prestados. A los empleados de Magueyes en especial a Marcos Rosado, Godo, Figueroa, Negrito, etc. A las secretarias Zulma, Lili y Tati por aliviarnos la vida.

Le doy gracias a todo el staff de Sea Grant en especial a Migdalia, Cana, Yuli y Lillian por todo su apoyo y cariño. También al Dr. Valdés y al Sr. Chaparro por brindarme la oportunidad de trabajo en Sea Grant. Agradezco al Programa Sea Grant por subvencionar este sueño con fondos del programa "SEED MONEY".

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1 INTRODUCTION

Heavy metal pollution in the marine environment is a serious problem and the reason for monitoring programs around the world. Bivalves, such as oysters and mussels, are widely used as sentinel organisms for monitoring the concentration of contaminants in coastal environments (Goldberg et al., 1978; Hang and Hung, 1990). Oysters have been identified as good bioindicators of pollution in aquatic environments in worldwide coastal areas (Jaffé et al., 1998) due to their ability to concentrate pollutants. As shellfish bioaccumulate pollutants, their consumption could be hazardous to human or wildlife. Ikuta (1968) reported that Cu accumulation is generally higher in oysters than in other Bivalvia. This trait turns oysters into suitable organisms for copper accumulation studies. Copper is an essential trace metal for animal metabolism but at high levels is a very toxic substance to aquatic life (Langston & Bryan, 1984).

The trace metal in body content of any organism results from the net balance between the process of metal uptake and metal loss (Rainbow et al., 1990). Since accumulation and depuration are two independent processes that influence metal concentrations in the organisms, is important to consider both processes when a monitoring study is conducted. This study examines the depuration capacity of *Isognomon alatus* (flat tree oyster) in its native environment. Laboratory accumulation and field depuration experiments were conducted to estimate the necessary time for each processes to occur under various Cu concentrations. The depuration rates and biological half-lives were determined to characterize the depuration process. Also, a baseline study of the copper concentrations in flat-tree oyster tissue at Bioluminescent Bay (Bahía Fosforescente) from May 2004 to June 2005 was conducted.

Isognomon alatus was used as an ideal bioindicator of pollution (biomonitor) because it is sessile, abundant in the study area, easy to identify and to sample throughout the year. In addition, this bivalve has sufficient tissue for analysis, is an efficient accumulator, and tolerates various copper concentrations which made them suitable for laboratory and transplantation experiments.

1.1 Motivation

There is limited scientific information concerning the use of *Isognomon alatus* as a bioindicator of metals in the environment. The *I. alatus* copper accumulation threshold and depuration capacity in Puerto Rico or in the rest of the Caribbean is unknown. Therefore, the purpose of this study is to increase our knowledge on the accumulation threshold of *I. alatus* and its depuration capacity in their natural environment. Furthermore, the capacity of this species as a bioindicator for monitoring studies will be evaluated. It will be especially useful where other indicator species are not present or when there is a need of transplantation to areas of unknown metal concentrations.

1.2 Literature Review

Factors affecting the bioaccumulation of heavy metals

Physico-chemical, environmental and biological factors affect the uptake and retention of heavy metals by marine organisms (Rainbow et al., 1990). In general, the soft tissue concentration of metals in marine organisms is several times greater than the concentration of metals in the surrounding seawater. Often, the metal level in the organism is proportional to the metal level in seawater so that the organism can be used as a biological indicator of metal pollution. The metal uptake rate by an organism may depend on the element chemical form, which may vary from site to site. Other factors such as pH, temperature, hardness and salinity may affect the rate of metal uptake as well (Rainbow et al., 1990; Ansari et al., 2004). The toxicity of most metals such as Cu was reported to increase with decreasing salinity. Hall and Anderson (1995 in Ansari et al., 2004).

There are different interactions between metals in the natural environment. Antagonistic interactions occur when metal pollutants may interact with each other i.e. when one metal inhibits the uptake of another metal by specific organisms. In contrast, synergistic interactions occur when accumulation is more pronounced in a mixture of two metals than the sum of individually accumulated elements (Connell et al., 1999). Biological factors that influence metal concentration are size, sex, age, growth rate, and reproductive cycle of the organisms (Rainbow et al., 1990; Geffar et al., 2002 Ansari et al., 2004).

Factors affecting heavy metal toxicity

Metal toxicity results from nonspecific metal binding, which can inactivate important regulatory enzymes by displacing essential metal ions from catalytic sites, thus altering the functional conformation of proteins. Mason and Jenkins (1996 in Conners et al., 2000). Geochemical factors of the environment and physiology of the organisms influence toxicity of heavy metals. Among geochemical factors are organic carbon, water hardness, temperature, pH, dissolve oxygen, sediment grain size, and hydrologic features of the system Elder and Collins (1991; Martoja et al., 1988; in Boening, 1999). Studies with metals such as copper have demonstrated that the toxicity and bioavailability of trace metals is highly dependent on their chemical form. Therefore it is important to consider the chemical speciation, that is, the chemical forms of an element (Ansari et al., 2004) in accumulation studies (Zamuda and Sunda, 1982). Usually the common chemical forms of trace metals in natural waters are free ions, inorganic and organic complexes, and the metals absorbed and/or incorporated into particulate matter (Stumm and Brauner, 1975; Sunda and Guillard, 1976; in Engel and Fowler, 1981). The forms of Cu that are the most toxic to aquatic organisms are the dissolved cupric species (free cupric ion; i.e. Cu ($H_2O_6^{2+}$). Factors which control cupric ion activity such as the level of organic and inorganic complexation will therefore control copper toxicity. For example trace metals can form complexes with inorganic ligands such as Cl-, CO_3^{2-}, OH^- and organic ligands (i.e., humates, hydroxamates) (Sunda and Lewis, 1978; in Engel and Fowler, 1981). Other important factor affecting biological responses to trace metals is the relationships between the chemical form of trace metals and its bioavailability. For example, food and water ingestion are sources or

pathways of trace metal incorporation into marine fauna. The chemical speciation of the metal in the aquatic environment should be considered when analyzing the metal pathways in the organisms. Also, the presence of various metals could produce antagonistic (reduction) or synergistic (increase) toxic effects.

Chemical speciation, environmental and biological factors could explain the seasonal variations in trace metal content that have been observed in oysters (Zamuda and Sunda, 1982). Environmental factors such as salinity, dissolved oxygen, temperature, pH, metabolic condition and physiological state of the aquatic organisms, can make them more or less susceptible to toxicity (Ansari et al., 2004). Accumulation can also be in function of different biological factors. The organisms sensitivity to the metals can vary with their life stages. In addition, several other factors can make organisms more or less sensitive to metal toxicity (i.e. sex, age, food availability, reproductive state, genotype, phenotype and feeding activity), causing different metal reaction effects among and within species (Boening, 1999).

Mechanisms of Trace Metal Detoxification

Various detoxifying mechanisms are responsible for limiting the effects of chronic exposure in marine organisms. Oysters have specialized cells called amoebocytes (granular acidophilic hemocytes Auffret (1989, in Ballan- Dufrancais et al., 2001) for storage of copper and zinc. Copper deposits in amoebocytes contribute to the preferential storage of copper as biochemical insoluble forms. The presence of metals in such structures is generally considered as evidence for the deposits being involved in metal metabolism and an indication of the vital role that amebocytes play in metal detoxification. Mason and Jenkins (1996, in Ballan-Dufrancais et al.,

2001). Molluscs, mammals, and crustaceans block heavy metals by complexation with metallothioneins (low molecular weight thio-proteins), and some metals can be immobilized by the formation of stable compounds by antagonistic elements. However, it is possible that the primary function of these low molecular weight metal-binding proteins in oysters is to storage or transport of other physiologically important elements, and only act secondarily in the detoxification of trace metals (Engel and Brouwer, 1982).

Many marine organisms are capable of sequestering substantial concentrations of trace metals in membrane-limited vesicles or granules Coombs and George (1978 in Jenkins and Brown, 1984). Accumulated metals may be concentrated in metal-rich granules, which may have the potential to be excreted. Many bivalves detoxify assimilated trace metals by forming granules in their kidneys (thus maintaining a high body metal concentration with minimal toxic effects), which may be available for excretion (George et al., 1980). Detoxification strategies involving temporary storage lead to raised body metal concentration. Such body concentrations increase steadily if the rate of excretion is constant or oscillate if excretion occurs in pulses, for example, the release of granules high in metals via the kidney. The rate of turnover of metalliferous granules in a bivalve kidney may therefore controls the net rate of metal accumulation in the body of the bivalve (George et al., 1980). George et al. (1980) investigated subcellular localization of heavy metals in polluted marine shellfish and found that the organisms are able to tolerate extreme high concentrations of heavy metals by isolating the potentially toxic metal within membrane-limited vesicles, thereby immobilizing and detoxifying it. These structures are particularly prominent in mollusks and much of the high metal burden observed in these

organisms is attributable to metals in membrane-limited vesicles rather than the free cytoplasm George and Piere (1980; in Jenkins and Brown, 1984). However, the detoxification capacity is limited, and under conditions of high metal uptake or severe stress the capacity to sequester these metals can be exceeded. If these conditions are present, excess metals would spill over and have direct toxic effects on specific sites, such as the enzyme-containing pool. Data reported for bivalves exposed to Cd or Cu is consistent with these observations Engel and Fowler (1979; in Engel et al., 1981).

According to Mason and Jenkins (1996), limiting metal accumulation is one of the strategies adopted by marine organisms to prevent metal toxicity. An alternative mechanism for detoxication of metals is compartmentation within subcellular organelles which prevent reaction with essential enzyme systems in the cytoplasm (George, 1982). Aquatic animals can control intracellular metal concentrations with several detoxication processes such as membrane pumps, transport, storage proteins, and compartmentation in intracellular vesicles. This detoxication processes also prevent the interaction of highly reactive metal ions with essential enzyme systems (George, 1982). Hummel and collaborators (1997) found that Cu in Baltic clams was slowly regulated as a consequence of storage in fractions in which Cu turn-over was slow compared with that of Cu highly exchangeable in the cytosolic fraction (Ballan- Dufrancais et al., 2001).

Also organelles such as the lysosomes are able to play a role in detoxifying. The presence of excess Cu in lysosomes of exposed specimens has been reported and indicates that these organelles can detoxify ions in excess in the medium through precipitation (Jeantet et al., 1997;

in Ballan-Dufrancais et al., 2001). In addition, the basal lamina and intracellular spaces have been described as a site of mineral bioaccumulation of Cu in mollusks.

In aquatic organisms, glutathione (a tripeptide, nonprotein thiol in biological systems (Kosower and Kosower, 1978) is believed to play a fundamental role in metal detoxification (Conners and Ringwood, 2000). Glutathione has been proposed to protect cells from metal-induced oxidative damage by scavenging oxy-radicals and by participating in detoxification reactions catalyzed by glutathione peroxidases (Viarengo, 1989; Winston and Di Giulo, 1991; Mason and Jenkins, 1996; in Conners and Ringwood, 2000).

In summary, oysters have several strategies of metal detoxification. The oysters have amoebocytes and metallothioneins, which play an important role in detoxification. Also, the compartmentalization of metals in different organelles or proteins helps to prevent toxicity. However, the oysters' detoxification capacity can be affected by an increase in metal concentrations or environmental stress.

Effects of Copper in Oysters

The heavy metals of greater concern in aquatic systems are copper, zinc, cadmium, mercury, and lead. The toxicity of these elements is concentration dependent. Copper is an essential nutrient, and a functional constituent of all cells. However, after mercury and silver, copper is one of the most toxic metals to the marine biota.

The main sources of copper pollution are domestic effluents, industrial wastes and antifouling paints (Sunda and Lewis, 1978; in Engel et al., 1981). Copper sulfate is used

throughout the world to kill and inhibit the growth of algae in municipal reservoirs, irrigation equipment and piping, swimming pools and industrial cooling systems. It is also used in animal feed additives and growth promoters, as well as for disease control in livestock and poultry (Grant et al., 1990).

Despite the existence of detoxifying and storage mechanisms, a considerable number of aquatic species are sensitive to copper in the concentration range of 1-10 μg/L (Bryan, G.W., 1976; in Ansari, 2004). Nelson et al. (1988) observed that 2μg/L had significant effects on young bay scallops and surf clams (acute toxicity/ EC50) (Ansari, 2004). Also certain copper concentrations could be toxic to organisms, producing significant histopathological and physiological alterations in gills, digestive gland and heart. The gills of oysters exposed to 200 μg Cu /L during 30 days reach a tissue concentration of 784 μg/g dry wt, and epithelium disorganization and hemocyte infiltration around the conjunctive tissue was observed, also the gill filaments show alteration of the apical portions of the cilia and loss of ciliature. Specimens exposed to 600 μg Cu /L reach 1650 μg/g dry wt tissue concentration after 15 days, and show hyperplasia with lamellar fusion and loss. Also the oysters tissues show increase cell damage with increase in copper accumulation. The digestive gland of oysters exposed to 200 μg/L reach 770 μg/g dry wt and show hemocyte infiltration and an increased in brown cells (which were scarce in control). In the oysters exposed to 600 μg/L (after 7 days= 638 μg/g dry wt) thinning of the digestive gland epithelium and occlusion in the lumen of some digestive diverticula, some individuals show focal necrosis of conjunctive tissue cells with massive necrosis in some cases. Also, an increased in the number of basophile cells of the digestive tubules with increase in time

of Cu exposure was observed. In the heart the histological alterations also increase with higher Cu concentration and increased in time exposure. In the heart was observed the thinning of auricular and ventricular epithelium, an increased of brown cells in auricular walls, distension of the muscular fibers, and necrosis of the connective tissue in auricular and ventricular epithelium (Sarasquete et al., 1992, 1997; Arellano et al., 1999; Ortiz-Delgado, 1999 in Rodríguez de la Rua et al., 2005). Although copper play an important role in structural proteins, enzymes and mammalian metabolism, large doses could be toxic and excessive ingestion could result in accumulation in the liver leading to haemolysis, the destruction of red blood cells (Iyengar, 1989).

Several biomarkers of environmental stressors in marine bivalves have been identified and its presence is related to Cu exposure to the organisms. Examples of biomarkers are heat shock proteins and glutathione. Sanders et al. (1991) observed the accumulation of heat shock protein 60 (hsp 60) in conjunction with a decrease in scope for growth (SFG) measurements in *Mytilus edulis* exposed to sublethal concentrations of copper (McDowell, 2005). Alterations in growth rates of bivalve mollusks occur as a result of reductions in feeding rates, reduced digestive efficiencies, and higher respiratory metabolism. Reductions in physiological measurements (e.g., respiration rates, carbon turnover, and scope for growth) have correlated with reduced growth rates measured for bivalve populations from contaminated habitats (Gilfillan et al., 1976; Gilfillan and Vandermeulen, 1978; Capuzzo and Sasner, 1977; in McDowell, 2005).

The glutathione occurs naturally in oysters for detoxifying metals, but low concentrations in bivalves may increase the toxic effects of copper in the organisms (Conners, et al., 2000).

Oysters exposed to metals could increase the depletion of cellular stores of glutathione and there is evidence that its depletion may enhance metal toxicity in aquatic organisms. Viarengo et al., (1990) found a significant correlation between glutathione concentrations and Cu-induced lipid peroxidation in the digestive glands of mussels *Mytilus galloprovincialis*. Doyette et al. (1997) observed that enhanced lipid peroxidation was concomitant with the depletion of antioxidant defenses and digestive glands of Cu exposed mussels (Conners and Ringwood, 2000). Ringwood and collaborators observed alterations in glutathione concentrations and lysosomal function in juvenile oysters (*Crassostrea virginica*) exposed to contaminated sediments with a mixture of trace metals. Alteration in lysosomal structure and function are consistent with observations of degeneration of digestive tubules, and degeneration of reproductive tissues (Lowe et al., 1981; in McDowell, 2005). The lysosomes of bivalve mollusks play an important role in immune responses. Lysosomes hydrolases are released out of cells to degrade foreign materials on phagocyte stimulation (Mohandas et al., 1985). Also, the hydrolases can be released into phagosomes, participating in the degradation of internalized foreign particles (Cheng, 1981; Mattozzo, 2001). Alteration of the integrity of lysosomal membranes may cause undesired release of hydrolases into the cytosol, with consequent cellular damage (Lowe et al., 1995). A reduction in lysosomal membrane stability has been reported in mussels and oysters exposed to heavy metal and proposed as an indicator of cell damage (Regoli, 1992; Ringwood et al., 1998; Mattozzo, 2001). In cell-mediated immune responses phagocytosis by circulating hemocytes is the main defense against pathogens and foreing materials (Cheng, 1981). Toxic effects on hemocytes potentially affect the survival of these animals. Alteration in oysters

inmunosurveillance have been reported for bivalve mollusks exposed to metals (Cheng and Sullivan 1984; Mattozzo, 2001).

Other responses of organisms to copper toxicity are shown in the metabolism. Elfwing and Tedengreen (2002) studied the effects of Cu on the metabolism of 3 species of tropical oysters, *Saccostrea cucullata, Crassostrea lugubris* and *C. belcheri* and observed that *C. belcheri* show a decreased in filtration. The data suggested that *C. belcheri* is more sensitive to Cu and the reduced filtration rate could be a result of gill damage. Variengo (1989) also recorded structural deformation of gill as one of the effects of sublethal concentrations of copper (Elfwing and Tedengreen, 2002). The deleterious effects of copper upon metabolism, filtration rates, and other biological responses could affect the survival of the species, and are indicators of metal toxicity.

Other detrimental copper effect for mariculture industry is the phenomenon called "green oysters". Oysters from Taiwan showing abnormally high copper content (1280- 4401 ppm dry wt) in their natural habitat near to a coal-fired power plant are an example of green oysters. When the Cu content in oyster was over 500 ppm (dry wt) usually the oyster color became green (Han and Hung, 1990). This effect could be deleterious to mariculture because the oysters have a bitter taste and could generate gastric problems in human consumers. All these studies strongly suggest that copper concentrations in the natural environment should be monitored, because high Cu concentrations could lead in a great detrimental effect(s) to oyster populations.

Previous studies of accumulation and elimination using *I. alatus*

Only a few studies related to *Isognomon alatus* capacity to assimilate and depurate heavy metals under field and laboratory conditions were available in the scientific literature. In Venezuela, Dominican Republic, and Puerto Rico the flat-tree oyster was used as a bioindicator of pollution (Table 1). During a 6 month-length study Saed et al., 2004 found that *I. alatus* Cu depuration in the field was a fast process. They also indicated that approximately 50% Cu depuration was achieved within a short period of time (from weeks to one month) in both field and laboratory experiments. During the first month about 53% of the Cu was lost ($p<0.05$). In the following months, copper concentrations went from 22 to 11μg g^{-1}, which indicate a slowing down in depuration rates. In the laboratory, the oysters collected from an unpolluted site were exposed to 100 μg L^{-1} for 2 weeks, and the Cu concentration went from 11 to 35 μg g^{-1}. Exposed animals achieved 46% decrease in Cu tissue content after 1 week depuration in aquaria with clean water (a decrease from 35 to 19 μg g^{-1}). Saed et al. (2001) also studied metal concentrations in oysters after exposure to pig farm effluents in Malaysia, by transplanted oysters during 5 months. At the end of experiment the copper concentrations were significantly greater than initial concentration. Both studies show that *I. alatus* has a strong capability of accumulation and depuration and it could be use as a suitable sentinel organism for monitoring programs, in areas were other populations of bivalve species have decreased.

Table 1. Estimates of copper concentration in *Isognomon alatus* tissue according to previous studies.

Study Site	Copper Concentrations (µg g^{-1}) in *I. alatus:*	Reference
Sepang Besar River, Malaysa	17-45 (wet wt) (*approximately 94-247.5 dry wt)	Saed K. et al., 2004
Sepang Kecil River, Malaysa	11(wet wt.) (*approx. 60dry wt)	Saed K. et al., 2004
Sepang Besar River, Malaysa	30.73 ± 0.78 (wet wt) (*approx.169 dry wt)	Saed K. et al., 2001
Sepang Kecil River, Malaysa	11.02 ± 0.51(wet wt) (*approx. 60 dry wt)	Saed K. et al., 2001
Morrocoy National Park, Venezuela	14-49 (dry, wt)	Jaffe, R. et al., 1998
Río San Juan, Dominican Republic	7.58 (dry, wt)	Sbriz, L. et al., 1998
Barahona, Dominican Republic	19.7 (dry, wt)	Sbriz, L. et al., 1998
Bahía Guayanilla, Puerto Rico	14.34-232.3 (dry, wt) (Mean=51.51)	Almodóvar, 1986
Bahía Fosforescente, La Parguera, Lajas- Puerto Rico	6.16-108 (dry, wt) (Mean=31.43)	Almodóvar, 1986
Bahía de Jobos (NERR), Salinas- Puerto Rico	13.06-84.85 (dry, wt) (Mean=31.82)	Almodóvar, 1986

*conversion wet wt to dry wt (wet wt. * 5.5= dry wt)

2 Material and Methods

2.1 Study Site

All specimens/samples of *Isognomon alatus* for the baseline study, accumulation in the laboratory and elimination/depuration experiments in the field were collected at the Bioluminescent Bay (East of La Parguera, Lajas, Puerto Rico [Fig.1]). Furthermore, the field depuration experiments were carried out in the east channel of Bioluminescent Bay (18°12'N, 67°8.5'W), which is less exposed to boat traffic and the red mangroves roots hold an abundant population of the bivalves (Fig.2).

The bay is surrounded by 23.42 hectares (57.88 acres) of mangroves. The mangrove species found are *Avicennia germinans* (black mangrove), *Laguncularia racemosa* (white mangrove), *Conocarpus erectus* (button mangrove), but mostly *Rhizophora mangle* (red mangrove). The bay is used for fishing and recreational activities including nightly boat traffic from local operators transporting tourists to the bioluminescence bay (http://www.ceducapr.com/ inventariodemanglares.htm).

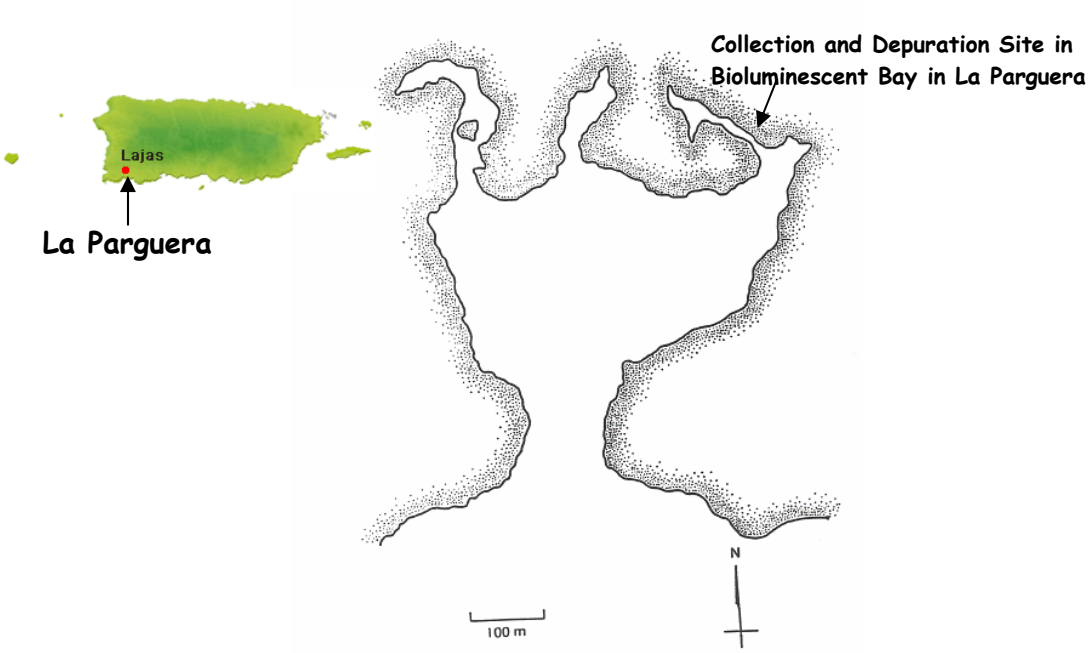

Figure 1. Study Site Bioluminescent Bay (Bahía Fosforescente) in La Parguera, Lajas-Puerto Rico. (PR map adapted from http://topuertorico.org/ accessed feb/19/07).

Figure 2. Oysters *Isognomon alatus* in red mangrove roots of the Bioluminescent Bay

2.2 Hydrographic Conditions

The Bioluminescent Bay does not have a permanent entrance of freshwater, and the salinity has slight variation (Almodóvar, 1986). The reported salinities generally range between 34.1-38.4‰, Coker and González (1960; in Almodóvar, 1986), although during November 2004 and May, 2005 salinities as low as 30.0‰ has been observed due to an increase in precipitation (S. Lebrón, pers. obs.). In the study of Soler (2006) the temperature reported for the Bioluminescent Bay was 26.3-30.0° C. This range was similar to water surface temperatures reported by Read

(1964) (25.5°C and 32°C). Tides at La Parguera are diurnal and in 1958 ranged from 0-1.1 ft. (Coker and González, 1960; in Read, 1964). Otero and Carbery (2005) reported similar tides values of 0.5m (1.65 ft.).

2.3 Summary of Work

Oysters were collected in triplicates of 8 individuals each for a baseline study in May 2004-September 2005. For copper treatments, the oysters were collected in duplicate of 8 individuals each. The metal content in oysters' tissue samples (collected for acclimation, accumulation, and elimination experiments), were analyzed by Flame Atomic Absorption Spectroscopy (FAAS) (Fig.3). The logistics of the different phases of the methodology is shown in Fig.3. The oyster collection includes all the oysters used in the different experimental phases, and analyzed with FAAS. In the acclimation part some oysters were used for copper analysis with FAAS, and others were used in the accumulation phase. After accumulation, some oysters were analyzed with FAAS, while a subset was used in the elimination/depuration phase and analyzed by FAAS as well.

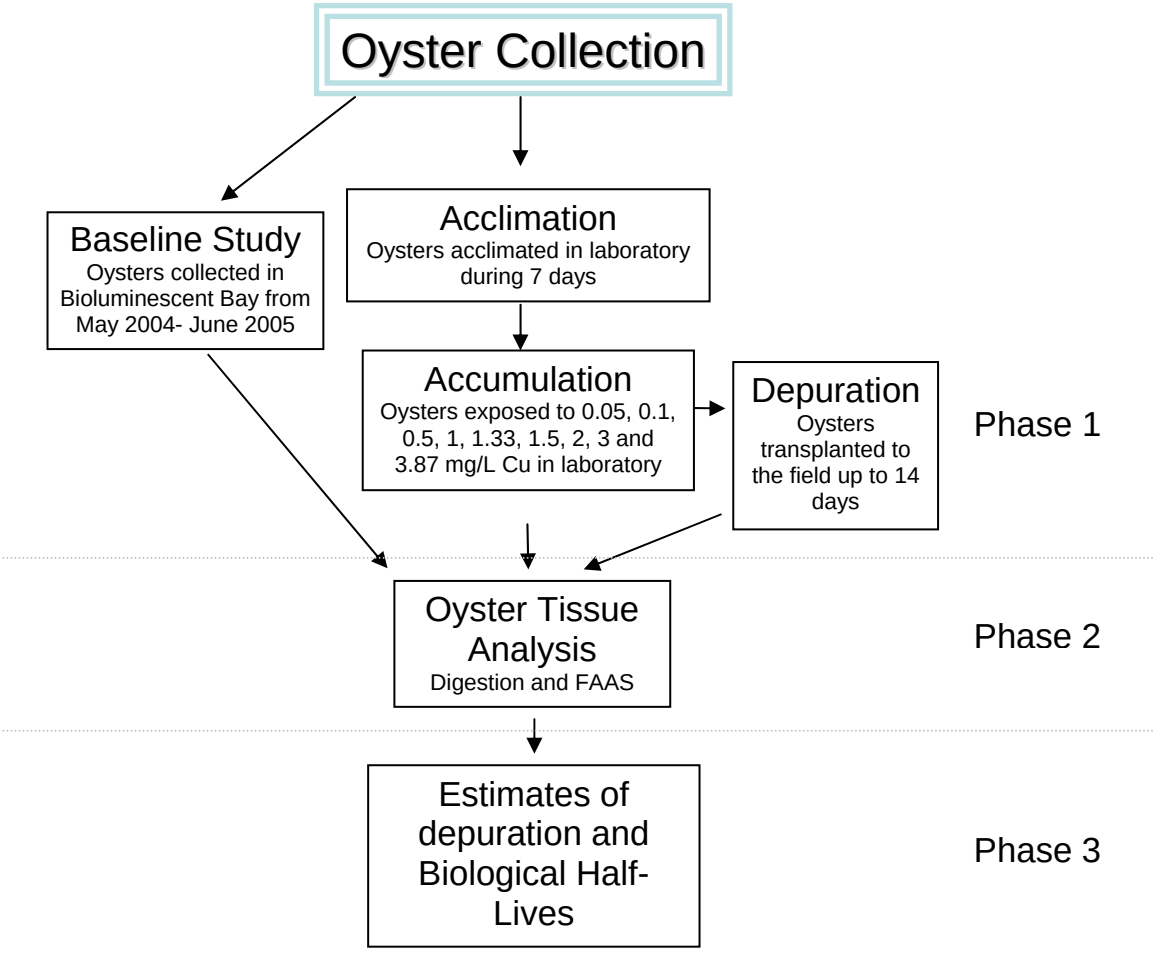

Figure 3. Summary of work conducted. Phase 1 represents sets of oyster samples used to determine oyster tissue content during a baseline study and during acclimation, accumulation and depuration stages of bioassays; Phase 2 include sample preparation and chemical analysis; Phase 3 involves the calculation of parameters related to Cu exposure.

Oyster Collection

Eight medium size (3.5-6.5 cm) oysters were sampled in triplicates during a baseline study of oysters´ tissue copper concentration. Oysters were placed in plastic bags and transported in a

cooler with ice and kept in the freezer until copper analysis. Oysters were also collected similarly for the acclimation, accumulation and elimination experiments.

Oysters Acclimation in the Laboratory

The oysters were transported alive in spat bags of 2.7mm (Aquatic Ecosystem Co.) to the laboratory located at the Department of Marine Sciences in Magueyes Island, La Parguera-Lajas (Fig.4). The samples were acclimated for 7 days in three aerated glass or plastic tanks with filtered natural seawater at ambient temperature (approximately 26.3-30° C) and salinity (≤35 ‰). In general, the tanks were filled with approximately 12L of seawater.

For each experiment, the oysters were subdivided in 3 tanks: one control system and two copper treatment replicates. Oysters were fed daily with a *Tetraselmis* suspension (approximately 5,000 cells/day), during the laboratory experiments. After the acclimation period, eight oysters from each tank were collected for Cu tissue analysis and kept in the freezer until further processing.

Figure 4. Spat Bag of 2.7mm (Aquatic Ecosystem Co.) for transplantation of contaminated oyster to the Bioluminescent Bay (Elimination experiments)

Oysters Accumulation Experiments in the Laboratory

After the acclimation period, the remaining oysters were used during Cu accumulation experiments under various exposure regimes. The same initial copper concentration was added from a stock solution of copper sulfate ($CuSO_4.5H_2O$, Fisher Scientific Co.) to duplicate treatment tanks in each experiment. Initial copper concentrations of 0, 0.05, 0.1, 0.5, 1, 1.33, 1.5, 2, 3, and 3.87 mg/L were used to study copper accumulation. In general, oysters were collected and frozen after the exposure period to determine Cu levels and survival rates. A detailed accumulation profile was obtained for oysters exposed to 0.5mg Cu/L (May 2005) with individuals collected on a daily basis. The remaining oysters were transplanted to the Bioluminescent Bay where Cu depuration/elimination experiment took place.

Copper Elimination by Oysters in Bioluminescent Bay

After exposure to Cu, alive oysters as well as the control group were transplanted back to the red mangrove roots where they were originally collected and kept in identified spat bags (one per each treatment and control). The field experiment was conducted for 5, 6 or 14 days. During the 5 and 6 day elimination treatment protocol, oysters were only collected at the end of the period. In contrast, 8 oysters were collected from each spat bag everyday during the fourteen days elimination experiment of May-June 2005 (0.5 mg Cu/L). Everyday analysis allowed us to obtain the biological half-life and depuration rates.

Depuration Analysis using Depuration Rates and Biological Half-lives

Depuration rates were calculated with the formula:

 Metal concentration at initial depuration time – metal concentration at final depuration time / total depuration time (days of depuration) (Han, 1993).

The biological half- lives ($B_{½}$) of a metal is the time required for half the accumulated trace metal to be lost as the result of biological processes (Okazaki et al., 1981). The $B_{½}$ was used to quantify the depuration rates during the depuration experiments. The $B_{½}$ was calculated using the natural logarithm of the average copper concentrations of two treatment tanks during the depuration period.

The $B_{½}$ was calculated based the method of Renfro (1973):

ln y= a + bx

y= % of the initial tissue concentration (depuration time 0)

b= slope of the line

x= time that experimental treatments took to reach control levels (days).

The slope was substituted in the equation $B_{½} = (\ln 2)/b$.

Oyster tissue analysis (Dry Ashing Procedure)

Oysters were cleaned of epibionts, and the shells were measure (length and width). A composite of eight oysters per sample provided enough material for analysis. The tissue analysis used in this study was a modified version of the Dry Ashing Procedure by FAAS. Approximately 5g wet weight of the composite sample was placed in crucibles. Some samples were used for quality control, adding a spike of 10 µL of Copper Reference Solution (1,000 mg/L ± 1%; Fisher Scientific Co.) to attain a final spike of 0.2 mg/L. The homogenized samples were placed in the oven at 110°C for 16-24 hr and the weight recorded. Dry ashing of samples was conducted in a muffle oven overnight at 500°C (Perkin Elmer, 2000). After the crucibles were cool at room temperature, 2 mL of HNO_3 was added to every sample and evaporated just to dryness on warm hot plate. The samples were maintained for 1 hr at 500°C in the muffle furnace to obtain clean, carbon-free ash. Approximately 10 mL of HCl 1N was added to the samples and heated on the hot plate to dissolve ash. The resulting solution was transferred to a 50mL volumetric flask where volume was completed with HCl 10%. The samples were transferred to 50mL disposable polypropylene centrifuge tubes (Fisherbrand) until analysis.

Flame Atomic Absorption Spectroscopy (FAAS)

Flame atomic absorption analysis was conducted on a Perkin Elmer AAnalyst 100 Spectrometer using hollow cathode lamps. The instrument was calibrated before every analysis using 5 copper standard concentrations (0.1, 0.2, 1, 2 and 4 mg/L) made from a 1,000 mg/L ± 1%Copper Reference Solution (Fisher Scientific Co.). After the calibration curve was obtained (Fig.5), one copper standard solution was routinely included with the rest of the samples to assess instrumental drift and accuracy. For quality control purposes, a random sample spiked with a known concentration of Cu was included in every sample set. This control sample was used to estimate the percentage of metal recovery. The oyster tissue copper concentration was calculated as:

Cu (µg/g) = Vol. 50mL* Cu (mg/L)/ Dry wt (g)

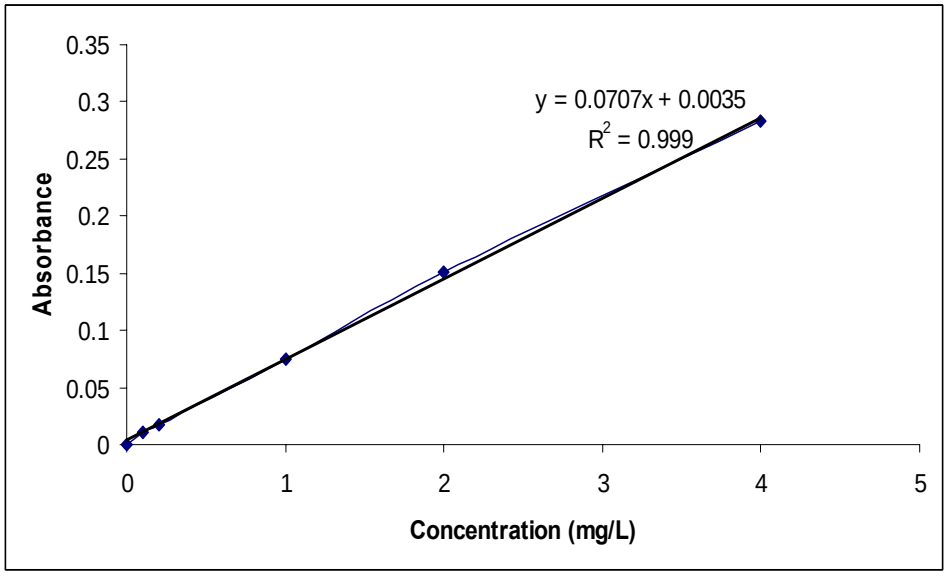

Figure 5. Example of a calibration curve obtained during FAAS optimization

Statistical Analysis

Statistical tests were conducted using Unistat 5.5 software. One-way analysis of variance (ANOVA) with Tukey-B test was ran to evaluate differences of copper concentrations during the baseline study at the Bioluminescent Bay. Multiple Comparisons with t-Distribution were ran to test for differences between 0.5 mg/L Cu accumulation and elimination experiments between February, April and May 2005.

3 RESULTS

Baseline Study of Oyster Tissue Copper Concentrations in the Bioluminescent Bay

Analysis of oyster's tissue samples collected in the field between May 2004 to June 2005 provided key information about the copper concentrations of the organisms in their natural environment (Fig.6). There were significantly differences ($p<0.01$) between the average Cu tissue concentration among sampling dates. The Tukey-B Test shows three significantly different groups with average Cu concentrations of 9.12, 11.9, and 16.7 μg/g dry wt (different groups are shown with different colors in Fig.6). Samples obtained during March and May 2005 show the lowest average Cu values (7.64-8.88 μg/g dry wt), and the highest values (16.5-17.1 μg/g dry wt) were observed in oysters sampled in September, October, and November 5, 2004.

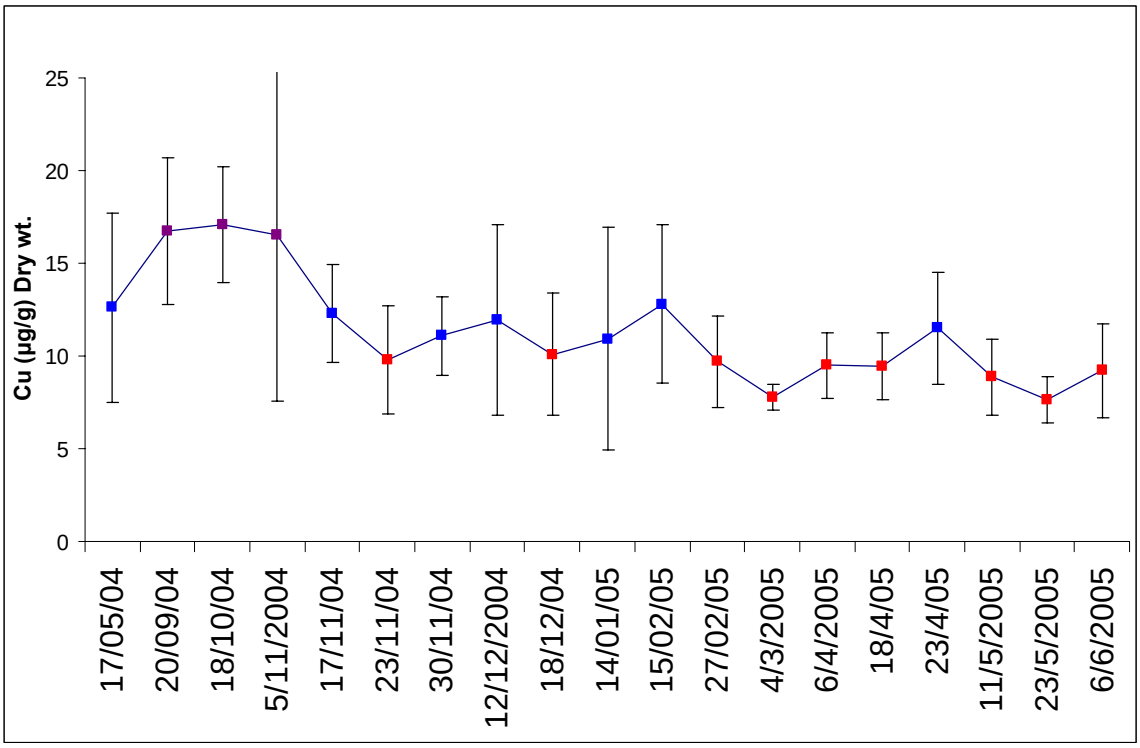

Figure 6. Baseline study of average copper concentrations in *I. alatus* tissues from May 2004- June 2005 of the Bioluminescent Bay (Bahía Fosforescente). Different colors denote significant differences (sampling dates with the same colors are not significantly different). Standard Deviations are shown in the graph. n=triplicates of 8 oysters.

Laboratory Accumulation Experiments

The relationship between 5 day exposure to Cu at 0.05, 0.1, 0.5, 1, 1.5, 2, and, 3 mg Cu/L versus Cu accumulation (control adjusted) in the oysters is shown in Fig.7. The polynomial regression was used to test for a correlation between the two variables (Cu accumulation and exposure Cu concentrations). Control adjustment refers to the net difference of experimental minus control subjects. The average oyster tissue Cu concentration for the control treatments was 8.46 µg/g dry wt and remained constant during the exposure period. The highest average Cu

concentration was observed in oysters exposed to copper regimes of 1.5, 2, and 3 mg/L, and the lower in oysters exposed to 0.05, and 0.1 mg/L. The oysters exposed to 1.5, 2 and, 3 mg/L reach average Cu concentrations of 115-362 µg/g dry wt, and suffered the highest mortality. The average Cu concentration of oysters exposed to 0.5-1.0 mg/L was 30-141 µg/g dry wt, and presented none or moderate mortality.

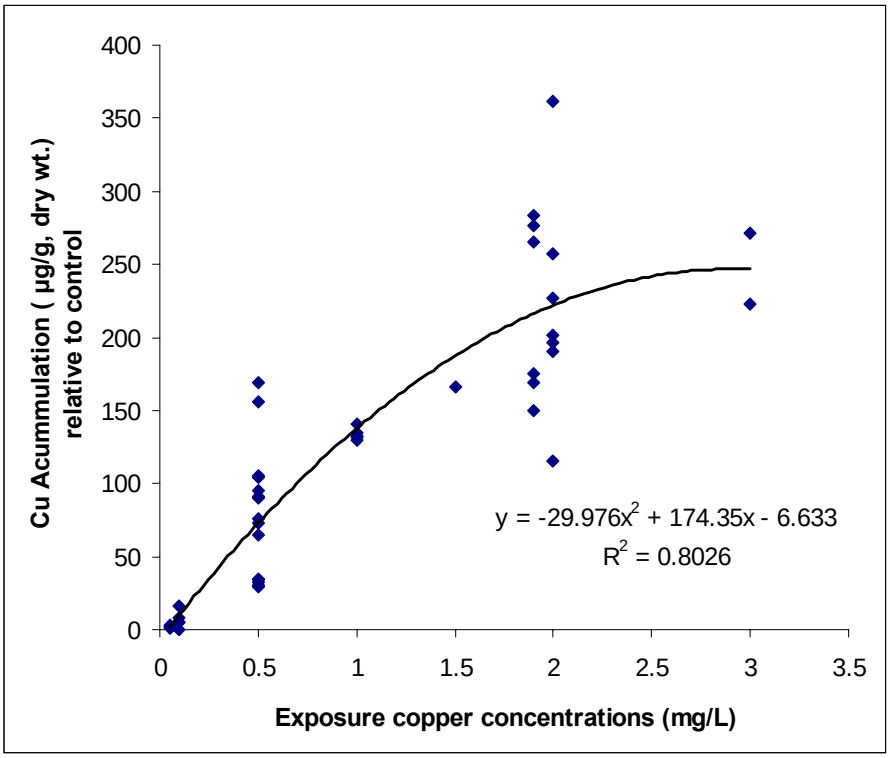

Figure 7. Cu accumulation in oyster tissues in relation to copper exposure during 5 days. Copper accumulation tissue concentrations obtained at day 5 are relative to that of control organisms incubated without copper addition for the same length of time. Copper concentration relative to control was obtained with the net difference of experimental minus control oysters.

Accumulation of Cu in Tissues of Oysters Exposed to 1.33 mg Cu /L

Oysters exposed to Cu levels of 1.33 mg/L in laboratory conditions accumulated significantly higher levels of Cu than the control population. Prior to initiation of the accumulation experiments, the oysters in Cu treatment tanks 1 and 2 had average Cu concentrations of 15.5 µg/g and 18.8 µg/g dry wt, respectively, while the control was 21.5 µg/g dry wt. After 5 days the oysters in the Cu treatment tanks 1 and 2 had average Cu concentrations of 103 µg/g Cu and 105 µg/g dry wt respectively, whereas, the control did not change (20 µg/g dry wt; Fig.8). All oysters survived the copper exposure.

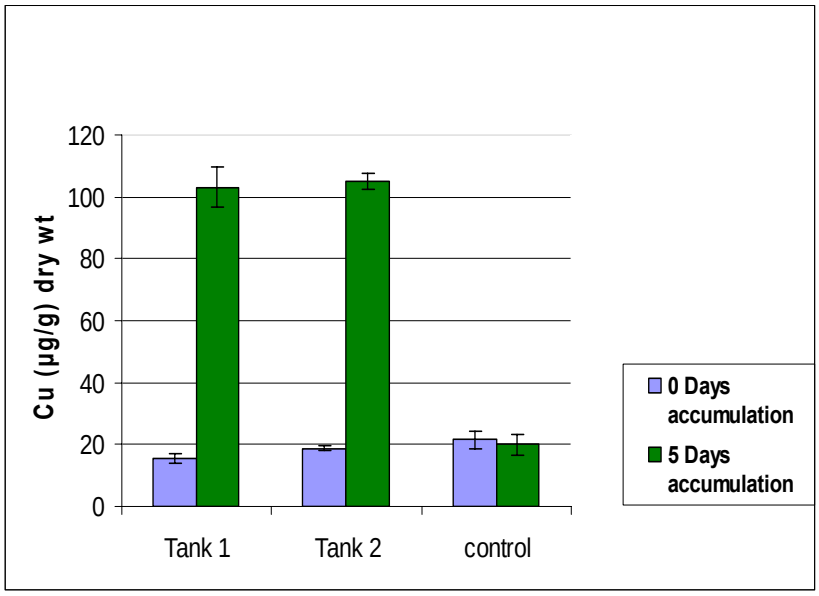

Figure 8. Copper concentrations in oysters' tissue exposed to 1.33 mg Cu /L during 5 days. The copper concentrations in oysters of treatment tanks 1 & 2 were significantly higher than controls at 5 days of accumulation.

Accumulation of Cu in Tissues of Oysters Exposed to 3.87 Cu mg /L

Prior to initiation of the accumulation experiments, the oysters average Cu concentrations were 20.1 µg/g dry wt, and 7.27 µg/g dry wt, in tanks 1 and 2, respectively, while in control oysters was 15.3 µg/g dry wt. After only 2 days of exposure the oysters exposed to 3.87 mg Cu /L presented 100% mortality. The oysters reached the highest values of all the exposure experiments with Cu concentrations of 1022 and 1259 µg/g dry wt in treatment tanks 1 and 2, respectively. Significantly lower Cu concentrations were observed in control oysters, at day 2 (average 17 µg Cu/g dry wt and 100% survival (Fig.9)).

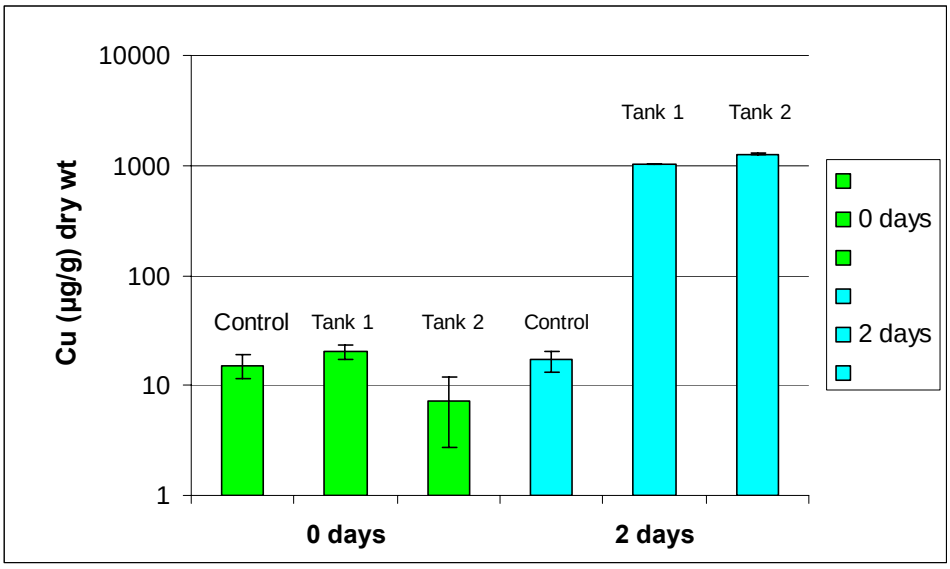

Figure9. Copper concentrations in oysters' tissue exposed to 3.87 mg Cu /L during 2 days. In the accumulation experiment the copper concentrations in oysters of treatment tanks 1 & 2 were significantly higher than controls.

Accumulation/Elimination Experiments

The following results present a separate set of assays where a series of individuals were acclimated in the laboratory and then exposed to Cu followed by depuration in the field. In one of the experiments the oysters were exposed to copper concentrations of 1.9 mg Cu/L, while in the other the oysters were exposed to 0.5 mg Cu/L. The maximum and minimum Cu concentrations where oysters showed accumulation and elimination with minimum mortality or complete survival were estimated.

Oysters Exposed to 1.9 mg Cu /L

Prior to initiation of the accumulation experiments, the oysters average Cu concentrations in tanks 1 and 2 were 26.3μg/g dry wt, and 18 μg/g dry wt, respectively, while control oysters were 10.8 μg/g dry wt. After 5 days of exposure, copper tissue levels of oysters in tanks 1 and 2, contained an average of 174 and 284 μg/g dry wt, respectively. Copper in control oysters remained at much lower levels (8.88 μg/g dry wt). Until this point the oysters' survival was 45 % and 32% in treatment tanks 1 and 2, and 100% in control tank.

After 6 days back in the field the oysters exposed to Cu regimes showed average depuration rates relative to control of 26.3 ($\mu g\ g^{-1}\ day^{-1}$) (Table 2). A rapid removal of 69.7 % of the initial Cu concentration was observed. The mean Cu concentration in oysters transplanted from tank 1 was 75.9 and 72.7 μg/g dry wt in tank 2, and only 12.4 μg/g dry wt in the control tank (Fig.10). Survival after 6 days of depuration was 40 and 29% in oysters from tanks 1 and 2,

and 100% in the control group. These results are similar to previous observations from accumulation assays.

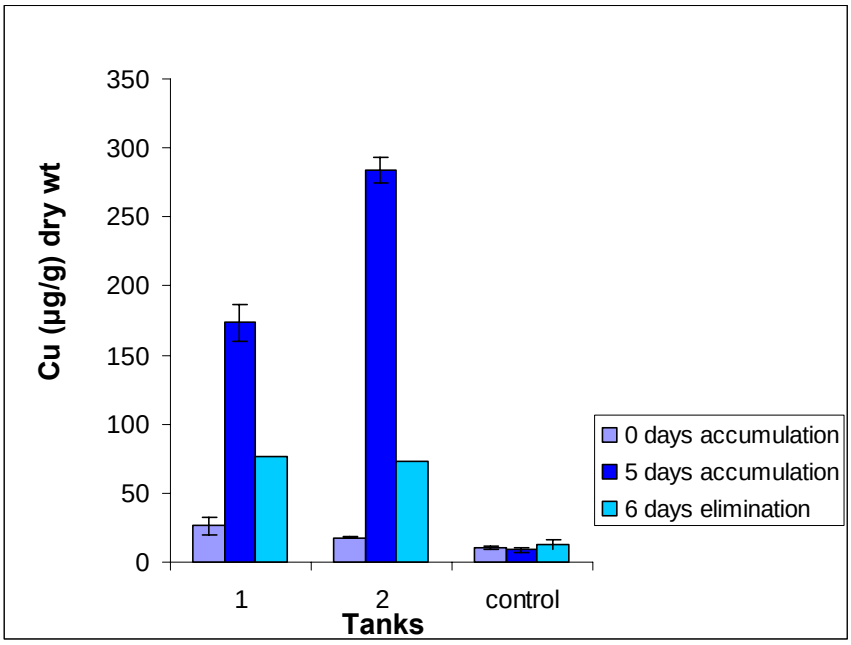

Figure 10. Copper concentration in tissue of *I. alatus* after 5 days of exposure (accumulation) to 1.9 mg Cu/L in laboratory aquaria and 6 days of depuration (elimination) in Bioluminescent Bay.

Table 2. Summary of depuration experiments during different occasions. *Isognomon alatus* was exposed to copper during 5 days at different concentrations after which elimination/depuration for 5 or 6 days was conducted at the Bioluminescent Bay. Depuration rates are relative to control.

Date of Exp.	Cu exposure	Depuration rate Cu ($\mu g\ g^{-1}\ day^{-1}$)	Depuration %
Dec-04	1.9 mg/L	26.3	69.7
Feb-Mar 05	0.5mg/L	19.7	79.4
Apr-05	0.5 mg/L	16.3	85.2
May-05	0.5 mg/L	3.3	50.7

*The time of depuration rate calculation was 6 days for 1.9 Cu mg/L exposure and 5 days for 0.5 Cu mg/L exposure.
**Depuration % = (copper conc. at day 0 – copper conc. at day 5 or 6 of depuration) / copper conc. at day 0 *100.

Oysters Exposed to 0.5 Cu mg/L

This detailed set of observations includes Cu analyses for *I. alatus* samples collected daily and encompassing exposure and depuration stages. Prior to the initiation of exposure/accumulation, oysters from both treatment and control tanks showed low Cu concentrations (8.66-10.7 µg/g dry wt). After 5 days of exposure to Cu at 0.5 mg/L in the laboratory, the oysters' average Cu concentration increased significantly. The oysters mean Cu concentrations after 1-day in tanks 1 and 2 were 71 and 64 µg/g dry wt, respectively, while the control oysters showed 9.24 µg/g dry wt. Metal accumulation continued until day 4, but a reduction of Cu concentrations occurred at day 5 in both experimental tanks (38-40 µg/g dry wt). The control oysters show a slight decrease in Cu (6.80 µg/g dry wt) at day 5. Survival of 100% was observed during the 5 days of accumulation. After this period, the oysters transplanted to the Bioluminescent Bay for elimination/depuration showed reduction in Cu concentrations. The

experimental oysters reached values similar to the control after 11 days of elimination (Fig.11), thus oysters were capable of eliminating Cu in a short period of time. The daily Cu depuration rate is presented in Fig.12. During the first elimination period of 11 days approximately 95% of the copper accumulated in the oysters exposed to 0.5 mg/L Cu treatment was depurated. The higher depuration rate (23.3 μg/g/day) corresponds to day 1 with an average Cu concentration decreasing from 62.6 μg/g dry wt at day 0 to 39.3 μg/g dry wt 24 hours later. The depuration rate was quick at the initial part of the elimination experiment, where the copper concentration was higher, and slowed down proportionally to lower Cu concentration. After 11 days of elimination, the copper concentration reached values similar to the control (12.8 μg/g dry wt), with an average depuration rate of 1.86 μg/g/day (Fig.12).

Different accumulation and elimination experiments conducted in February, April and May 2005 showed significant differences between the oysters in control and Cu treatments. The duration of the experiments was 5 days for accumulation and 5 days for elimination. Multiple Comparisons with t Distribution showed that in the 3 different experiments the mean control values (9.27 μg/g dry wt) were significantly different from those of the Cu treatments with mean values of 36.34-177.61 μg/g dry wt. The oysters showed mean values of 20.81 μg/g dry wt and 32.74 μg/g dry wt after 5 days of elimination (Fig.13).

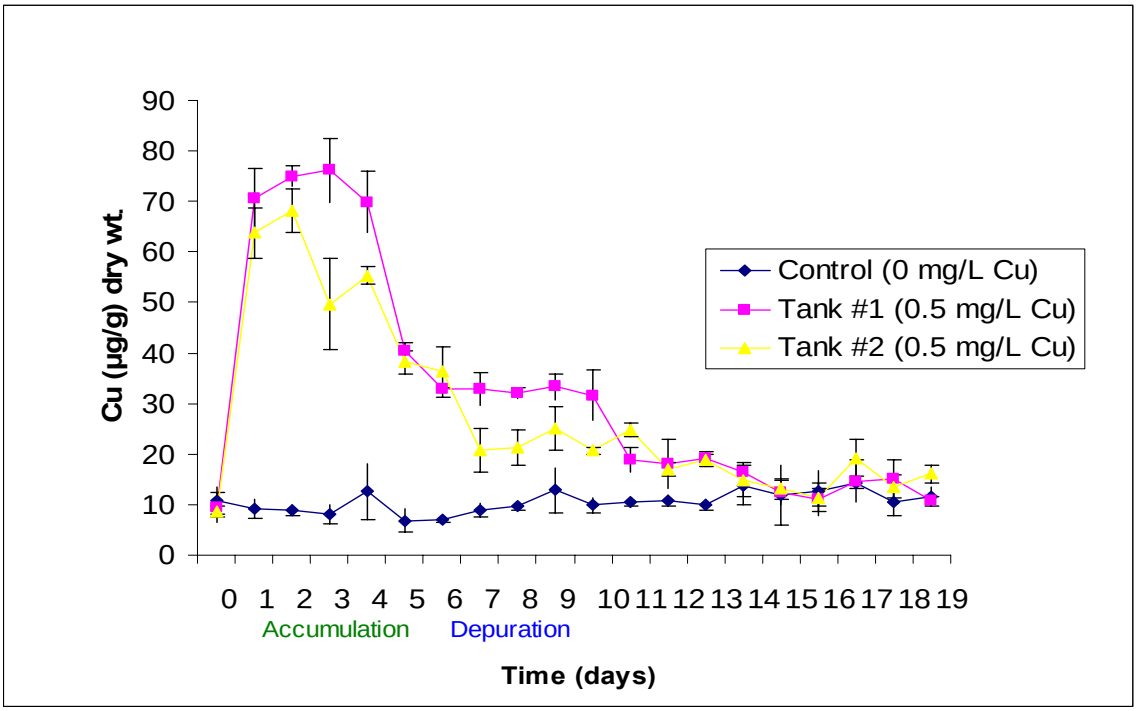

Figure 11. Average copper content in oyster tissue prior accumulation (day o) and during exposure of 0.5 mg Cu/L during 5 days (1-5 days) followed by 14 days of depuration (6-19 days). n=8 experimental and control oysters. The standard deviation (σ) is shown in the graph.

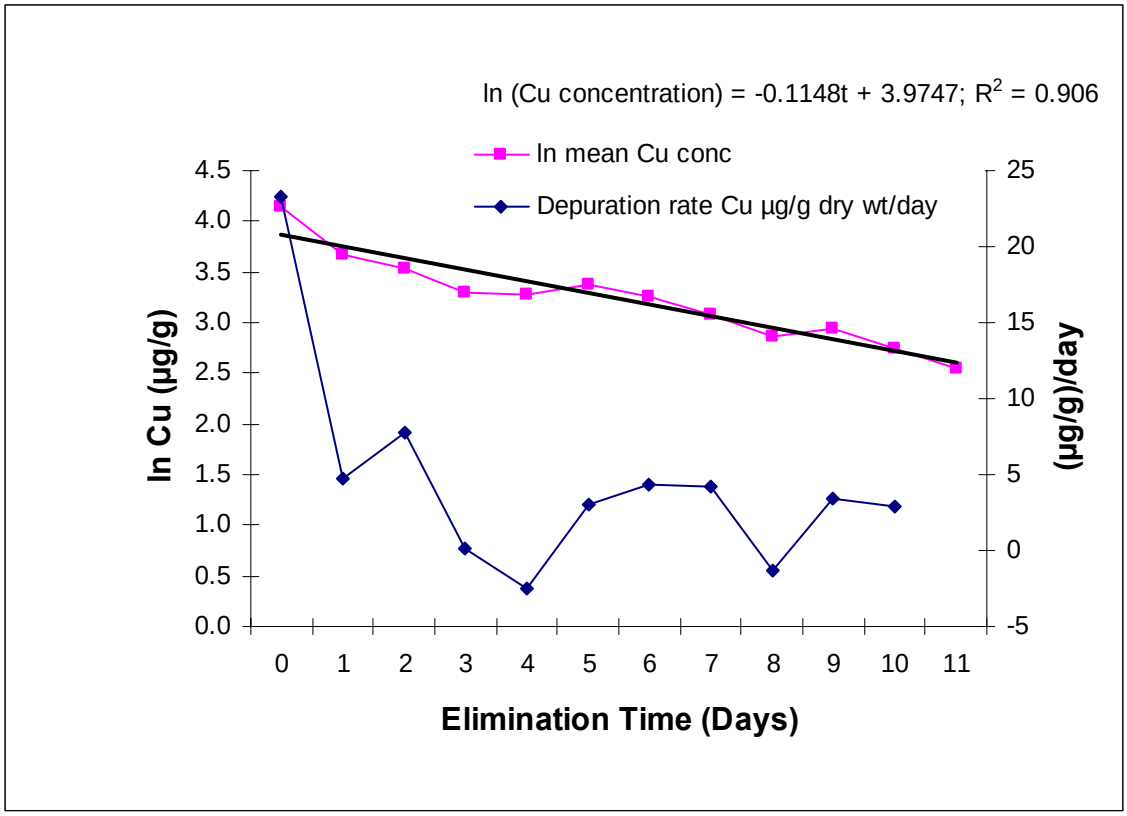

Figure 12. Correlation between average copper concentrations (ln) in oyster tissue and depuration rates during 11 days of elimination. The depuration rate is variable during all depuration time. The oysters were exposed to 0.5 Cu mg/L during 5 days prior elimination in the field. The depuration start at day 5 of accumulation (day 1).

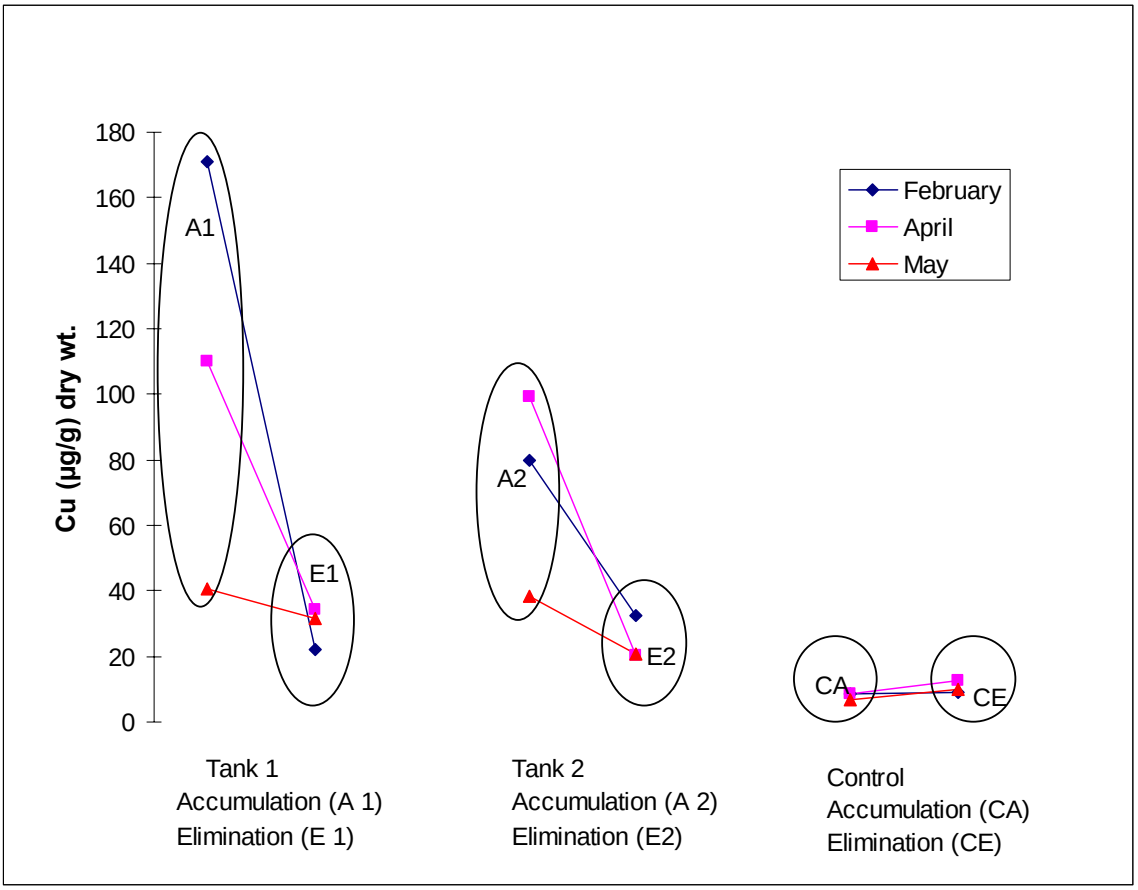

Figure 13. Accumulation and elimination during 5 days at different months and its relation with oyster tissues Cu concentrations. The lines between accumulation and elimination show a tendency of decreased copper concentrations in all the treatment oysters, after 5 days of elimination in different experiments. The controls in every experiment did not present a significant change in copper concentration.

4 DISCUSSION

The observed copper concentrations in *Isognomon alatus* (9.12, 11.9, and 16.8 μg/g dry wt (Fig. 6)), were lower than those previously reported by Almodóvar (1986) from oysters collected at Guayanilla, the Bioluminescent Bay in La Parguera, Lajas and Jobos Bay Salinas, PR (Table 1). To this date, his study was the only report for Puerto Rico using *I. alatus* for metal monitoring in coastal waters. Additional studies done with *I. alatus* in Dominican Republic, found Cu concentrations (19.7 μg/g dry wt) similar to our results (Table 1). However, in Venezuela and Malaysia, Cu concentrations were higher than in Puerto Rico (Table 1). Previously, low copper concentrations have been observed in the Bioluminescent Bay (Almodóvar, 1986); and also in the current study, contrasting to higher Cu values in Malaysia, where oysters received effluent discharged from pig farms among other anthropogenic impacts. The low copper concentrations found in oysters from La Parguera, in comparison with oysters from other studies, show that oysters in the Bioluminescent Bay are subjected to low copper concentrations.

Various research groups have used other species of oysters for biomonitoring, accumulation, and depuration studies of metals (Almodóvar, 1986; Cantillo et al., 1999; Wallner-Kersanach et al., 2000). The mangrove oyster, *Crassostrea rhizhophorae*, which share the same habitat with *I. alatus*, has been used in Puerto Rico and other locations in South America. The "National Status & Trend Program (NS&T) Mussel Watch" used *C. rhizophorae* for monitoring metal concentrations in PR. The NS&T study was conducted during 1986-1997 in Boquerón, Montalva, and Jobos Bay, Puerto Rico. The overall median concentration for all three sites and

the 85th nationwide percentile copper concentration were 140 and 290 µg/g dry wt, respectively. The copper concentrations at Boquerón were below the median (40-130 µg/g dry wt) during the one year sampling effort, while Cu concentrations at Bahía Montalva were above the median, 190 and 220 µg/g dry wt during 1992 and 1994, respectively. In 1993, data for Bahía Montalva were more extreme (330 µg/g dry wt) and above the 85th percentile (an index of high concentrations; Cantillo et al., 1999). Furthermore, in 1993 the Cu concentrations in oysters from Jobos Bay were above the median with values of 197 µg/g dry wt and 155 in 1997. The NS&T program reported higher copper concentrations in oysters than those observed in the Bioluminescent Bay during the baseline study of May 2004 until June 2005 (Fig.6).

Crassostrea rhizophorae has been used in Brazil for accumulation and elimination experiments. Field transplantation studies were performed to determine accumulation and depuration rates of Cu and other metals. An increase in oysters copper concentrations from 27 µg g^{-1} to 380 µg g^{-1} dry wt was observed after 60 days of transplantation from a clean to a contaminated site. Although the exposure time during the transplant was longer than in this study (60 days), the Cu concentrations in transplanted oysters did not reach the elevated values found in native oysters from the contaminated site. Oysters returned from the contaminated to the clean site showed a significant increase in depuration rate after 30 days (30% decrease from 377 µg Cu/g dry wt) relative to oysters native to the contaminated site (9% decrease from 1,567 µg Cu/g dry wt; Wallner-Kersanach et al., 2000). These experiments suggest that oysters exposed to increased Cu concentrations during different periods of time have different depuration capacities with occasional requirements of lengthy periods of time to achieve tissue copper content close to

background levels. This observation is compatible to previous studies (Almodóvar, 1986; Cantillo (NS&T), 1999; and Wallner-Kersanach et al., 2000) using *C. rhizophorae* that indicate the oyster's adaptability to high copper concentrations. Therefore, *C. rhizophorae* could be a good indicator of temporal variations over long periods of time.

Our study showed a high Cu accumulation capacity by *I. alatus*. A primary steady state concentration was reached after only 1 day of exposure to 0.5 mg Cu/L (Fig.11). Under exposure to higher concentrations *I. alatus* was able to accumulate over 1000 µg/g dry wt. However, a high mortality rate was observed during these extreme exposure treatments. Although uncertain interspecies differences are present between *I. alatus* and *C. rhizophorae*, both species are suitable for monitoring metal concentrations in the environment. However, the fast fluctuations in Cu tissue content as a response to drastic changes in exposure to Cu suggest that *I. alatus* may integrate environmental conditions at short periods of times by reaching equilibrium conditions within days (Fig 11).

In our elimination study *I. alatus* showed a high depuration capacity as well. *Isognomon* exposed to 0.5 mg Cu/L in laboratory followed by transplantation to the field for elimination, reached Cu concentrations similar to control specimens in only 11 days of elimination representing a decrease of 95% (Fig.11). In contrast, *C. rhizophorae* tissue Cu concentration decreased only 30% from a maximum of 378 µg g^{-1} dry wt after 30 days of depuration in uncontaminated waters of Brazil (Wallner-Kersanach et al., 2000). The higher depuration capacity of *I. alatus* could be advantageous for monitoring studies in which identifying uncontaminated sites for depuration studies is necessary.

Several factors could explain the differences in accumulation and depuration between the two oyster species, such as environmental (i.e. availability of copper in water, salinity, temperature, others) or biological differences of the organisms (sex, age, food, reproductive state, genotype, phenotype, and others). Differences in body size between *I. alatus* and *C. rhizophorae* might as well explain variability among the depuration capacity of these species. Almodóvar (1986) found that metal concentrations in *Crassostrea* were independent of size, and *Isognomon* copper concentrations were lower with increase in size and dry weight, showing that metal concentrations in smaller organisms were less variable than in larger ones. The metal concentrations found in different indicator organisms could be indicative not only of the environmental concentrations of the metal in the water or food, but also of the capacity of accumulation and depuration of the organisms. Therefore, *I. alatus* and *C. rhizophorae* could both be used as indicator species in the Caribbean, but is important to understand their biological differences for better understanding in future monitoring program.

Previous studies documented differences in depuration rates among a variety of oyster species. For example, Han and coworkers (1993) studied *Crassostrea gigas* in Taiwan after observations of green coloration in oysters due to high copper content. This group reported a depuration rate of 351 $\mu g\ g^{-1}d^{-1}$, or 67% Cu elimination within the first 6 days in the laboratory conditions. Depuration rates generally decreased until a steady state is reached between the copper level in the incubation water and the oyster tissue (i.e. lack of net change in Cu tissue content; Han et al., 1993). A more speedy depuration rate was observed in the present study,

where the tissue content of Cu decrease continuously during the first eleven days, when levels reached control values and 95% Cu removal.

Okazaki and Panietz (1981) conducted transplantation studies with *C. gigas* and *C. virginica*, and found reduction of trace metals in both species during a depuration period in natural clean water, but with differences in the depuration rates and biological half-lives. *C. gigas* lost 45-82% of copper with a $B_{½}$ of 33 days, while *C. virginica* loss 22-44% of Cu with a $B_{½}$ of 156 days (Table 3). In France, Geffard and collaborators (2002) found feasibility for decontamination of oysters impacted by metal contaminants with a higher initial depuration rate followed by depuration desacceleration. The Cu concentrations in *C. gigas* from a contaminated site were 110 µg/g wet wt and after 7 months of transplantation to a clean site for depuration were 79 µg/g wet wt with $B_{½}$ of 430 days (Table 3). Other studies found that after 12 weeks of exposure in laboratory, Cu concentrations in *C. virginica* increased from 363 to 702 µg/g wet wt. The copper concentrations begin decreasing after 24 weeks in clean water. However higher decreased was observed after 30 to 48 weeks in clean water, where Cu concentrations decreased to 200 ± 50 µg/g wet wt (Greig and Wenzloff, 1978). In our study we found that the oysters reached normal values in a shorter period of time (approximately 11 days), resulting in extremely low $B_{1/2}$ (Table3) in comparison with previous studies (Greig and Wenzloff, 1978; Zaroogian, 1979; Okazaki et al., 1981; Han et al., 1993; Wallner-Kersanach et al., 2000, and Geffard et al., 2002). Calculations based on the results from the only study available that examined *I. alatus* (Saed et al., 2004) showed high $B_{1/2}$ under field depuration experiments, but under laboratory conditions showed results similar to those presented here (Table 3).

Table 3. Biological half-lives (days) for copper in different species of oysters. The $B_{½}$ were calculated to quantify the depuration process with the formula $B_{½} = ln/b$ (In the current study $ln = -0.1148 + 3.9747$).

Oyster	$B_{½}$ (days)	Study Site	Reference
Crassostrea gigas	32.9	San Francisco, USA	Okazaki et al., 1981.
C.virginica	156.2	San Francisco, USA	Okazaki et al., 1981.
C. gigas	11.6	Erhjin chi estuary, Taiwan	Han et al., 1993.
C. gigas	25.1	Charting coastal area, Taiwan	Han et al., 1993.
C. gigas	430	France	Geffard et al., 2002.
Isognomon alatus	85	Malaysia	Saed et al., 2004
Isognomon alatus	7.9	Malaysia	Saed et al., 2004
Isognomon alatus	6*	Bioluminescent Bay, PR	Current study.

***The $B_{1/2}$ was calculated with the average between two treatment tanks.**

Our study suggests Cu exposure thresholds for *I. alatus* populations showing oysters sensitivity to different copper concentrations. The oysters exposed to 3.87 mg/L presented a 100% of mortality after only 2 days of exposure, while oysters exposed to 0.05 mg/L exhibited 100% survival after 5 days of exposure. We observed an increase in mortality by oysters exposed to 1.5 mg Cu/L, which could indicate that at this Cu concentration oysters reach their survival

threshold. Oysters exposed to 0.1 mg Cu/L showed accumulation while oysters exposed to 0.05 were similar to control. This indicates that 0.1 mg Cu/L is the threshold concentration for accumulation. Those oysters exposed to low Cu concentrations (0.05 mg/L), at the end of the accumulation experiment resulted in levels of (10.75 μg g^{-1} dry wt), similar to the control population (9.22 μg g^{-1} dry wt.). On the contrary, studies conducted by Silva and Qasim (1979) with *Crassostrea cucullata* exposed to 0.05 mg/L, found higher Cu concentrations after 7 weeks of uptake (63.97 μg g^{-1} wet wt/approximately 352 dry wt) and after 7 weeks of depuration were 40.58 μg g^{-1} wet wt. In the study of Silva and Qasim (1979), the initial Cu concentration (20.63 μg g^{-1} wet wt) was higher than the Cu concentration found in *I. alatus* from Bioluminescent Bay (the maximum level was 17.07 μg g^{-1} dry wt). This research group suggested a lower accumulation threshold for *C. cucullata* in comparison to *I. alatus*. The divergence could be attributed to biological differences between species.

The metal accumulation in oysters is not a continuous process, and differences have been observed in oysters from different studies. In the heavy metals accumulation study by Saed et al. (2001) with oysters exposed to pig farm effluents, the Cu accumulation process was not continuous with drops in uptake rates at moments. A similar occurrence was observed in our study with the exposure of oysters to 0.5mg Cu /L for 5 days (day 5; Fig.11). Drops in metal uptake have been associated to metabolic control of the internal metal burdens in oysters (Saed et al., 2001). Oysters utilize different metabolic strategies to sequester and partition accumulated metals depending upon whether they are to be detoxified or used in routine metabolism (Engel, 1999). One mechanism for internal Cu control is the formation of intracellular inclusions in

amebocytes and excretion by diapedesis (removing excess). Therefore oysters most probably have multiple mechanisms for Cu regulation (i.e. amebocytes and metallothioneins), especially under high levels of Cu Ruddell and Rains (1975; in Engel, 1999).

Therefore, different mechanisms of oysters for Cu regulation cause differences in Cu loss and depuration rates in oysters. Saed et al., (2004) found that copper depuration in the field was faster during the first month with approximately 53% of Cu loss, but the depuration rate slowed down during the following months. In contrast, *Isognomon* showed a high depuration capacity with Cu almost complete removed within 11 days. Several studies show that oysters with initial low copper concentrations subjected to high copper concentrations depurate relatively faster than oysters long term exposed to increased concentrations of the element. This could be due intracellular copper deposits found in oyster exposed to high metal concentrations for a long time, which could reduce its depuration capacity (Engel, 1999). This could explain the differences in depuration rates from our study where initial Cu concentrations were lower than in other studies. In addition, the differences can be attributed to the depuration medium (laboratory or field). Depuration in laboratory was found to be faster for reducing the metal contents of oysters than in the field (Saed et al., 2004). However our depuration study was conducted in the field and depuration was faster than laboratory and field depuration from the other studies. The lower biological half-lives that we found demonstrate a faster depuration capacity of *Isognomon* in our study (Table 3). In the literature we found a variety of explanations for the differences in depuration rates and biological half lives (Okazaki and Panietz, 1981; Saed, 2004). For example, changes associated to the depuration capacity of the organism, differences in copper exposure,

and other environmental factors. Moreover, several categories of trace metal elimination have been identified, such as raises in $B_{1/2}$ when the body burden of the trace metal increase; stable $B_{1/2}$ when the rate of metal loss reach equilibrium and decrease in $B_{1/2}$ with increase in body burden of a trace metal Cunningham and Tripp (1975; in Okazaki and Panietz, 1981).

In summary, the fluctuations in the copper concentrations of different oysters' species during accumulation and depuration could be a combination of several internal processes of the organisms, to avoid toxicity of excess metals. The process of assimilation and elimination of metals is part of a balance; and sometimes the oysters used in these studies could have reach a steady state and therefore the accumulation and/or depuration could be slower. In contrast, when the organisms are receiving an abnormal high concentration of the metal are going to increase the body burden or decreases the metal concentration if the excess is over its accumulation capacity.

It is important to understand the accumulation and depuration dynamics of the bioindicator species, in despite of differences between species, sites, mediums and environmental conditions. Our study demonstrates that *I. alatus* is capable to depurate all the accumulated copper in a very short period of time (11 days). Therefore, presenting the lower biological half-lives found in the scientific literature. The ability of *I. alatus* to depurate in a short period of time could be use for managing mariculture areas where there is a need to identified unpolluted areas. Furthermore, the oyster ability to reflect environmental changes in a short period is important for the detection of contaminated areas in monitoring studies. The survival capacity of the oysters at

different copper concentrations is as well an important trait to be consider in future monitoring studies.

5 CONCLUSIONS

• *I. alatus* has great depuration capacity, as noted by their Cu removal efficiency and short depuration time; this is confirmed by the differences in the biological half-lives between different studies. In our study the $B_{1/2}$ of *I. alatus* (6 days) is lower than in other oysters.

• *I. alatus* is well distributed around Puerto Rico, easy for collection, accessible, and individuals with medium size has enough tissue material for analysis, therefore, this oyster is a good bioindicator of Cu exposure and perhaps, useful for other metals.

• This species is capable of rapid depuration in the field and could be use as a tool for monitoring programs where there is a need for detection of recent changes in the environment.

• The oysters are feasible for transplantation experiments which could be an advantage in areas where the metal concentrations are unknown or indicator organisms are not available.

• In their natural environment, *I. alatus* at the Bioluminescent Bay is not exposed to high Cu concentrations as demonstrated by the low copper concentrations found during 2004-2005 survey.

• *I. alatus* can not tolerate high copper concentrations (>1.5mg Cu/L); the mortality increase dramatically with levels above this concentration. Only a few individuals were tolerant to 1.0 mg Cu/L. We found an accumulation threshold for *I. alatus* between 0.1 mg Cu/L to 1.5 mg Cu/L. The tolerance to Cu concentrations higher than those typically detected in their natural environment made this species a good sentinel organism.

• *I. alatus* could be use for laboratory bioassays or field experiments due to their facility to manipulate, resistance to laboratory conditions as well as capacity to tolerate transplantation experiments.

• Overfishing/overexplotation of comestible oysters like *Crassostrea rhizophorae* have resulted in a population decline in many areas of Puerto Rico. The flat-tree oysters now are the most abundant species of comestible oysters and in some areas the only available one.

• Low tissue sample than previously required in the original method of Dry ashing can provide enough biomass for Cu detection by FAAS. This is a key aspect to reduce the amount of oysters need it for monitoring purposes.

• The accumulation dynamic of *I. alatus* is rapid at the initial exposure time. We observed a higher copper concentration (7 times higher) in only 1day of exposure at 0.5 mg Cu /L.

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