EXPERIMENTAL CADMIUM CONTAMINATION OF THE ECHINOID *Stomopneustes variolaris* (ECHINODERMATA: ECHINOIDEA): INFLUENCE OF DOSAGE AND DISTRIBUTION OF THE METAL IN THE ORGANISM

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Submitted in part fulfillment for the degree of Master of Science in the Discipline of Civil Engineering in the School of Engineering in the Faculty of Science and Engineering at the University of Durban-Westville

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DECLARATION

The Registrar (Academic)

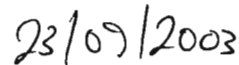UNIVERSITY OF DURBAN-WESTVILLE

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I, Santosh Bachoo, do hereby declare that the dissertation/thesis entitled "Experimental cadmium contamination of the echinoid *Stomopneustes variolaris* (Echinodermata: Echinoidea): Influence of dosage and distribution of the metal in the ortganism" is the result of my own investigation and has not been submitted in part or in full for any other degree or to any other University.

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ACKNOWLEDGEMENTS

ABSTRACT

Cd levels were measured in three different body compartments of the echinoid
Stomopneustes variolaris after exposure to concentrations of 5 µg l^{-1}, 20 µg l^{-1} and 50 µg
l^{-1} Cd for a period of two weeks. The body compartments investigated included the
intestine, gonads and skeleton. The gonads did not exhibit dose-dependent
bioaccumulation. The levels of cadmium in the intestine and skeleton were significantly
different between the treatments, suggesting that dose dependent bioaccumulation had
occurred in these compartments (except in the skeleton where higher levels were
recorded for those exposed to 20 µg l^{-1} than those exposed to 50 µg l^{-1}) The levels
recorded in the intestine were higher than those recorded in the gonads and skeleton at the
higher Cd exposures of 20 and 50 µg l^{-1}. The levels accumulated in the gonads and
skeleton were not significantly different from each other. A separate group, exposed to 20
µg l^{-1} Cd over a period of two weeks, was placed in uncontaminated seawater to
determine if the echinoids were capable of bioremediation. Bioremediation was found to
lower the Cd levels in the gonads, but not in the intestine and skeleton. Cadmium levels
for the three body compartments were also recorded from specimens collected from the
field, indicating the presence of this element in the environment. In addition to using
atomic absorption spectroscopy (AAS), energy dispersive X-ray analysis (EDX) was used
as a comparative technique to detect the presence of Cd in the skeleton of the urchin.
Cadmium was detected in the skeleton with AAS, but not with EDX. *S. variolaris* proved
to be a capable biomonitor of Cd contamination.

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1. INTRODUCTION

The most important aspects of heavy metal contamination in the marine environment are the hazards posed to human beings consuming contaminated fish and invertebrates and the damage caused to marine organisms and ecosystems. O'Donoghue and Marshall (2003) reviewed the status of marine pollution research in South Africa and found that this type of research rose sharply from the 1960s, peaked in the early 1980s and is sharply declining to low present day levels. It was found that the Western Cape undertook the most of these studies, followed by the Eastern cape and then KwaZulu-Natal. This highlights a dire need for such research to be undertaken along the KwaZulu-Natal coast. Heavy metals are considered as conservative contaminants i.e. they are neither biodegradable nor dissipative and are understandably issues of concern. Although there has been concern over the introduction of heavy metals into the marine environment caused by human activities, it was the tragic events in Mimata Bay, Japan, which drew attention to the severity of the problem (Waldichuk 1974, Bishop 1983, Khristoforova *et al* 1984). Between 1953 and 1960, 111 people were poisoned by eating fish that had accumulated mercury in their bodies. The mercury had come from industrial discharges into the bay. Another tragedy struck the Japanese where many more people were poisoned by eating fish contaminated with cadmium (Bishop 1983).

1.1 Terminology

The term "bioaccumulation" is often used in a very general sense to include all measurements of contaminants in tissues of organisms, without regard to source or mechanism of introduction to the organism. Bioaccumulation was measured to "protect the environment" in some undefined sense, but gradually it came to be measured for two reasons: to determine existing conditions and to monitor changes in them (Peddicord 1984). Thus it became necessary to identify suitable biological indicators of pollution to quantify and monitor changes in the environment brought on by harmful human practices and take remedial action not only to repair damage already done, but to prevent such occurrences in future.

Martin and Coughtrey (1982) (in Phillips and Rainbow 1993) recognized the importance of separating the terms *biological indicators* and *biological monitors* as most authors used them synonymously. They proposed that the two terms are in fact distinct and should not be used interchangeably.

1. Monitoring Organisms

These are used primarily to quantify pollution levels. These organisms have special characteristics which induce responses that can be measured. Biological monitors provide the means for regular surveillance and may also be used to quantify the amount of contaminants present in a particular environment.

2. Indicator Organisms

These organisms are used primarily to identify rather than to measure environmental changes brought on by the presence of a pollutant. According to Rainbow & Phillips (1993), all organisms exhibit a defined tolerance to an environmental stimulus and can exist up to a certain threshold of tolerance. Within this defined threshold, increased exposure to contaminants or natural stressors (e.g. fluctuating temperatures and/or salinities) may be met by compensatory mechanisms. Signs of toxicity are likely to occur as this threshold is exceeded. Tolerant indicator species may thrive in pollution resulting in high population densities in polluted areas, whereas sensitive indicator species indicate the presence of pollution by their absence. Also, changes in species diversity and population density in apparently suitable ecosystems may serve as an indication of the presence of pollution (Butler *et al.* 1972). Certain aquatic species have been widely documented as very useful pollution indicators e.g. it has been shown that an increase in abundance of the polychaete *Capitella capitata* is a reliable indicator of organic enrichment in the sediments of temperate marine ecosystems (Rainbow & Phillips 1993).

Martin and Coughtrey (1982) (in Phillips and Rainbow 1993) used a flow diagram (Fig. 1) to illustrate the potential for use of organisms as *biological indicators* or *biological monitors*.

1.2   Attributes of a Suitable Biological Monitor of Pollution

The choice of a suitable biomonitor of pollution rests in the species having most of the following requirements (Martin and Severeid 1984):

1. The species must integrate pollutants over time. That is, at higher concentrations, the pollutant burden increases; at lower concentrations, the pollutant burden decreases.

2. The species should be sessile or sedentary to be representative of a particular geographic area.

3. The species should be common and abundant for ease of collection.

4. The species should be large enough to provide sufficient tissues for analysis.

5. The age (or size) should be sufficient to allow sampling of more than one year class.

6. The species should be tolerant of laboratory conditions.

7. The species should be tolerant of lower salinity and higher temperatures (estuarine adaptation).

8. There should be a correlation between water concentration and the organism's body burden (i.e., bioconcentration factor).

9. The body burden of the species should rapidly reach equilibrium and consistently conform to water concentrations, regardless of location or condition.

1.3 Why Use Biomonitors?

Traditional methods used for monitoring contaminants included water and sediment analysis, but these have had their drawbacks.

1.3.1 Water Analysis

Contaminant concentration in fresh and salt water is extremely low in most cases, ranging from around the nanogram per litre (part per trillion/10^{12}) to about the milligram per litre (part per million). Despite these low levels, sensitive techniques have been developed to accurately determine the level of contaminants in salt and fresh waters. The disadvantages inherent with water analysis relate to the low concentrations of these contaminants – the low levels renders the analysis technically difficult, contamination of the sample during handling (purpose-built "clean laboratories" have been constructed for

this purpose) as well as contaminant loss (trace metals are known to adsorb to the walls of containers during sampling, leading to their loss prior to analysis). Because of these experimental errors, it became widely accepted that data on metals in seawater produced prior to the mid 1970's should not be relied on. Also, contaminants levels in water vary with time, especially in rivers and estuaries where contaminant abundance may be affected by different factors e.g. fluctuating tides, flow rate, varying times of contaminant discharge, salinity etc. The greatest disadvantage of water analysis is its inability to elucidate any useful correlations between the concentrations of contaminants present in the water and their biological availability. Because contaminant bioavailability cannot be inferred from water analysis, it has been concluded that organisms should be used for monitoring tasks (Phillips and Rainbow 1993).

1.3.2 Sediment Analysis

Sediment analysis presents certain advantages over traditional water analysis. Certain trace metals such as aluminium, iron, lead and manganese are generally found to associate with particles, with only very low concentrations remaining in solution. On the other hand, arsenic, cadmium and selenium are generally present in solution. These properties can be taken advantage of when designing monitoring schemes. Sediment analysis can be used to define locations of contaminant sources, where the level of the contaminants increase as the source gets closer. However, the bioavailability of the contaminants cannot be determined with such analyses. Sediments act as a final sink for contaminants and while remobilization may occur, a significant portion may be lost to the fauna through their irreversible binding in sediments and their final burial (Phillips and Rainbow 1993).

Experimental cadmium contamination of *S. variolaris*: Influence of short-term exposure via the waterborne route
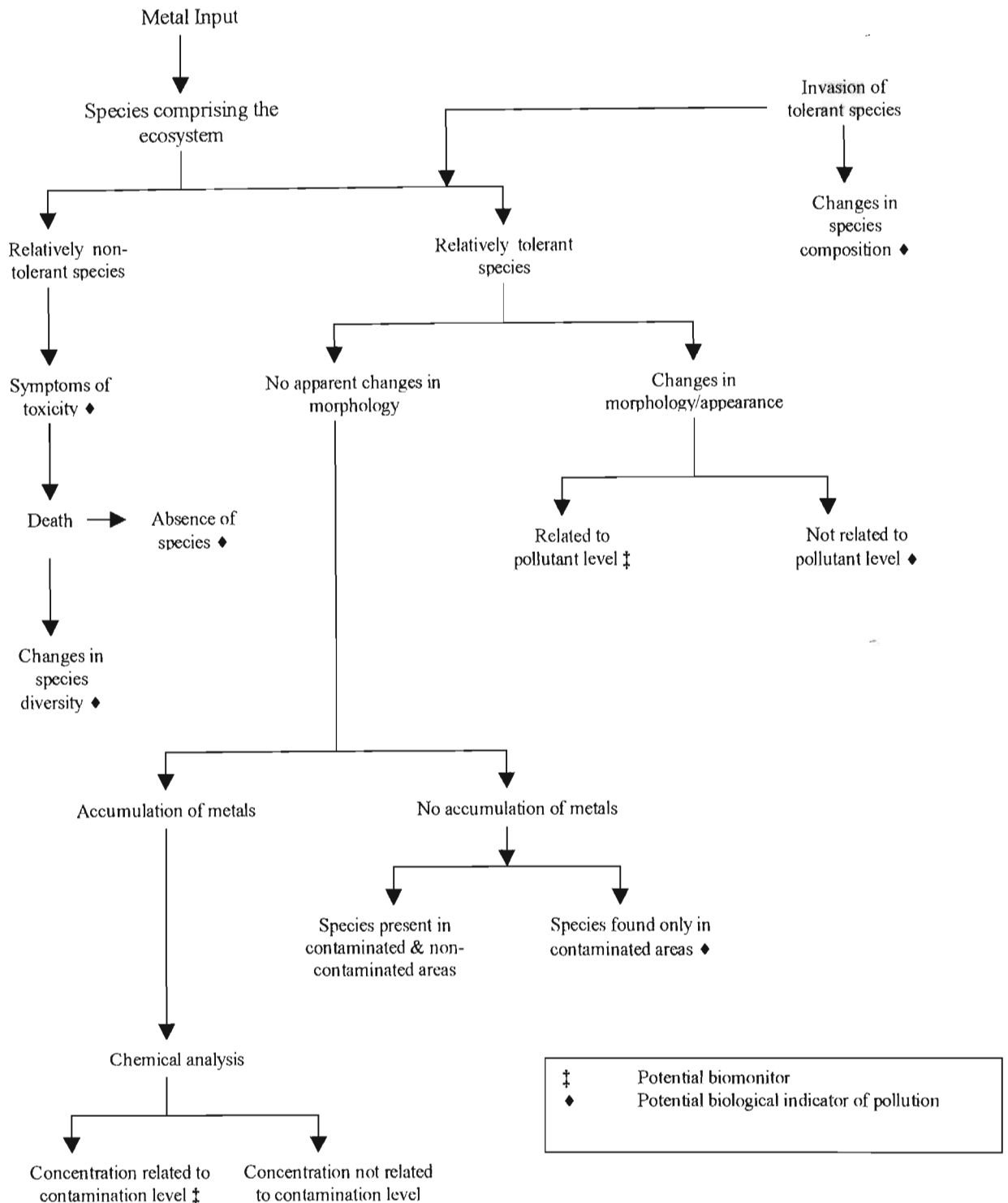
Fig.1: Relationships between the responses of organisms and metal contamination, showing the potential for use of organisms as biological indicators or biomonitors. After Martin and Coughtrey (1982) (after Phillips and Rainbow 1993).

1.4 The Dangers of Cadmium

Heavy metals have entered the marine environment naturally through the weathering of the earth's crust and this has given rise to steady-state background concentrations of metals in seawater. However, human activities have introduced large quantities of metals to localized areas of the sea, in some instances, upsetting the natural steady-state balance (Bishop 1983). The presence of an element in the marine environment does not necessarily mean that there is a potential for that element in living tissues and structures. The form a metal occurs in and its oxidation state play a role in determining its toxicity to aquatic organisms. Whether it is present in ionized form or in an oxidized or reduced state, forming complexes with other organic chemicals, or adsorbed on inorganic or organic matter, is a factor determining its uptake by marine organisms and its toxicity to them (Waldichuk 1974). Cadmium is considered as one of the most toxic heavy metals in the marine environment. On the basis of the 48h LC_{50} bioassay, the pollution hazard for fish were ranked as follows (Bishop 1983):

$$Hg^{2+} > Cd^{2+} > Cu^{2+} > Ag^{+} > Ni^{2+} > Pb^{2+} > As^{3+} > Cr^{3+} > Sn^{2+} > Zn^{2+}.$$
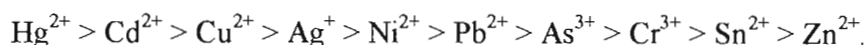
Hellawell (1988) (in Phillips and Rainbow 1993) ranked the toxicity of trace metals in Table 1, in which cadmium is listed as the third most toxic metal:

Table 1: The approximate order of the toxicity of trace metals.

Highly toxic										Less toxic	
Hg	Cu	Cd	Au	Ag	Pt	Ba	Mn	Li	Ca	Sr	Na
		Zn	Sn	Al	Fe^{3+}		Co	K		Mg	
				Ni	Fe^{2+}						

Cadmium, used extensively in electroplating, is also found in industrial paints and may represent a hazard when paint is applied. It is used in the manufacture of batteries (nickel-cadmium batteries) and in the production of protective coatings for electroplating metal parts. The largest quantity of this metal is used in compound form as a pigment and as a stabilizer for plastic production (PVC). *Itai itai* disease was caused by the release of

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cadmium-rich effluents from a zinc mine operated by the Makioki Company, situated adjacent to the Jintsu river, Japan. The receiving downstream basin was used for the irrigation of rice fields and the resulting rice crop was severely contaminated with levels an order of magnitude greater than those recorded elsewhere in Japan. The uptake of cadmium was suspected as being the chief cause of the disease in those affected. (Phillips and Rainbow, 1993). There is a global concern over the potential adverse public health impacts for cadmium. The maximum tolerable uptake for humans weekly is 400-500 μg, but these levels have been known to be approached and/or even exceeded by many populations in the world. In certain regions, cadmium contamination is the result of previous industrial activities or because of the presence of shales and other geological strata which are naturally rich in cadmium. Those placing themselves at high risk by eating unusually large amounts of the element in contaminated seafood and/or offal would undoubtedly suffer with liver and kidney complications. Due to these concerns, cadmium has been included in the "Black List" (Table 2) of contaminants for controls in Europe (Phillips and Rainbow, 1993).

Table 2: List I or "Black List" substances as originally published by the European Economic Commission (after EEC, 1976) (in Phillips and Rainbow 1993).

1. organohalogen compounds and substances which may form such compounds in the aquatic environment,
2. organophosphorous compounds,
3. organotin compounds,
4. substances in respect of which it has been proved that they possess carcinogenic properties in or via the aquatic environment,
5. mercury and its compounds,
6. cadmium and its compounds,
7. persistent mineral oils and hydrocarbons of petroleum origin,
8. persistent synthetic substances which may float, remain in suspension or sink and which may interfere with any use of the waters.

Following the "Black List" was the publication of the "Red List" (Table 3) in the United Kingdom in 1988 which listed cadmium and cadmium compounds as priority

contaminants (Phillips and Rainbow, 1993). Symptoms of cadmium poisoning include brittleness of the bones (osteomalacia and osteoporosis), accompanied by pain, tenderness, muscular weakness and loss of appetite. It also has a serious effect on the human circulatory system and is known to cause hypertension. Exposure can be both acute and chronic (Waldichuk 1974).

Acute Exposure : Metal fume fever may result from acute exposure with flu-like symptoms of weakness, fever, headache, chills, sweating and muscular pain. Acute pulmonary edema usually develops within 24 hours and reaches a maximum by three days. If death from asphyxia does not occur, symptoms may resolve within a week.

Chronic Exposure : The most serious consequence of chronic Cd poisoning is cancer (lung and prostate). The first observed chronic effect is generally kidney damage, manifested by excretion of excessive (low molecular weight) protein in the urine. Cadmium may also cause anaemia, teeth discoloration (Cd forms CdS) and loss of smell (anosmia).

Cadmium is therefore an extremely toxic metal that perhaps should be afforded more notoriety, as is the case with mercury and lead.

Table 3: The "Red List" of priority contaminants of the United Kingdom (after Phillips and Rainbow 1993).

1. Mercury and its compounds
2. Cadmium and its compounds
3. γ-Hexachlorocyclohexane
4. DDT
5. Pentachlorophenol
6. Hexachlorobenzene
7. Hexachlorobutadiene
8. Aldrin
9. Dieldrin
10. Endrin
11. Polychlorinated biphenyls
12. Dichlorvos
13. 1,2-Dichloroethane
14. Trichlorobenzene
15. Atrazine
16. Simazine
17. Tributyltin compounds
18. Triphenyltin compounds
19. Trifluralin
20. Fenitrothion
21. Azinphos-methyl
22. Malathion
23. Endosulphan

1.5   Echinoderms as Biological Monitors of Pollution

Mussel species are generally considered good indicators of heavy metal pollution, and are used worldwide as part of the international "mussel watch" program. However, studies conducted on the mussel *Perna perna* (a common bivalve occurring on the coast of KwaZulu-Natal) exposed to mercury showed that it was able to eliminate much of the accumulated metal and repair the tissues in a short space of time, thus casting doubt on the usefulness of this species as a bioindicator of heavy metal pollution (Gregory *et al* 2002). Also, mussel species are not distributed in all ecosystems, and so additional indicator species are needed in communities where mussels are absent (Warnau *et al* 1995a). More importantly, an effective characterization of the contamination status of an ecosystem must rely on the use of several biomonitor species (Warnau *et al* 1996). New techniques for the monitoring of aquatic contamination have replaced basic methods employed previously. Traditional methods of water sampling and their analysis for minute concentrations of contaminants have largely been replaced by a reliance on organisms to monitor pollution (Phillips and Rainbow 1993).

Due to their complex symmetry, echinoderms appear to be an especially suitable group in order to produce specimens with spectacular abnormalities (Catoira Gómez and Míguez Rodríguez 1998). Echinoderms form a major component of the macrobenthos in most marine habitats and tend to be sensitive to many forms of pollutants (Newton and McKenzie 1998). Most of the echinoderm classes have been used in ecotoxicological studies. Ophiuroids such as *Ophiothrix fragilis* and *Amphiura chijei* were used to measure responses to environmental stressors by measuring the 'righting' time after being inverted, 'avoidance' responses where the arms were withdrawn into the sediments and 'surfacing' response where the ophiuroid emerged from the sediment (Newton and McKenzie 1998). The use of asteroids in ecotoxicology has also met with great success, especially the asteroid *Asterias rubens* which proved capable of accumulating a diverse array of heavy metals including cadmium, lead, zinc, copper, iron and titanium thereby making it a suitable biomonitor species (Temara *et al* 1994, Temara *et al* 1995, Temara *et al* 1996a,b, Temara *et al* 1997, Temara *et al* 1998a,b). The loss of cushion stars *Asterina phylactica* was used as a measure to quantify the damage caused to the Pembrokeshire coastline caused by the oil tanker *Sea Empress* running aground on rocks at the mouth of

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Milford Haven (Crump and Emson 1998). Holothuroids such as *Thyone briareus* were also used in heavy metal studies involving mercury uptake (Farmanfarmaian *et al* 1982).

Few investigators have utilized adult echinoids for toxicity testing, in part because of the utility of gametes and embryos for such testing and because of the general impression that sea urchins tend to survive in areas known to be polluted especially by organic-rich sewage effluents. It was found that the adult *Echinometra mathaei* was the least sensitive indicator of toxicity (Dinnel *et al* 1988). Most tests conducted on adult sea urchins involve chronic exposures, and assess a variety of sublethal endpoints, including behavioral modifications, growth impairment, bioaccumulation or effects on gametes and/or embryonic development following spawning (Dinnel *et al* 1988). The echinoid *Paracentrotus lividus*, endemic to the Mediterranean Sea, is known to bioaccumulate cadmium very effectively, thus establishing its role as an effective biomonitor of cadmium pollution (Warnau *et* al 1995a,b,c, Warnau *et al* 1998a,b). Other echinoid species also proved to be valuable bioindicators of pollution e.g. *Diadema setosum* (Flammang *et al* 1997), *Echinus esculentes* (Catoira Gómez and Míguez Rodríguez 1998) and *Tripneustes gratilla* (Dafni 1980). Pathological effects exhibited by sea urchins as a result of pollutant exposure can be observed at the macroscopic, microscopic or biochemical level. Specimens of *T. gratilla* collected from the Gulf of Eilat (Aqaba) showed gross abnormalities and deformations in the skeleton. The collection site was shown to be extremely polluted, with heavy metals such as iron and copper being key ingredients in the toxic soup (Dafni 1980). Echinoids have also been used in assessing the effects of agricultural pesticides runoff. Organic phosphates represents a component of pesticides that was shown to inhibit feeding, fecal production, nutrient absorption, growth and righting behavior at sub-lethal concentrations in the sea urchin *Lytechinus variegates* (Böttger *et al* 2001). The green sea urchin *Strongylocentrotus droebrachiensis* has been used with great success in assessing the effects of chemical wastes from salmon aquaculture sites in Canada (Chou *et al* 2003). Reports on echinoderms used in ecotoxicological research in South Africa appear to be lacking, except for a single report by Moodley (1997). Skeletal elements (spicules) of the holothurian *Pseudocnella sykion* were found to accumulate aluminium when analysed by energy dispersive X-ray analysis (EDX) (Moodley, 1997). Toxicity tests, in which percentage fertilization was determined,

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were undertaken by the Council for Scientific and Industrial Research (CSIR - Environmentek) using the gametes of sea urchins viz. *Echinometra mathaei*, *Tripneustes gratilla*, or *Stomopneustes* to assess the toxicity of solid waste effluent (CSIR 2000).

1.6 *Stomopneustes variolaris* as a Biological Monitor of Pollution

Stomopneustes variolaris (Fig. 2) is a long-spined echinoid widely distributed in the Indo-Pacific. In southern Africa, it inhabits the high energy intertidal rocky shores on the east coast, between Inhambane and Preslies Bay (Drummond 1993). On the KwaZulu-Natal coast, it is a sedentary, and frequently cryptic, drift feeder. It is found within the intertidal region of the KwaZulu-Natal coast, usually the wave-swept intertidal shelves of the lower Balanoid zone, deep pools in the low and mid-shore and channels and gullies extending into the mid and upper-shore (Drummond 1993). It fulfills most of the requirements outlined previously by Martin and Severeid (1984) for being categorised as a suitable biomonitor species. However, no data on dose-dependent bioconcentration is available for this species.

1.7 Study Objectives

This investigation will examine the extent to which *Stomopneustes variolaris* accumulates Cd via the waterborne route during short-term exposure to the metal. This information would be useful especially if effluent containing Cd is released in pulses into the marine ecosystem.

Three different tissues were intended for examination:

a) Skeleton : This tissue was examined because it represents the first physical barrier to prevent the entry of pollutants.

b) Intestine : Examination of the intestine was also undertaken because it represented the first route of entry of heavy metal-laden water into the body cavity.

c) Gonads : Sea urchins are harvested commercially for their gonads. As sea urchin roe is considered a delicacy in many parts of the world. Ingestion of cadmium

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contaminated gonads by humans could lead to severe consequences as already described.

The study will also attempt to determine whether the urchins are capable of bioremediation; a factor which may have application in rendering the organisms relatively safe for human consumption.

Furthermore, the study intends to investigate how two methods, namely atomic absorption spectroscopy (AAS) and energy dispersive x-ray analysis (EDX) compare with regard to the accumulation of Cd in the skeleton of the urchins.

In order to determine whether *S. variolaris* constitutes a valuable biomonitor of heavy metal (i.e. Cd) contamination in its environment, the present investigation aims to elucidate answers to the following questions:

1.   If *S. variolaris* does bioaccumulate, what levels of Cd are found in the skeleton, gonads and the gut?

2.   Is the bioaccumulation of cadmium dose-dependant?

3.   Are contaminated specimens capable of bioremediation when exposed to uncontaminated seawater?

4.   Since energy dispersive x-ray analysis (EDX) is a simpler tool to use as opposed to atomic absorption spectroscopy (AAS), how do these two methods compare with regard to detection of cadmium in the skeleton?

Fig. 2: The sea urchin *Stomopneustes variolaris* (Echinoidea: Echinodermata)

2. MATERIALS AND METHODS

2.1. Specimen collection and maintenance

Fifty specimens of *S. variolaris* were collected from the intertidal zone at Park Rynie beach on the south coast of KwaZulu-Natal (30°31'S, 31°E). The specimens were divided into five groups (A, B, C, D and E) comprising 10 specimens each. Group A served as the control group i.e. those that were collected from the field and analysed to determine background Cd levels . Groups B, C and D (experimental) were be placed into three separate aquarium tanks containing 5, 20 and 50μg l^{-1} Cd respectively, where they remained for 2 weeks. These are sub-lethal levels that fall into the range used by several authors studying bioaccumulation effects in echinoderms (Khristoforova *et al* 1984, Warnau *et al* 1995a, Warnau *et al* 1995c, Temara *et al* 1996, Warnau *et al* 1997). A review of the literature revealed no LC$_{50}$ studies done using echinoids. The final concentrations were obtained by weighing CdCl$_2$, taking into account the weight of the chlorine portion of the salt. For example, to attain a final concentration of 5μg l^{-1} Cd, 5 g of Cd needed to be weighed out, dissolved in 1 l of seawater and diluted serially to obtain a final concentration of 5 μg l^{-1} Cd. To obtain 5 g of Cd from CdCl$_2$, 8.15 g CdCl$_2$ needed to be weighed out as this amount also takes into account the weight of the chlorine. This amount was obtained by multiplying 5 g Cd by 183.3 (MW CdCl$_2$) and dividing by 112.4 (MW Cd). The same procedure was used to weigh out 20 and 50 g Cd. Purified seawater from the Oceanographic Research Institute (ORI) was used for the duration of the experiments. Background metal levels in the seawater was not investigated.

The water was changed every three days to maintain the Cd concentrations, so as to eliminate the need to test the Cd levels in the water. The level of contamination remained as described above for the exposure period. Group E was used for bioremediation studies. Throughout the experiments, the animals were not fed. This was done to determine the effects of Cd contamination on the urchins via seawater alone.

2.2. Determination of cadmium levels in tissues

After the exposure, the specimens were removed from the tanks and placed in a freezer. After thawing, the specimens were dissected and 0.5 g pieces of tissue from the gonads and skeleton and 0.1 g from the intestine were excised to test for the presence of cadmium. Atomic absorption spectroscopy (AAS) was used to determine cadmium levels in soft tissues and the skeleton whereas energy dispersive X-ray analysis (EDX) was used to detect cadmium levels in the skeleton only. The AAS was calibrated using 1.00 and 3.00 μg Cd ml^{-1} solutions (Tables 9-13)

2.2.1 Determination of cadmium levels in soft tissues

Tissues from the three different regions of each echinoid were individually processed. The soft tissues (gut and gonads) were dried overnight in an oven set at 90°C. Once dried, 0.5g of dried tissue was digested in 6ml concentrated nitric acid at room temperature for approximately 12 hours before slow heating began. For the intestine, 0.1 g dried tissue was used because of its low yield after rinsing and drying. Samples were heated on a hot plate in a fume hood to constant boiling until dryness. The contents of the flask were then cooled and diluted with 20 ml deionized distilled water. After dilution, the samples had a clear to slight yellow tint. The inside of the flasks were then rinsed with deionized water to ensure complete transferral of the acid digest. Digested samples were brought to volume with deionized water. These samples were then subjected to AAS analysis. No blanks were used as the AA spectrometer was calibrated using solutions of 1.00 and 3.00 μg Cd ml

2.2.2 Determination of cadmium levels in the skeleton

Two methods were employed to investigate the presence of cadmium in the skeleton of *S. variolaris*.

2.2.2.1. Atomic absorption spectrometry

The skeletal elements were cleansed of non-calcified tissues using a 1% proteinase k solution incubated at 37°C (Dubois and Jangoux 1984). This was

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done to ensure that soft tissue which could potentially contain cadmium was removed from the skeleton. The tissues were then washed three times in deionised distilled water. Once again, 0.5g of the skeleton was used for acid digestion which was accomplished successively at 20°C, 40°C, 60°C and 80°C for 12h, 24h, 12h and 12h respectively (Warnau *et al.* 1995). Digests were allowed to cool, brought to volume with deionised water and filtered on Whatman filters (Number 4). Cadmium levels were measured as for soft tissue using AAS.

2.2.2.2. *Energy dispersive X-ray analysis*

Skeletal elements were treated as above to remove traces of soft tissue. The samples were then dried, carbon-coated and analysed for cadmium using a Noran "Voyager 2100" EDX system interfaced with a JEOL scanning electron microscope. Analyses were performed at a magnification of 640X with a working distance of 15 mm at 25 kV. Dead time was maintained between 25 and 30% and live time at 120 seconds. The inner, outer and cross-sectional surfaces of the skeleton were scanned for Cd.

2.3   Bioremediation Studies

Group E (n=10) was exposed to $20\mu g\ l^{-1}$ Cd for 2 weeks. They were then rinsed and placed into a tank containing uncontaminated seawater and after a further 2 weeks, the specimens were analysed to determine cadmium levels in the gut, gonads and skeleton.

2.4   Data Analysis

Data collected was statistically analysed using GRAPHPAD INSTAT® for Windows® 9X. The tests used included a One-way Analysis of Variance (ANOVA) and a Tukey-Kramer Multiple Comparison Test to determine if levels of cadmium in the three different tissue types were significantly different for the three exposure modes. An unpaired t-test was used to determine if the levels of

cadmium in the three tissue types dropped significantly after the specimens were bioremediated.

3.   RESULTS

The tissues were analysed for cadmium bioaccumulation using AAS and the results are presented in Table 4.

Table 4: *Stomopneustes variolaris*. Bioaccumulated cadmium levels (μg g^{-1} dry weight; means $\pm$ standard error) in the different tissues of the echinoids. n = number of samples.

Treatment	n	Gonads	Intestine	Skeleton
Field specimens	10	6.17 $\pm$ 0.86	6.88 $\pm$ 0.45	4.98 $\pm$ 0.34
5 μg l^{-1}	10	5.80 $\pm$ 0.71	1.88 $\pm$ 0.62	4.36 $\pm$ 0.17
20 μg l^{-1}	10	5.96 $\pm$ 1.07	12.78 $\pm$ 2.22	6.20 $\pm$ 0.12
50 μg l^{-1}	10	6.69 $\pm$ 1.31	20.04 $\pm$ 1.22	4.99 $\pm$ 0.22
Bioremediated specimens	10	2.90 $\pm$ 0.57	8.66 $\pm$ 0.95	6.40 $\pm$ 0.11

S. variolaris does possess the ability to accumulate cadmium (Table 4). In the intestine, the level of Cd appeared to increase appreciably in a dose-dependent fashion (Table 4). Cadmium levels in the gonads also appeared to have increased in response to increasing concentrations of the metal, but differences observed here were not as marked as those recorded for the intestine. Of particular concern are the levels of Cd recorded in specimens taken directly from the field – these background levels indicate the presence of the metal in the environment.

3.1 Bioaccumulation Experiments

The cadmium levels in the gonads, intestines and skeleton of the specimens after exposure to three different levels of cadmium (5, 20 and 50 µg l^{-1}) were compared and the results analysed using ANOVA.

In the intestine, the level of Cd appeared to increase appreciably in a dose-dependent fashion (Table 4). Cadmium levels in the gonads also appeared to have increased in response to increasing concentrations of the metal, but the differences observed here were not as marked as those recorded for the intestine. Although the skeleton accumulated Cd, the accumulation was not dose dependent.

Table 5: *S. variolaris*. ANOVA conducted to assess dose-dependent bioaccumulation in the intestine and skeleton. The results for the gonads are not presented here because of the high P value obtained (P>0.05).

Comparison	Mean Difference	q	P value
INTESTINE			
5 µg l^{-1} vs. 20 µg l^{-1}	-10.900	7.236 ***	P<0.001
5 µg l^{-1} vs.50 µg l^{-1}	-8.560	5.683 **	P<0.01
20 µg l^{-1} vs.50 µg l^{-1}	2.340	1.553 ns	P>0.05
SKELETON			
5 µg l^{-1} vs. 20 µg l^{-1}	-1.836	10.447 ***	P<0.001
5 µg l^{-1} vs. 50 µg l^{-1}	-0.6320	3.596 *	P<0.05
20 µg l^{-1} vs. 50 µg l^{-1}	1.204	6.851 ***	P<0.001

a) Gonads

Dose-dependent bioaccumulation was not demonstrated in the gonads of this echinoid. Although there appeared to be an increase in Cd levels in the gonads in response to increasing Cd dosages, these differences were found to be insignificant (P>0.05).

b) Intestine

The intestine showed a marked increase in Cd levels in response to increasing Cd levels in the water. In this instance, the difference in Cd levels recorded from those specimens exposed to 5 µg l^{-1} Cd and 20 µg l^{-1} Cd were found to be highly significant (Table 5;

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P<0.001). Those specimens exposed to 5 μg l^{-1} and 50 μg l^{-1} Cd also showed significant differences in the levels of Cd in the intestine (Table 5; P<0.01). The levels recorded in the intestine of those specimens exposed to 20 μg l^{-1} and 50 μg l^{-1} was not significantly different (Table 5; P>0.05).

c) Skeleton

Table 5 show that there are significant differences in the levels of cadmium accumulated in the skeleton for each of the different treatments considered. The results obtained for the levels in the skeleton, however, are somewhat anomalous as those specimens exposed to 20 μg l^{-1} Cd recorded higher levels in the skeleton than those exposed to 50 μg l^{-1}. Dose dependency was therefore exhibited only in those animals exposed to 5 and 20 μg l^{-1} Cd.

Cadmium levels in the three body regions i.e. gonads, intestine and skeleton were also compared within each treatment group to determine if the differences in the metal levels recorded there were significantly different from each other. The results are presented in Table 6.

Table 6: *S. variolaris*. ANOVA conducted to assess significance between cadmium levels in the three body regions for each treatment.

Comparison	Mean Difference	q	P value
5 μg l^{-1}			
Gonads vs. Intestine	3.920	7.069 ***	P<0.001
Gonads vs. Skeleton	1.440	2.597 ns	P>0.05
Intestine vs. Skeleton	-2.480	4.472 *	P<0.05
20 μg l^{-1}			
Gonads vs. Intestine	-6.820	4.792 **	P<0.01
Gonads vs. Skeleton	-0.2360	0.1658 ns	P>0.05
Intestine vs. Skeleton	6.584	4.626 **	P<0.01
50 μg l^{-1}			
Gonads vs. Intestine	-3.755	3.598 *	P<0.05
Gonads vs. Skeleton	1.693	1.622 ns	P>0.05
Intestine vs. Skeleton	5.448	5.220 **	P<0.01

Specimens Exposed to 5 μg l^{-1} Cd

Those specimens exposed to 5 μg l^{-1} (Table 6) showed that the gonads had significantly higher Cd levels than the intestine (P<0.001) and the skeleton had significantly higher levels of Cd than the intestine (P<0.05). The differences between the gonads and skeleton were not significant.

Specimens Exposed to 20 μg l^{-1} Cd

Specimens exposed to 20 μg l^{-1} Cd had significantly higher levels of Cd in the intestine than in the gonads and skeleton (P<0.01) (Table 6). The differences between Cd levels in the gonads and skeleton were not significant (P>0.05).

Specimens Exposed to 50 μg l^{-1} Cd

Specimens in this treatment group exhibited significantly higher Cd levels in the intestine as compared to the gonads (P<0.05) and skeleton (P<0.01) (Table 6).

The general trend evident here is the gonads and skeleton showing similar Cd uptake at each exposure i.e. there are no significant differences in the Cd levels in these body regions at each concentration.

3.2   Bioremediation Experiment

Cadmium levels between those specimens exposed to 20 µg l^{-1} and those that were bioremediated for two weeks were compared using an unpaired t-test. The results are shown in Table 7.

Table 7: Statistical analyses of the Cd levels recorded in the intestine, gonads and skeleton to gauge the efficacy of bioremediation.

Comparison	Mean Difference	t	F	P value
Gonads – 20 vs. Bioremediation	-3.064	2.523	3.493	0.0213
Intestine – 20 vs. Bioremediation	-4.120	1.708	5.449	0.1048
Skeleton – 20 vs. Bioremediation	0.2040	1.274	1.173	0.4080

a) Gonads

There is a significant decrease in cadmium levels in the gonads from 5.96 µg Cd g^{-1} dry weight to 2.90 µg Cd g^{-1} dry weight tissue ($P<0.05$) after exposure to uncontaminated seawater.

b) Intestine

The decrease in Cd levels in the intestine from 12.78 to 8.66 µg Cd g^{-1} dry weight is not significant ($P>0.05$) after exposure to uncontaminated seawater.

c) Skeleton

The high P value of 0.4080 ($P>0.05$) (Table 7) suggests that the difference in cadmium levels between specimens exposed to 20 µg l^{-1} and bioremediated specimens is not significant. It can therefore be concluded that there was no cadmium loss from the skeleton when non-contaminating conditions prevailed.

3.3   Energy Dispersive X-ray Analysis (EDX)

Cadmium levels in the skeleton were undetectable using EDX (Appendix II) . A scan of the skeleton exposed to 50 μg l^{-1} Cd is shown in Fig. 3. The skeleton is comprised primarily of calcium carbonate, and the double calcium peak recorded in the scan relates to the abundance of the element in the skeleton. Appendix II provides the printout of the elemental analysis from the EDX system. Whilst some of the printouts indicate the presence of Cd (weight %), these should not be regarded as the weight percentage error column shows that the percentage error is greater than the elements weight percentage composition.

Fig. 3: Surface scan of the skeleton of *S. variolaris* using EDX indicating the abundant elements present in the skeleton (% composition). Note that Cd levels are undetectable.

4. DISCUSSION

4.1 Metal Uptake by Aquatic Organisms

Aquatic organisms take up metals by solution and/or food. These animals are constantly bathed with this contaminated water in concentrations ranging from nanograms to micrograms per litre. Metals have a high affinity for proteins and other macromolecules and it is thought that their uptake across permeable surfaces is a passive process requiring no expenditure of energy (Phillips and Rainbow 1993).

4.2 Bioaccumulation in *Stomopneustes variolaris*

Surveys undertaken by the Council for Scientific and Industrial Research (CSIR) in 1999 at various sampling stations situated along the east coast of KwaZulu-Natal from the Durban Harbour to Isipingo beach showed sediment levels to have an average of 0.14 μg g^{-1} Cd (this average was well within the range of California "pristine" sediments), with the highest being 2.74 μg g^{-1} Cd (CSIR 2000). The CSIR report also investigated the levels of Cd bioaccumulated in bivalve molluscs viz. the mussel *Perna perna* and the oyster *Crassostrea* spp., and levels here were found to be 5.7 μg g^{-1} dry mass, much higher than the amount present in the sediment. The levels in the bivalve molluscs were considered high according to standards set by the U.S. "Mussel Watch" programme (CSIR 2000) and are comparable to the levels of Cd found in the gonads, intestine and skeleton of *S. variolaris* (Table 4). Although the *P. perna* specimens used by CSIR was not from the same site as *S. variolaris*, both these animals are exposed to similar effluent being discharged along the KwaZulu-Natal coastline. This supports the use of echinoids as biomonitors of Cd contamination as well. The difference in Cd levels between the bivalve molluscs and sediments as reported by the CSIR should provide further impetus for the use of additional pollution biomonitors. Whilst sediment show low levels of Cd, it is important to determine the fraction of Cd that is biologically available because:-

a)   it is this fraction that would end up in the food web and become biomagnified at higher trophic levels and

b) cadmium is generally present in solution (Phillips and Rainbow 1993), therefore levels of this metal present in sediment is not a good indicator of the overall contamination of the site.

Table 8: Comparison of the mean Cd concentrations (μg g^{-1} Cd) in the gonads, intestine and skeleton of different sea urchin populations from the literature with *S. variolaris*.

Sites	Organ	Cd level	Species	Reference
Park Rynie	gonads	6.17	*S. variolaris*	present study
	intestine	6.88		
	skeleton	4.98		
Armorique (Breast Bay)	gonads	2.9	*Sphaerechinus granularis*	Guillou *et al* (2000)
	intestine	5.4		
Marloux (Brest Bay)	intestine	3.83		
Ischia Island, Bay of Naples (Italy)	gonads	0.45	*P. lividus*	Warnau *et al* (1995a)
	intestine	4.65		
	skeleton	0.13		
Singapore coral reefs (8 study sites)	gonads	11.34	*D. setosum*	Flammang *et al* (1997)
		6.76		
		10.15		
		5.86		
		6.48		
		4.49		
		8.61		
		5.03		
Calvi Bay (Corsica)	gonads	0.24[a]	*P. lividus*	Warnau *et al* (1998a)
	intestine	5.02[a]		
Marseille, France	gonads	0.45[a]	*P. lividus*	Warnau *et al* (1998a)
	intestine	5.93[a]		
Clyde Sea (Scotland)	gonads	0.4	*Echinocardium cordatum*	Buchanan *et al* (1980) in Guillou *et al* (2000)
	intestine	1		
Saudi Arabia (coastal)	gonads	0.11	*Echinometra mathaei*	Sadiq *et al* (1996)
	intestine	0.07		
	skeleton	0.80		

[a] – Average Cd level taken over seven months

Other studies using sea urchins as biomonitors of Cd pollution worldwide are compared to the levels found in *S. variolaris* for this study and is summarized in Table 8. The levels of Cd recorded in the intestines in the species listed in Table 8 are much higher than the levels in the other body compartments. The level of Cd in the intestine of field samples of *S. variolaris* is not significantly higher than the levels in the gonads and skeleton.

S. granularis (Table 8) demonstrated its capability to monitor Cd levels in its environment. The authors found that the accumulation of Cd in *S. granularis* were much higher in the heavier polluted sites of Armorique and Marloux as compared to their reference (healthy) site (Guillou *et al* 2000).

The numerous heavy metal studies with *P. lividus* has more than adequately demonstrated its capability to be used as a biomonitor of such pollution. Warnau *et al* (1998a) described the Mediterranean sea as an almost closed sea which was of great concern given the level of industrialization along the northwestern part. The seagrass (*Posidonea oceanica*), a major dietary component of the echinoid *P. lividus*, is threatened by the coastal pollution (Warnau *et al* 1998a). It was shown that the intestine of these animals accumulated high Cd levels (Table 8) during periods of maximum productivity of *P. oceanica* and its epiphytic algae (Warnau *et al* 1998a).

Flammang *et al* (1997) made use of the echinoid *D. setosum* to assess the status of heavy metal contamination threatening coral reef ecosystems around the Southern Islands, Singapore. Sources of contamination were from port and shipping facilities and various petroleum industries. The authors here noted a positive correlation between the levels of Cd in gonads of *D. setosum* to the proximity of the industries i.e. Cd levels were the highest – 11.34 µg l^{-1} (Table 8) in the vicinity of the industries. The lowest levels of Cd were found in those echinoids taken from the sampling station furthest from the industrial activity (Flammang *et al* 1997).

Sea urchins (*Echinometra mathaei*) were also used to assess the impacts to the Saudi coast of the Arabian Gulf caused by the 1991 oil spill and Gulf War activities (Sadiq *et al* 1996). Elevated Cd levels were also found in the tissues of this echinoid (Table 8).

Table 8 shows that echinoids are being used to assess Cd contamination in many areas worldwide, thus supporting the use of *S. variolaris* in assessing the Cd

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contamination along the KwaZulu-Natal coast. *S. variolaris* also showed comparatively high Cd levels as compared to the levels in the above echinoids (Table 8). The Cd levels in the field samples in the three body compartments were found to be statistically similar. This is a departure from the usual trend where other studies have shown the intestine to accumulate much higher Cd levels. This is probably due to the ingestion of Cd contaminated food material. Even though the intestine also represents a site of excretion, the continual consumption of Cd contaminated material from the polluted site would account for higher Cd levels. Warnau *et al* (1995c) stated that *P. lividus* showed a decrease in Cd uptake in the intestine when exposed to Cd via seawater and food. It is possible that a similar mechanism is operating in *S. variolaris*, but this would require verification in future experiments. This type of bioaccumulation comes closest to the type displayed by *Echinometra mathaei*, in which the Cd levels in the skeleton and gonads were significantly higher than the intestine (Sadiq *et al* 1996).
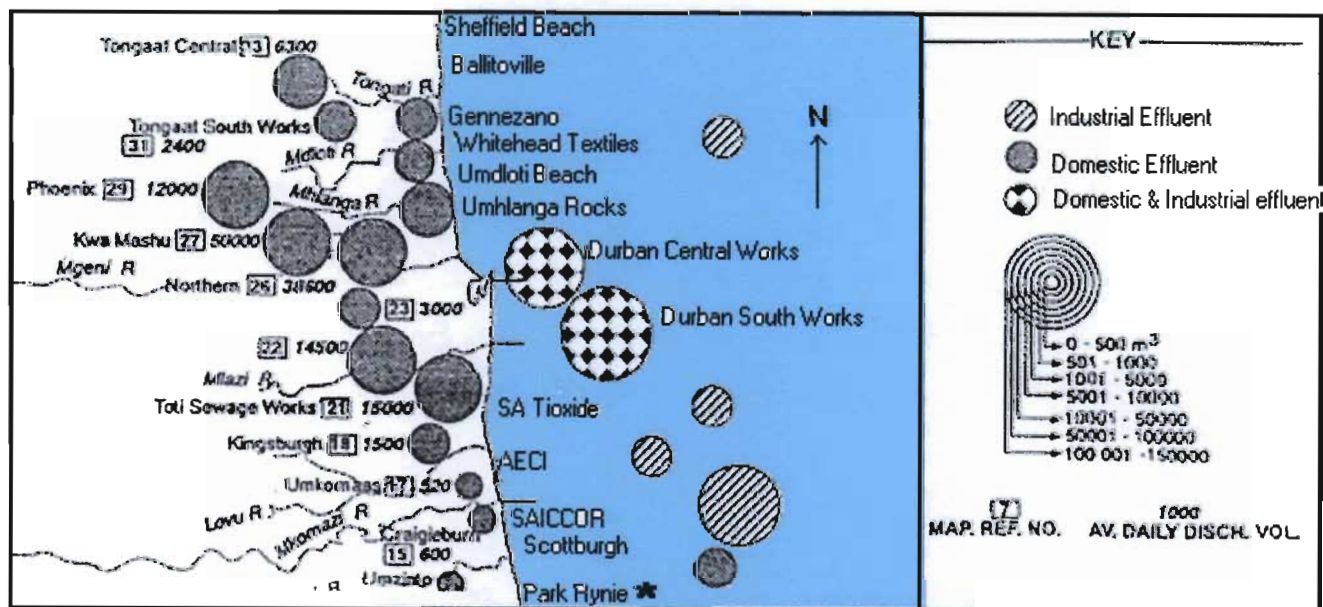
Fig. 4: Sites of domestic and industrial discharge sites along the KwaZulu-Natal coastline.

* - Park Rynie (Collection site of field specimens).

From http://www.ceroi.net/reports/durban/issues/Marine/pressure.htm#pipeline

The Cd in the three body compartments in the field specimens could be attributed to the proximity of the collection site with disposal facilities (Fig. 4). Wastewater and ambient water samples typically contain complex mixtures of trace organic and metal

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components, including Cd which is commonly present in effluent (Phillips *et* al 2003). There are eighteen point sources (including sewerage works outflows and marine pipelines that discharge effluent into the sea around the Durban Metropolitan Area (DMA). Together, the Durban Central outfall and the Durban Southern outfall carry in excess of 200 000 m^3 of domestic and industrial effluent, including sewage sludge, into the sea every day. The southern metropolitan region, around the Umlaas and Reunion canals, is the most heavily polluted marine area in the Durban Metropolitan Area. At least 14 500 m^3 per day of mainly domestic effluent finds its way into the sea from these canals. Industrial outfalls include the SA Tioxide, AECI and SAPPI-SAICCOR pipelines (The SAPPI SAICCOR pipeline discharges at Umkomaas which is not included in the Metropolitan Area. The effluent does, however, impact on the waters of the DMA, therefore it was included here). These pipelines extend 2 to 4 km out to sea. AECI pumps 3 500 m^3 of calcium and sodium salts, which include trace levels of metals, through their pipeline into the sea every day. The adjacent SA Tioxide pipeline adds a further 5000 m^3 of sulphuric acid and ferrous sulphate each day. The SAPPI-SAICCOR pipeline carries approximately 90 000 m^3 of lignosulphate into the sea daily (Durban Metro Website). Such pollution could impact negatively on marine life, given the large volumes being discharged into the marine environment on a daily basis. This could probably account for the high levels of Cd in *S. variolaris* given the proximity of the collection site to the major discharge sites (Fig. 4).

Sea urchins do indeed possess the capability to store metals and can be used independently to quantify heavy metal pollution in the field as the above authors have indicated OR be used in conjunction with other monitor species e.g. bivalve molluscs to effectively determine the pollution status of an ecosystem, as suggested by Warnau *et al* (1996).

Dose dependent bioaccumulation is one of the criteria a good biomonitor of pollution should possess i.e. a simple relationship between contaminant level in the environment and the organism's tissue (Warnau *et al* 1995a). The results for dose-dependent Cd bioaccumulation was met with rather limited success in this study. The

gonads of *S. variolaris* did not exhibit dose-dependent bioaccumulation as the differences in cadmium levels after each treatment were not significant (P>0.05). The skeleton showed significant differences in cadmium levels for those specimens exposed to 5 and 20 µg l^{-1} Cd and 5 and 50 µg l^{-1} Cd (Table 5). Those exposed to 20 µg l^{-1} showed levels that were higher than those exposed to 50 µg l^{-1}. This probably relates to the inability of the skeleton to accumulate higher levels of Cd. Table 1 shows that the skeleton had lower Cd levels than the intestine at higher Cd exposures, and this trend is evident in many echinoid species studied (Table 8).   This is due to the ability of the intestine to bioaccumulate Cd very effectively. Cadmium levels in the intestine were significantly different for each treatment except for the comparison of those specimens exposed to 20 and 50 µg l^{-1} (Table 5). The intestine showed a significant increase in cadmium concentration from 5 to 20 µg l^{-1} and 5 to 50 µg l^{-1} (Table 5) and this points to a simple relationship that exists between environmental cadmium levels and those occurring in the intestine of *S. variolaris*. A similar relationship was found with *Paracentrotus lividus* (Warnau *et al* 1995a) with respect to the intestine. Dose-dependent bioaccumulation experiments with *P. lividus* showed significant increases in all body compartments studied i.e. intestine, gonads, body wall and skeleton in specimens exposed to 20 µg l^{-1} and 50 µg l^{-1} (Warnau *et al* 1995a). The spermatozoa of the sea urchin *Anthocidaris crassispina* exhibited a good dose-dependent response to increasing Cd levels. It was found that sperm motility declined when exposed to increasing Cd concentrations of 1, 5 and 10 mg l^{-1} (Au *et al* 2000). Au *et al* (2000) attributed the decreased sperm motility to the Cd enlarging the sperm midpiece (mitochondria), affecting the streamlining and integrity of the sperm cell. The damage became more pronounced as the concentration of Cd increased. At the highest concentrations, the mitochondrial membranes and cristae became disorganized, thereby disrupting normal respiratory processes and ATP supply required for sperm movement. The study by Au *et al* (2000) showed that the sea urchin spermatozoa was more sensitive than the mussel (*Perna viridus*) spermatozoa to both Cd and phenol, thus strengthening the case for the use of sea urchins to monitor heavy metal pollution. It is fortunate that these abnormalities occur at Cd levels that are much higher than the levels present along the KwaZulu-Natal coastline. Overall, *S. variolaris* does

possess a limited capability to display dose-dependent bioaccumulation. This, however, is only restricted to the intestine.

BIOACCUMULATION OF CADMIUM IN THE THREE BODY COMPARTMENTS

Overall, analysis of the gonads, intestine and skeleton of *S. variolaris* showed higher cadmium levels in the intestine than in the gonads and skeleton at higher exposures (Table 4; Table 6), an observation that corresponds with cadmium accumulation in *P. lividus* (Warnau *et al* 1995a,c; 1996; 1998a) as well as with *Sphaerechinus granularis* (Guillou *et al* 2000). The differences in Cd levels between the gonads and the skeleton were found to be non-significant (Table 6) for all the treatments considered.

Cadmium Accumulation in the Intestine

Warnau *et al* (1998a) found certain heavy metals to be selectively distributed in the body cavity of the echinoid *Paracentrotus lividus*. One of the observations was that with the exception of lead, metal concentrations (including cadmium) were always higher in non-calcified compartments e.g. the intestine, which could be attributed to the high metabolic activities associated with this body compartment (Warnau *et al* 1995b). Warnau *et al* (1996, 1995a) identified the digestive tract wall of the echinoid *P. lividus* as a useful biomonitor as it displayed the highest concentration factor for cadmium, and this trend is displayed in *S. variolaris* at higher Cd contamination levels. Warnau *et al* (1995a,c) stated that the digestive tract wall accumulates heavy metals very rapidly and efficiently. In their experiments, *P. lividus* fed on the seagrass *Posidonia oceanica* contaminated with cadmium while exposed to cadmium-contaminated sea water resulted in **decreased** uptake of cadmium in the digestive tract as compared to contamination via the water column alone, which showed higher uptake of cadmium. It was suggested that the decreased uptake of cadmium from seawater could be due to enhanced echinoid excretion which would augment metal elimination. In the present study, cadmium was introduced via the water column only, and the intestine showed the highest cadmium uptake levels at higher exposures as compared to the gonads and skeleton (Table 4). It would have been

interesting to see the effects of a combination of contaminated seawater and algae on the uptake of Cd in the intestine of *S. variolaris*. Another mechanism suggested to decrease bioavailability of cadmium is mucus production in the intestine which would inhibit uptake from dietary sources. It appears that there are mechanisms in place to quarantine toxic heavy metals in the digestive tract so that most of it can be voided rather than entering the coelom (Warnau *et al* 1995a,c). Mucus production was also reported to occur in the sea urchin *Lytechinus variegatus* when exposed to both inorganic and organic phosphates. These animals increased digestive mucus production resulting in a mucal coating on the food boluses, thereby reducing the absorption of contaminated food (Böettger *et al* 2001).

Cadmium Accumulation in the Gonads

The accumulation in the gonads can have serious implications, not only for those species whose gonads are harvested commercially for human consumption, but also problems associated with the production of non-viable gametes. It has been shown that cadmium chloride injected into rat testes and ovaries resulted in hemorrhagic necrosis (Khristoforova *et al* 1984). Injections of other cations, including Hg were not followed by such changes. Chronic exposure of the mature sea urchin *Strongylocentrotus intermedius* to cadmium at and above 0.1 mg l^{-1} leads to the formation of anomalous sex cells and production of non-viable offspring (Khristoforova *et al* 1984). In *S. intermedius*, cadmium concentrations of 0.5 mg l^{-1} resulted not only in the resorption of ovocytes, but also a temporary increase in ovogonium number, the latter being an adaptive response to cadmium stress. There was also a decreased percentage of fertilized egg cells reported in the urchins after 10 days of cadmium exposure, suggesting a low quality of sex products (Khristoforova *et al* 1984). Warnau *et al* (1995a) found that the gonads, along with the intestine of *P. lividus*, accumulated the highest levels of Cd as compared to the body wall and skeleton. In *S. variolaris*, it was only the intestine that recorded the highest levels of Cd at the higher concentration. There were no significant differences between the levels detected in the gonads and skeleton (Table 6). This probably relates to the ability of the intestine to sequester Cd, thereby preventing it from being made available in large

quantities to gonads or the action of the coelomocytes which could have aided detoxification.

It has also been found that chronic exposure to sub-lethal concentrations of inorganic and organic phosphates resulted in significantly decreased gonad indices in the sea urchin *L. variegatus* because of reduces lipid and carbohydrate absorption. This resulted in a delay of the onset of gametogenesis. Such a delay could result in delayed spawning, less optimal planktonic development and reduced juvenile recruitment (Böettger *et al* 2001).

Cadmium Accumulation in the Skeleton

Skeletal abnormalities in the skeleton were often reported as random phenomena arising as a result of genetic failure or from damage caused by predators and parasites (Dafni 1980). However, Dafni (1980) attributed the skeletal abnormalities in the echinoid *Tripneustes gratilla* to the thermohaline and heavy metal ion effluent plume it was exposed to. Abnormalities in the skeleton included irregular bulging of the aboral half of the skeleton, a wider peristome aperture, larger Aristotle's lantern and fewer interambulacral plates than a normal urchin of the same diameter (Dafni 1980). No such deformities were observed in the skeleton of *S. variolaris*.

4.3 Bioremediation in *S. variolaris*

Detoxification mechanisms are in place to assist in the bioremediation process. Metallothioneins are proteins known to sequester heavy metals making them unavailable for biological processes. Metallothioneins are characterized by their low molecular weight, high cysteine content, lack of aromatic amino acids and high affinity for metals including silver, gold, cadmium, copper, mercury and zinc (Jenkins and Brown 1984). These proteins are located in the cytosol and accumulate in large quantities in tertiary lysosomes which are recognized in the tissues as being metal rich (Phillips and Rainbow 1993). Also, many marine organisms are capable of sequestering substantial concentrations of these metals in membrane-bound vesicles or granules, making it biologically unavailable for essential processes (Jenkins and Brown 1984). It was these

granules that came under scrutiny in a study initiated by Adams and Shorey (1998) which involved the direct analysis of the composition of these granules. Another method involved in voiding heavy metals from the body is coelomocytes. Coelomocytes have been suggested to assist mercury detoxification in the asteroid *Lepasterias polaris* after being exposed to contaminated food (Warnau *et al* 1995c). There are mechanisms within the animal that also lowers cadmium concentration within the organs, thus accounting for decreased cadmium levels in organs other than the intestine. The results indicated that for *S. variolaris*, there were no significant differences in the levels accumulated in the gonads and skeleton. Only the intestine showed increased uptake at higher exposures. Although marine organisms have the capability to detoxify, it should be noted that this capability is limited and under conditions of high metal concentrations or stress, the organisms may lose this capability altogether.

Bioremediation studies were undertaken to determine the extent of cadmium loss from body regions of the urchins after exposure to clean, uncontaminated seawater. The results indicate a significant drop in the levels of Cd in the gonads, a non-significant drop in the intestine and no significant reduction in the Cd levels of the skeleton (Table 7).

The significantly reduced levels in the gonads from 5.96 Cd to 2.90 µg Cd g^{-1} dry weight tissue were especially encouraging, especially with respect to a possible fishery for this resource. This loss of cadmium associated with bioremediation would render the organs suitable for human consumption should a fishery develop for this species. This drop in Cd could be attributed to the action of detoxification mechanisms e.g. coelomocytes as well as metallothioneins.

The non-significant drop in the levels recorded for the intestine could be a function of its ability to compartmentalize the cadmium in that region and its ability to accumulate cadmium. It has been shown that there is a net decrease in cadmium uptake in the intestine of *P. lividus* when exposed to cadmium via seawater and food as opposed to those individuals exposed to waterborne cadmium only (Warnau *et al* 1995c). In this experiment, the seawater constituted the sole source of cadmium in the aquaria. The presence of food material contaminated with cadmium would have probably decreased cadmium uptake as was the case in *P. lividus*, but this needs verification for *S. variolaris*.

Bioremediation experiments, over a two-week period had no effect in lowering cadmium levels in the skeleton (Table 7). This could be attributed to the physical and chemical properties of the skeleton. Metal elements like lead, cadmium and chromium can become trapped in the calcified compartment (Warnau *et al* 1995d). Lead and cadmium are able to substitute for calcium cations and are known to have high affinity for calcium-containing skeletons. *P. lividus* skeletons effectively accumulate cadmium when the echinoids are experimentally exposed to cadmium in seawater, and no subsequent cadmium elimination is detected when echinoids are returned to an uncontaminated environment (Warnau *et al* 1995a). *S. variolaris* seems to conform to this pattern, as there were no significant differences in Cd levels between specimens exposed to 20 μg l^{-1} and the bioremediated specimens. Warnau *et al* (1997) also stated that Cd has a long biological half-life. This, coupled with the affinity of metal ions to the calcified compartments could account for the retention of Cd in the skeleton of *S. variolaris* after exposure to uncontaminated seawater. The starfish *Asterias rubens* was found to retain cadmium in its skeleton when non-contaminating conditions prevailed (Temara *et al* 1996a). This property could make the skeleton of sea urchins more attractive biomonitors of pollution as compared to mussels, which are utilized worldwide for this purpose. The mussel *Perna perna* has been shown to eliminate accumulated mercury and repair its gill filaments in a short space of time after exposure to clean, uncontaminated seawater (Gregory *et al.* In press), thus casting doubt on the effectiveness of *P. perna* as a bioindicator of heavy metal pollution. This would not be the case in sea urchins as they would retain the heavy metals in the matrix of their skeleton.

4.4 Energy Dispersive X-ray Analysis (EDX)

Energy dispersive X-ray analysis (EDX) is particularly useful in detecting the presence of a particular element(s) in solid materials. Fig. 3 shows the elements present in the skeleton of *S. variolaris*. The major elements (carbon, oxygen, calcium and magnesium) was expected because these structures are composed primarily of magnesium-rich calcite ($CaCO_3$) (Temara *et al* 1995). Phosphorous is commonly found in bone-like structures,

and this would explain its presence in the skeleton (Stricker 1986). Sodium and chlorine are elements associated with the normal composition of seawater, and these could have been incorporated into the skeleton directly from seawater. The presence of silicon and sulphur in the skeleton could not be accounted for. EDX was unable to detect cadmium even though surfaces (internal and external) as well as cross-sections of the skeleton were scanned (Fig. 3, Appendix II). Reasons for this could be attributed to the complex surface morphology of an echinoid skeleton (a flat surface is usually favoured for these types of scans) and the cadmium levels in the skeleton being lower than the detection threshold of the apparatus. Because EDX has poor sensitivity with respect to trace elements, it becomes impossible to quantify the amount of a particular element with less than 1% weight composition in any particular specimen or groups of specimens. EDX is, however, a valuable tool and has met with success in ecotoxicological studies. There have been successes detecting heavy metals with EDX. Moodley (1997) scanned spicules from the holothurian *Pseudocnella sykion* and detected heavy metals such as aluminium and iron, but no cadmium. Adams and Shorey (1998) made use of a Philips CM12 transmission electron microscope fitted with an energy dispersive X-ray detection system (EDAX 9900) in the scanning transmission electron microscope (STEM) mode. Ultra-thin sections of the mantle of the mussel *Hydriella depressa* were prepared and 1.2 μm granules in the tissue were scanned to determine their metal composition. Metals detected included Cu, Zn, Pb, Al, Mg, P, Ba, Ca, Mn and Fe. The authors considered this study advantageous as it allowed the mantle tissue to be removed without destroying the organism, with special reference to the worldwide decline of freshwater mussels. The disadvantage associated with this technique is that it is unable to quantify the metal concentration in the tissue i.e. its *bioaccumulation*, only the percentage composition of the metal as related to the total number of elements present in the scan, thus providing an incomplete picture of the level of contamination in the environment. Bioaccumulation data provide direct information on the actual bioavailability of contaminants (Jenkins and Brown 1984). As a screening process though, EDX proves to be very useful.

4.5 Waste Minimization Measures

Mitigation measures should be put in place to reduce the metal output from industry. Pre-treatment of wastewater before release into receiving waters should be given high precedence. Although this is enforced in South Africa, accidental spillages can release voluminous quantities of pollutants into waterways which invariably find their way to a marine coastline. Commercially important species including sea urchins can become threatened. An example of this is the commercially important sea urchin *Echinus esculentes* of Galicia. Harbour installations in the area release hydrocarbons, organochlorinated and heavy metals into the water. Not only has deformities been reported for this species as a result of pollutant exposure, but also elevated metal and hydrocarbon levels in the gonads and intestine. It is rather fortunate that this species is not exploited in Galicia (Catoira Gómez and Míguez Rodríguez 1999). Metals have certain properties which allow for its treatment and/or removal from waste water discharging into the receiving waterways. Cation exchangers are one of the methods that can be utilized in decreasing the metal load of wastewater. Dissolved metals can also form highly insoluble sulphides that is precipitated from solution. Metals have a tendency to form complexes. Because of this, they can be chelated by chelating agents such as Ethylenediaminetetraacetic acid (EDTA) and the sodium salt of Nitrilotriacetic acid (NTA). These agents have been found to render the heavy metals less toxic. Nature has its own detoxifying mechanisms as well. Organic substances found in waters of high humic acid content also appears to render heavy metals less toxic. Sewage has its own organic compounds that appear to render heavy metals less toxic than they would be in pure, uncontaminated water (Waldichuk 1974).

5. CONCLUSION

It is generally recognized that the use of a single species in biomonitoring studies is not ideal from an ecological point of view (Warnau *et al* 1996). The present work supports the use of *S. variolaris* as a biomonitor species of Cd contamination on the South African East coast. Both the digestive tract as well as the skeleton would prove especially valuable as biomonitors of cadmium bioaccumulation. The intestines of this species accumulated cadmium in a dose dependent fashion, thereby fulfilling an important criterion for being a suitable biomonitor of cadmium contamination. The specimens from the field proved the ability of the gonads, intestine and skeleton to accumulate cadmium effectively and the skeleton proved capable of retaining cadmium in non-contaminating situations. This is particularly useful as it would indicate the presence of a cadmium "fingerprint" in non-contaminating conditions. *S. variolaris* should be employed to establish acceptable water quality standards.

6. FURTHER RESEARCH

There lies scope for further work that can be undertaken from here on. Histological analysis of the intestine and gonads, at both the light and electron microscope level can be performed to assess that damage done by cadmium at the cellular level. Also, histochemical analysis of the intestine to investigate the production of a mucous layer in response to increased cadmium loading may also prove interesting. The minimum duration for bioremediation remains to be established, especially if there is a potential fishery for this resource. Cadmium is not the only toxic metal in the environment. It is therefore important to consider the effects of other heavy metals on *S. variolaris* as well as the effect of combinations of heavy metals on this species as was done for *P. lividus* (Warnau *et al* 1998). Other routes of cadmium uptake such as seawater and food vs. food alone, as was done with *P. lividus* (Warnau *et al* 1995c) need to be investigated to determine if uptake rates in the various organs are affected by multiexposure experiments.

Sea urchins have the potential to be bioaccumulate of a range of contaminants (Guillou *et al* 2000) in addition to heavy metals, and these should be tested for in *S. variolaris*. Bioassays using sex cells of *S. variolaris* is also an avenue worth exploring for future research. The sex cells of *P. lividus* has been used to assess the effects of neurotoxic insecticides which are widely used for agricultural purposes (Pesando *et al* 2003) as well as ingredients found in cosmetics that could be introduced into the aquatic environment (Amouroux *et al* 1999). The sex cells are versatile and such bioassays has the advantages of being rapid and sensitive to a wide range of toxicants and more sensitive to complex effluents than are tests with adult animals (Larrain *et al* 1999).

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8.   APPENDICES

1.   APPENDIX I   :   ATOMIC ABSORPTION SPECTROSCOPY

DATA

2.   APPENDIX II   :   ENERGY DISPERSIVE X-RAY ANALYSIS

DATA

8.1 Appendix I

Results obtained for atomic absorption spectroscopy. Please note that the final cadmium levels were obtained in μg Cd ml^{-1} and was converted to μg Cd/g dry weight tissue in the final column. Highlighted rows indicate standards that were used for calibration purposes. Concentration of standards used were 1.00 μg Cd ml^{-1} and 3.00 μg Cd ml^{-1}.

KEY

| 1.00 µg Cd ml⁻¹ | 3.00 µg Cd ml⁻¹ |

Table 9: Cd levels in *S. variolaris* - Field specimen data

SAMPLE ID	µg ml^{-1}	% RSD	MEAN ABSORBANCE READINGS				µg Cd/g Dry weight tissue
GONADS							
Sample 001	0.315	13.2	0.0085	0.0097	0.0083	0.0075	7.875
Sample 002	0.135	3.3	0.0036	0.0036	0.0036	0.0038	3.375
Sample 003	0.175	8.4	0.0047	0.0051	0.0048	0.0043	4.375
Sample 004	0.183	5.2	0.0049	0.0047	0.0052	0.0049	4.575
Sample 005	0.258	3.2	0.007	0.0071	0.0067	0.0071	6.45
Sample 006	0.972	1.5	0.0263	0.026	0.0267	0.0262	24.3
Sample 007	0.244	7.5	0.0066	0.0071	0.0063	0.0063	6.1
Sample 008	0.464	2.4	0.0125	0.0123	0.0128	0.0124	11.6
Sample 009	0.123	8.9	0.0033	0.0036	0.0033	0.003	3.075
Sample 010	0.372	3.1	0.01	0.0101	0.0103	0.0097	9.3
Sample 011	0.198	4.6	0.0053	0.0053	0.0051	0.0056	4.95
Sample 012	2.854	11.5	0.0889	0.0955	0.0941	0.0771	
SKELETON							
Sample 013	0.874	1.7	0.0236	0.0236	0.024	0.0232	21.85
Sample 014	1.19	0.5	0.0324	0.0322	0.0325	0.0325	29.75
Sample 015	1.002	2.1	0.0271	0.0265	0.0272	0.0277	25.05
Sample 016	0.471	4.7	0.0127	0.0132	0.012	0.0128	11.775
Sample 017	1.203	0.7	0.0327	0.0324	0.0328	0.0329	30.075
Sample 018	1.054	1.8	0.0286	0.0291	0.0283	0.0282	26.35
Sample 019	1.151	1.5	0.0313	0.031	0.031	0.0318	28.775
Sample 020	1.042	1.2	0.0282	0.0285	0.0279	0.0284	26.05
Sample 021	1.013	1.1	0.0274	0.0276	0.0276	0.0271	25.325
Sample 022	0.954	1.6	0.0258	0.0261	0.0253	0.0259	23.85
Sample 023	1.068	2.2	0.0289	0.0296	0.0283	0.0289	26.7
Sample 024	1.058	0.3	0.0287	0.0286	0.0288	0.0285	
INTESTINE							
Sample 025	0.239	4.6	0.0064	0.0061	0.0067	0.0064	5.975
Sample 026	0.228	7.1	0.0061	0.0066	0.0061	0.0057	5.7
Sample 027	0.282	7.8	0.0076	0.0078	0.0081	0.0069	7.05
Sample 028	0.237	11.2	0.0064	0.006	0.0072	0.006	5.925
Sample 029	0.245	2.2	0.0066	0.0066	0.0068	0.0065	6.125
Sample 030	2.896	0.9	0.0908	0.0903	0.0905	0.0918	72.4
Sample 031	0.316	7.2	0.0085	0.0086	0.0091	0.0079	7.9
Sample 032	0.416	4.8	0.0112	0.0114	0.0106	0.0116	10.4
Sample 033	0.254	6.9	0.0069	0.0067	0.0065	0.0074	6.35
Sample 034	0.243	3.4	0.0066	0.0066	0.0068	0.0063	6.075
Sample 035	0.295	29.5	0.008	0.0106	0.0073	0.006	7.375
Sample 036	0.19	14.8	0.0049	0.0059	0.0049	0.0041	

Table 10: Cd levels in *S. variolaris* – Specimens exposed to 5µg l⁻¹

SAMPLE ID	µg ml⁻¹	µg Cd/g Dry weight tissue
GONADS		
Sample 001	0.163	6.52
Sample 002	0.115	4.6
Sample 003	0.116	4.64
Sample 004	0.089	3.56
Sample 005	0.15	6
Sample 006	0.179	7.16
Sample 007	0.065	2.6
Sample 008	0.23	9.2
Sample 009	0.231	9.24
Sample 010	0.112	4.48
SKELETON		
Sample 011	0.111	4.44
Sample 012	0.122	4.88
Sample 013	0.085	3.4
Sample 014	0.109	4.36
Sample 015	0.102	4.08
Sample 016	0.103	4.12
Sample 017	0.126	5.04
Sample 018	0.129	5.16
Sample 019	0.101	4.04
Sample 020	0.102	4.08
INTESTINE		
Sample 021	0.008	1.6
Sample 022	0.002	0.4
Sample 023	0.007	1.4
Sample 024	0.018	3.6
Sample 025	0.002	0.4
Sample 026	0.001	0.2
Sample 027	0.013	2.6
Sample 028	0.033	6.6
Sample 029	0.005	1
Sample 030	0.005	1

Table 11: Cd levels in *S. variolaris* – Specimens exposed to 20 µg l^{-1}

SAMPLE ID	µg ml^{-1}	% RSD	MEAN ABSORBANCE READINGS				µg Cd/g Dry weight tissue
SKELETON							
Sample 001	0.148	1.2	0.0225	0.0223	0.0223	0.0228	5.92
Sample 002	0.148	2.8	0.0224	0.0231	0.0218	0.0222	5.92
Sample 003	0.147	0.9	0.0222	0.0223	0.022	0.0224	5.88
Sample 004	0.175	0.9	0.0265	0.0263	0.0264	0.0268	7
Sample 005	0.161	2.8	0.0243	0.0245	0.0249	0.0236	6.44
Sample 006	0.154	0.6	0.0232	0.0231	0.0233	0.0233	6.16
Sample 007	0.148	1.3	0.0223	0.0224	0.022	0.0226	5.92
Sample 008	0.164	1.9	0.0248	0.0244	0.0253	0.0248	6.56
Sample 009	0.156	3.4	0.0236	0.0244	0.0229	0.0234	6.24
Sample 010	0.148	3.3	0.0224	0.0217	0.0232	0.0222	5.92
Sample 011	0.972	0.6	0.1458	0.1460	0.1464	0.1462	
INTESTINE							
Sample 012	0.142	0.6	0.0215	0.0215	0.0216	0.0213	28.4
Sample 013	0.034	7.3	0.0052	0.0051	0.0056	0.0048	6.8
Sample 014	0.046	4.8	0.0069	0.0073	0.0067	0.0068	9.2
Sample 015	0.046	6.3	0.0069	0.0065	0.007	0.0073	9.2
Sample 016	0.087	2.7	0.0131	0.0133	0.0127	0.0133	17.4
Sample 017	0.1	3.8	0.0151	0.0157	0.0149	0.0146	20
Sample 018	0.054	1.1	0.0082	0.0081	0.0083	0.0082	10.8
Sample 019	0.032	9.6	0.0048	0.0045	0.0053	0.0046	6.4
Sample 020	0.053	4.3	0.0081	0.0082	0.0083	0.0077	10.6
Sample 021	0.045	10.2	0.0068	0.0075	0.0062	0.0065	9
Sample 022	0.978	0.5	0.1474	0.1475	0.1466	0.148	
GONADS							
Sample 023	0.086	8.1	0.013	0.0142	0.0126	0.0123	4.3
Sample 024	0.084	2.1	0.0126	0.0126	0.0129	0.0124	4.2
Sample 025	0.163	1.9	0.0246	0.0242	0.0245	0.0251	8.15
Sample 026	0.1	2.8	0.0152	0.0149	0.0156	0.0149	5
Sample 027	0.073	3.6	0.0111	0.0106	0.0113	0.0113	3.65
Sample 028	0.089	4.1	0.0135	0.014	0.0134	0.0129	4.45
Sample 029	0.108	3.1	0.0164	0.0169	0.0161	0.0161	5.4
Sample 030	0.072	2.9	0.0109	0.0112	0.0108	0.0106	3.6
Sample 031	0.121	3.2	0.0183	0.018	0.0178	0.0189	6.05
Sample 032	0.296	0.5	0.0448	0.0445	0.0449	0.045	14.8
Sample 033	2.601	0.2	0.4011	0.4002	0.4014	0.4017	

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Table 12: Cd levels in *S. variolaris* – Specimens exposed to 50 µg l⁻¹

SAMPLE ID	µg ml⁻¹	% RSD	MEAN ABSORBANCE READINGS				µg Cd/g Dry weight tissue
GONADS							
Sample 001	0.36	3.1	0.0544	0.0563	0.0533	0.0537	18
Sample 002	0.071	4.5	0.0107	0.0112	0.0102	0.0106	3.55
Sample 003	0.132	1.6	0.02	0.0197	0.0199	0.0203	6.6
Sample 004	0.07	3.2	0.0106	0.0105	0.0104	0.011	3.5
Sample 005	0.114	3.8	0.0172	0.018	0.0169	0.0167	5.7
Sample 006	0.13	3.7	0.0196	0.0201	0.0199	0.0188	6.5
Sample 007	0.134	2.8	0.0203	0.0208	0.0203	0.0197	6.7
Sample 008	0.102	2.3	0.0154	0.015	0.0156	0.0157	5.1
Sample 009	0.122	2.3	0.0185	0.019	0.0183	0.0182	6.1
Sample 010	2.914	0.3	0.4152	0.4139	0.4155	0.4161	
Sample 011	0.998	0.5	0.1499	0.149	0.1498	0.1501	
Sample 012	0.102	3.1	0.0154	0.0159	0.0149	0.0155	5.1
INTESTINE							
Sample 013	0.925	1.1	0.1474	0.1499	0.1475	0.149	
Sample 014	0.053	0.9	0.0872	0.0881	0.0869	0.0866	10.6
Sample 015	0.056	4.3	0.0089	0.0085	0.0093	0.009	11.2
Sample 016	0.035	11.8	0.0056	0.0064	0.0052	0.0053	7
Sample 017	0.05	1.4	0.008	0.0079	0.0079	0.0081	10
Sample 018	0.024	14.8	0.0038	0.0042	0.0032	0.004	4.8
Sample 019	0.035	9.2	0.0056	0.0061	0.0056	0.0051	7
Sample 020	0.093	4.7	0.0149	0.0154	0.0141	0.0153	18.6
Sample 021	0.069	3.9	0.011	0.0115	0.0108	0.0107	13.8
Sample 022	0.057	4	0.0091	0.0087	0.0091	0.0095	11.4
Sample 023	0.05	3.6	0.008	0.0082	0.0077	0.0081	10
Sample 024	0.902	0.4	0.1442	0.1445	0.1435	0.1445	
SKELETON							
Sample 025	0.156	4.8	0.0252	0.0265	0.0249	0.0241	6.24
Sample 026	0.142	3	0.0228	0.0236	0.0226	0.0222	5.68
Sample 027	0.137	3.7	0.022	0.0228	0.022	0.0211	5.48
Sample 028	0.136	4.3	0.0218	0.0226	0.0221	0.0208	5.44
Sample 029	0.125	6.9	0.02	0.0214	0.02	0.0186	5
Sample 030	0.116	2.8	0.0186	0.018	0.0188	0.0189	4.64
Sample 031	0.114	3	0.0183	0.0178	0.0181	0.0189	4.56
Sample 032	0.116	3.4	0.0186	0.0193	0.0185	0.0181	4.64
Sample 033	0.107	2.1	0.0171	0.0168	0.0172	0.0175	4.28
Sample 034	0.099	2.2	0.0159	0.0161	0.0155	0.0161	3.96

Table 13: Cd levels in *S. variolaris* – Bioremediated Specimens

SAMPLE ID	µg ml^{-1}	% RSD	MEAN ABSORBANCE READINGS				µg Cd/g Dry weight tissue
SKELETON							
Sample 001	0.17	9.7	0.0274	0.0303	0.0268	0.0251	6.8
Sample 002	0.163	1.2	0.0263	0.0264	0.0265	0.0259	6.52
Sample 003	0.158	3.1	0.0255	0.0262	0.0256	0.0247	6.32
Sample 004	0.145	0.3	0.0233	0.0233	0.0233	0.0232	5.8
Sample 005	0.16	2.9	0.0258	0.0267	0.0253	0.0255	6.4
Sample 006	0.162	2.2	0.0261	0.0266	0.0263	0.0255	6.48
Sample 008	0.149	2.7	0.024	0.0232	0.0244	0.0242	5.96
Sample 009	0.173	1.8	0.0279	0.0281	0.0284	0.0274	6.92
Sample 010	0.156	2.5	0.025	0.0257	0.0244	0.025	6.24
Sample 011	0.164	2.2	0.0264	0.0268	0.0266	0.0257	6.56
Sample 012	0.926	1	0.1475	0.1494	0.1475	0.1464	
INTESTINE							
Sample 013	0.063	7.5	0.0101	0.011	0.0097	0.0096	12.6
Sample 014	0.043	7.7	0.0069	0.007	0.0074	0.0063	8.6
Sample 015	0.038	2.4	0.0061	0.0063	0.006	0.006	7.6
Sample 016	0.046	4.8	0.0073	0.0069	0.0076	0.0073	9.2
Sample 017	0.039	0.8	0.0063	0.0063	0.0062	0.0063	7.8
Sample 018	0.074	3.5	0.0118	0.0123	0.0116	0.0116	14.8
Sample 019	0.036	5.6	0.0057	0.006	0.0056	0.0054	7.2
Sample 020	0.032	5.1	0.005	0.0051	0.0048	0.0053	6.4
Sample 021	0.04	1	0.0063	0.0064	0.0064	0.0062	8
Sample 022	0.022	7.8	0.0035	0.0035	0.0038	0.0032	4.4
Sample 023	0.925	1.1	0.1474	0.1459	0.1475	0.149	
GONADS							
Sample 024	0.902	0.4	0.1442	0.1445	0.1435	0.1445	
Sample 025	0.156	4.8	0.0252	0.0265	0.0249	0.0241	2.72
Sample 026	0.142	3	0.0228	0.0236	0.0226	0.0222	1.68
Sample 027	0.137	3.7	0.022	0.0228	0.022	0.0211	1.44
Sample 028	0.136	4.3	0.0218	0.0226	0.0221	0.0208	2.16
Sample 029	0.125	6.9	0.02	0.0214	0.02	0.0186	0.52
Sample 030	0.116	2.8	0.0186	0.018	0.0188	0.0189	6.04
Sample 031	0.114	3	0.0183	0.0178	0.0181	0.0189	3.2
Sample 032	0.116	3.4	0.0186	0.0193	0.0185	0.0181	5
Sample 033	0.107	2.1	0.0171	0.0168	0.0172	0.0175	4.72
Sample 034	0.099	2.2	0.0159	0.0161	0.0155	0.0161	1.48

8.2 Appendix II

Results obtained for Energy Dispersive X-ray analysis. Due to the low sensitivity of EDX, it was decided to investigate the levels of Cd in the skeletons of those individuals exposed to 50 μg l^{-1}, the highest exposure mode.

SCANS OF THE CROSS-SECTION OF THE SKELETON

Tty

Cross Section 1 ᴜ

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00676	3.368	3.71	2.28	+/- 0.32
Mg-K	0.01955	2.314	6.97	4.52	+/- 0.18
P -K	0.01213	1.306	1.91	1.58	+/- 0.36
S -K	0.04536	1.152	6.10	5.23	+/- 0.19
Cl-K	0.01278	1.154	1.56	1.47	+/- 0.30
Ca-K	0.80050	1.047	78.29	83.83	+/- 0.81
Cd-L	0.00000	1.081	0.00	0.00	+/- 0.00
Si-K	0.00551	1.473	1.08	0.81	+/- 0.28
Al-K	0.00145	1.899	0.38	0.28	+/- 0.15
Total			100.00	100.00	

Cross section 2

Chi-sqd = 2.54   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.06179	2.314	19.88	14.30	+/- 0.54
Mg-K	0.02712	2.155	7.69	5.84	+/- 0.35
P -K	0.04027	1.317	5.47	5.30	+/- 0.31
S -K	0.17583	1.229	21.55	21.61	+/- 0.43
Cl-K	0.14465	1.453	18.95	21.01	+/- 0.48
Ca-K	0.23386	1.247	23.27	29.17	+/- 0.89
Cd-L	0.00000	1.503	0.00	0.00	+/- 0.00
Si-K	0.01064	1.465	1.77	1.56	+/- 0.44
Al-K	0.00648	1.848	1.42	1.20	+/- 0.30
Total			100.00	100.00	

Cross section 3

Chi-sqd = 1.66   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01761	3.263	9.11	5.75	+/- 0.31
Mg-K	0.02468	2.386	8.83	5.89	+/- 0.18
P -K	0.00661	1.336	1.04	0.88	+/- 0.23
S -K	0.02877	1.163	3.80	3.34	+/- 0.14
Cl-K	0.03058	1.143	3.59	3.49	+/- 0.14
Ca-K	0.76058	1.052	72.74	79.98	+/- 0.71
Cd-L	0.00000	1.086	0.00	0.00	+/- 0.00
Si-K	0.00175	1.530	0.35	0.27	+/- 0.10
Al-K	0.00199	1.989	0.53	0.40	+/- 0.12
Total			100.00	100.00	

Command :

Tty

Cross Section 1

Chi-sqd = 1.77   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.03766	3.005	17.10	11.32	+/- 0.53
Mg-K	0.02825	2.453	9.90	6.93	+/- 0.19
P -K	0.00571	1.370	0.88	0.78	+/- 0.20
S -K	0.02773	1.185	3.56	3.29	+/- 0.13
Cl-K	0.07547	1.169	8.65	8.83	+/- 0.17
Ca-K	0.63003	1.083	59.15	68.26	+/- 0.44
Cd-L	0.00000	1.153	0.00	0.00	+/- 0.00
Si-K	0.00129	1.580	0.25	0.20	+/- 0.09
Al-K	0.00193	2.069	0.51	0.40	+/- 0.11
Total			100.00	100.00	

Cross Section 2

Chi-sqd = 1.25   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01104	3.213	5.61	3.55	+/- 1.18
Mg-K	0.03266	2.279	11.13	7.44	+/- 0.59
P -K	0.00105	1.408	0.17	0.15	+/- 0.41
S -K	0.02197	1.202	2.99	2.64	+/- 0.42
Cl-K	0.02106	1.165	2.51	2.45	+/- 0.40
Ca-K	0.72150	1.059	69.31	76.42	+/- 1.23
Cd-L	0.01361	1.123	0.49	1.53	+/- 0.87
Si-K	0.00791	1.609	1.65	1.27	+/- 0.39
Al-K	0.02309	1.969	6.13	4.55	+/- 0.51
Total			100.00	100.00	

Cross section 3

Chi-sqd = 1.22   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00152	3.652	0.96	0.56	+/- 0.39
Mg-K	0.00247	2.408	0.97	0.60	+/- 0.32
P -K	0.00375	1.262	0.60	0.47	+/- 0.27
S -K	0.00657	1.100	0.89	0.72	+/- 0.14
Cl-K	0.00738	1.052	0.87	0.78	+/- 0.15
Ca-K	0.95673	1.010	95.38	96.65	+/- 1.40
Cd-L	0.00000	1.010	0.00	0.00	+/- 0.00
Si-K	0.00000	1.425	0.00	0.00	+/- 0.00
Al-K	0.00124	1.841	0.33	0.23	+/- 0.13
Total			100.00	100.00	

Command :

Tty

Cross section 1
Chi-sqd = 1.14   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00725	3.398	3.99	2.46	+/- 0.37
Mg-K	0.03418	2.341	12.25	8.00	+/- 0.23
P -K	0.00005	1.295	0.01	0.01	+/- 0.14
S -K	0.01095	1.156	1.47	1.27	+/- 0.15
Cl-K	0.00343	1.107	0.40	0.38	+/- 0.12
Cd-L	0.00037	1.030	0.01	0.04	+/- 0.33
Si-K	0.00108	1.552	0.22	0.17	+/- 0.13
Al-K	0.00232	2.021	0.65	0.47	+/- 0.16
Ca-K	0.84892	1.027	81.00	87.21	+/- 0.63
Total			100.00	100.00	

Cross section 2
Chi-sqd = 2.00   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00700	3.429	3.92	2.40	+/- 0.22
Mg-K	0.02782	2.355	10.1?	6.55	+/- 0.21
P -K	0.00273	1.328	0.44	0.36	+/- 0.16
S -K	0.01217	1.146	1.63	1.40	+/- 0.17
Cl-K	0.00890	1.101	1.04	0.98	+/- 0.07
Cd-L	0.00000	1.034	0.00	0.00	+/- 0.00
Si-K	0.00064	1.523	0.13	0.10	+/- 0.07
Al-K	0.00092	1.985	0.25	0.18	+/- 0.09
Ca-K	0.85674	1.027	82.46	88.03	+/- 0.53
Total			100.00	100.00	

Cross section 3
Chi-sqd = 1.23   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00667	3.422	3.72	2.28	+/- 0.36
Mg-K	0.02936	2.346	10.63	6.89	+/- 0.21
P -K	0.00343	1.328	0.55	0.46	+/- 0.14
S -K	0.01494	1.149	2.01	1.72	+/- 0.14
Cl-K	0.00712	1.107	0.83	0.79	+/- 0.12
Cd-L	0.00000	1.036	0.00	0.00	+/- 0.00
Si-K	0.00104	1.522	0.21	0.16	+/- 0.12
Al-K	0.00000	1.892	0.00	0.00	+/- 0.00
Ca-K	0.85274	1.029	82.05	87.71	+/- 0.88
Total			100.00	100.00	

Command :

SCANS OF THE OUTER SURFACE OF THE SKELETON

Surface 1

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.02691	2.826	11.39	7.60	+/- 0.58
Mg-K	0.02947	2.205	9.21	6.50	+/- 0.35
P -K	0.03359	1.320	4.93	4.44	+/- 0.32
S -K	0.12037	1.212	15.67	14.59	+/- 0.41
Cl-K	0.06563	1.330	8.48	8.73	+/- 0.39
Ca-K	0.48526	1.144	47.69	55.52	+/- 1.12
Cd-L	0.00535	1.299	0.21	0.70	+/- 0.84
Si-K	0.00450	1.493	0.82	0.67	+/- 0.25
Al-K	0.00664	1.895	1.60	1.26	+/- 0.29
Total			100.00	100.00	

Surface 2
Chi-sqd = 4.57   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.05989	2.534	21.40	15.18	+/- 0.63
Mg-K	0.03573	2.326	11.08	8.31	+/- 0.39
P -K	0.03031	1.382	4.38	4.19	+/- 0.29
S -K	0.10132	1.251	12.82	12.68	+/- 0.36
Cl-K	0.09604	1.347	11.82	12.93	+/- 0.70
Ca-K	0.38368	1.168	36.23	44.80	+/- 0.91
Cd-L	0.00000	1.329	0.00	0.00	+/- 0.00
Si-K	0.00531	1.576	0.97	0.84	+/- 0.25
Al-K	0.00529	2.035	1.29	1.08	+/- 0.32
Total			100.00	100.00	

Surface 3
Chi-sqd = 1.51   Livetime = 60.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01422	3.242	7.31	4.61	+/- 0.36
Mg-K	0.02696	2.331	9.44	6.29	+/- 0.20
P -K	0.01119	1.326	1.75	1.48	+/- 0.33
S -K	0.03453	1.165	4.58	4.02	+/- 0.19
Cl-K	0.04086	1.154	4.85	4.72	+/- 0.18
Ca-K	0.73776	1.061	71.24	78.25	+/- 0.55
Cd-L	0.00000	1.108	0.00	0.00	+/- 0.00
Si-K	0.00209	1.514	0.41	0.32	+/- 0.13
Al-K	0.00159	1.965	0.42	0.31	+/- 0.15
Total			100.00	100.00	

Command :

Tty

Surface 4

Chi-sqd = 5.04   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.02010	3.080	9.57	6.19	+/- 0.36
Mg-K	0.02678	2.298	9.00	6.15	+/- 0.22
P -K	0.01676	1.331	2.56	2.23	+/- 0.16
S -K	0.07631	1.184	10.02	9.04	+/- 0.20
Cl-K	0.03704	1.232	4.57	4.56	+/- 0.18
Cd-L	0.00027	1.189	0.01	0.03	+/- 0.40
Si-K	0.00322	1.516	0.62	0.49	+/- 0.14
Al-K	0.00526	1.941	1.35	1.02	+/- 0.37
Ca-K	0.64610	1.088	62.31	70.28	+/- 0.66
Total			100.00	100.00	

Surface 5

Chi-sqd = 6.60   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.05889	2.474	20.45	14.57	+/- 0.49
Mg-K	0.03878	2.265	11.67	8.78	+/- 0.32
P -K	0.02283	1.378	3.28	3.15	+/- 0.23
S -K	0.11294	1.234	14.03	13.93	+/- 0.54
Cl-K	0.12552	1.352	15.46	16.98	+/- 0.33
Cd-L	0.00000	1.379	0.00	0.00	+/- 0.00
Si-K	0.00665	1.563	1.19	1.04	+/- 0.20
Al-K	0.00581	2.010	1.40	1.17	+/- 0.26
Ca-K	0.33955	1.190	32.53	40.39	+/- 0.43
Total			100.00	100.00	

Surface 6

Chi-sqd = 3.19   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01479	3.236	7.55	4.79	+/- 0.28
Mg-K	0.03457	2.336	12.04	8.08	+/- 0.28
P -K	0.00772	1.350	1.22	1.04	+/- 0.10
S -K	0.02658	1.174	3.53	3.12	+/- 0.22
Cl-K	0.02898	1.149	3.41	3.33	+/- 0.11
Cd-L	0.00000	1.088	0.00	0.00	+/- 0.00
Si-K	0.00197	1.548	0.39	0.31	+/- 0.09
Al-K	0.00116	2.023	0.32	0.23	+/- 0.12
Ca-K	0.75093	1.053	71.54	79.10	+/- 0.54
Total			100.00	100.00	

Command :

SCANS OF THE INNER SURFACE OF THE SKELETON

Tty

Underside 1

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00770	3.374	4.20	2.60	+/- 0.22
Mg-K	0.03032	2.331	10.81	7.07	+/- 0.14
P -K	0.00790	1.335	1.27	1.05	+/- 0.20
S -K	0.02324	1.162	3.13	2.70	+/- 0.21
Cl-K	0.00315	1.130	0.37	0.36	+/- 0.08
Cd-L	0.00000	1.048	0.00	0.00	+/- 0.00
Si-K	0.00228	1.526	0.46	0.35	+/- 0.08
Al-K	0.00129	1.987	0.35	0.26	+/- 0.10
Ca-K	0.82759	1.035	79.41	85.62	+/- 0.55
Total			100.00	100.00	

Underside2

Chi-sqd = 2.00   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00597	3.453	3.38	2.06	+/- 0.18
Mg-K	0.02665	2.355	9.75	6.28	+/- 0.11
P -K	0.00297	1.325	0.48	0.39	+/- 0.14
S -K	0.01268	1.145	1.71	1.45	+/- 0.08
Cl-K	0.00331	1.100	0.39	0.36	+/- 0.07
Cd-L	0.00171	1.051	0.06	0.18	+/- 0.18
Si-K	0.00087	1.519	0.18	0.13	+/- 0.06
Al-K	0.00110	1.978	0.30	0.22	+/- 0.08
Ca-K	0.86746	1.025	83.75	88.92	+/- 0.50
Total			100.00	100.00	

Underside 3

Chi-sqd = 1.98   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.00771	3.405	4.27	2.63	+/- 0.19
Mg-K	0.02872	2.350	10.39	6.75	+/- 0.11
P -K	0.00476	1.332	0.77	0.63	+/- 0.18
S -K	0.01344	1.153	1.81	1.55	+/- 0.08
Cl-K	0.01054	1.109	1.23	1.17	+/- 0.07
Cd-L	0.00137	1.067	0.05	0.15	+/- 0.18
Si-K	0.00154	1.525	0.31	0.24	+/- 0.15
Al-K	0.00084	1.989	0.23	0.17	+/- 0.08
Ca-K	0.84096	1.031	80.94	86.72	+/- 0.49
Total			100.00	100.00	

Command :

Underside 4
Chi-sqd = 3.45   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01786	2.914	7.91	5.20	+/- 0.46
Mg-K	0.02606	2.164	8.11	5.64	+/- 0.25
P -K	0.04333	1.309	6.40	5.67	+/- 0.25
S -K	0.12001	1.221	15.97	14.65	+/- 0.31
Cl-K	0.04237	1.333	5.57	5.65	+/- 0.28
Cd-L	0.00104	1.273	0.04	0.13	+/- 0.62
Si-K	0.00993	1.455	1.80	1.45	+/- 0.20
Al-K	0.00572	1.841	1.36	1.05	+/- 0.23
Ca-K	0.53624	1.129	52.82	60.56	+/- 0.83
Total			100.00	100.00	

Underside 5
Chi-sqd = 2.83   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.01114	3.189	5.62	3.55	+/- 0.32
Mg-K	0.02161	2.260	7.32	4.88	+/- 0.18
P -K	0.02352	1.309	3.62	3.08	+/- 0.17
S -K	0.07453	1.179	9.97	8.78	+/- 0.37
Cl-K	0.02618	1.222	3.28	3.20	+/- 0.17
Cd-L	0.00000	1.145	0.00	0.00	+/- 0.00
Si-K	0.00712	1.468	1.35	1.04	+/- 0.13
Al-K	0.00359	1.878	0.91	0.67	+/- 0.16
Ca-K	0.69366	1.078	67.92	74.78	+/- 0.46
Total			100.00	100.00	

Underside 6
Chi-sqd = 3.23   Livetime = 120.0 Sec.

Element	k-ratio (calc.)	ZAF	Atom %	Element Wt %	Wt % Err. (1-Sigma)
Na-K	0.03383	2.763	13.84	9.34	+/- 0.45
Mg-K	0.02135	2.232	6.68	4.77	+/- 0.28
P -K	0.03959	1.312	5.71	5.19	+/- 0.27
S -K	0.12450	1.217	16.10	15.16	+/- 0.33
Cl-K	0.07549	1.344	9.74	10.14	+/- 0.32
Cd-L	0.00000	1.300	0.00	0.00	+/- 0.00
Si-K	0.00838	1.465	1.49	1.23	+/- 0.21
Al-K	0.00530	1.860	1.25	0.99	+/- 0.24
Ca-K	0.46113	1.153	45.19	53.18	+/- 0.52
Total			100.00	100.00	

Command :

```
Chi-sqd = 2.21              Livetime = 60.0 Sec.
 Element   k-ratio    ZAF    Atom %   Element   Wt % Err.
           (calc.)                     Wt %    (1-Sigma)
   Na-K    0.01599    1.913    4.86     3.06    +/- 0.26
   Mg-K    0.07086    1.499   15.97    10.63    +/- 0.18
   P -K    0.00383    1.144    0.52     0.44    +/- 0.14
   S -K    0.01343    1.048    1.60     1.41    +/- 0.15
   Cl-K    0.00269    1.047    0.29     0.28    +/- 0.14
   Ca-K    0.82531    1.019   76.69    84.14    +/- 0.64
   Cd-L    0.00000    1.181    0.00     0.00    +/- 0.00
   Si-K    0.00042    1.210    0.07     0.05    +/- 0.11
   Total                     100.00   100.00
```

Fig 1 Spot A

```
Chi-sqd = 3.19              Livetime = 60.0 Sec.
 Element   k-ratio    ZAF    Atom %   Element   Wt % Err.
           (calc.)                     Wt %    (1-Sigma)
   Na-K    0.02503    1.869    7.38     4.68    +/- 0.26
   Mg-K    0.05130    1.496   11.45     7.68    +/- 0.29
   P -K    0.00751    1.126    0.99     0.85    +/- 0.15
   S -K    0.03691    1.040    4.34     3.84    +/- 0.19
   Cl-K    0.06342    1.060    6.87     6.72    +/- 0.21
   Ca-K    0.73741    1.032   68.86    76.12    +/- 0.61
   Cd-L    0.00034    1.241    0.01     0.04    +/- 0.49
   Si-K    0.00062    1.186    0.09     0.07    +/- 0.12
   Total                     100.00   100.00
```

Fig 1 spot b

```
Chi-sqd = 4.67              Livetime = 60.0 Sec.
 Element   k-ratio    ZAF    Atom %   Element   Wt % Err.
           (calc.)                     Wt %    (1-Sigma)
   Na-K    0.09060    1.617   21.02    14.65    +/- 0.47
   Mg-K    0.04797    1.498    9.75     7.18    +/- 0.49
   P -K    0.01672    1.125    2.00     1.88    +/- 0.24
   S -K    0.06369    1.046    6.86     6.66    +/- 0.32
   Cl-K    0.25651    1.101   26.29    28.24    +/- 0.80
   Ca-K    0.38149    1.085   34.07    41.38    +/- 0.64
   Cd-L    0.00000    1.375    0.00     0.00    +/- 0.00
   Si-K    0.00000    1.155    0.00     0.00    +/- 0.00
   Total                     100.00   100.00
```

Command :