The Development of Sea Sponges (*Hymeniacidon perlevis* and *Amorphinopsis sp*) as Novel Models for Genotoxicity Assessment and Environmental Monitoring of Pollutants in the Aquatic Environment

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Abstract

The measurement of chemical levels of pollutants and their biomarkers in resident sessile biota has been suggested for reliable aquatic monitoring and environmental risk assessment. This thesis proposes the use of the comet assay in sponges as a suitable model to complement analytical methods and other established invertebrate aquatic sentinels in aquatic pollution monitoring and risk assessment. A novel '*in vivo'* exposure sponge cell model was optimised for assessment of DNA strand breaks (DSB) using the alkaline comet assay and reactive oxygen species (ROS) formation using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) assay. Concentration-dependent increase in DSB and ROS formation were observed in sponge cells exposed to aluminium (0, 0.1, 0.2, 0.3 and 0.4mg/L), cadmium (0,0.1, 0.3, and 0.4mg/L), chromium (0,0.1, 0.3 and 0.4mg/L), and nickel (0, 0.1, 0.3 and 0.4mg/L) in laboratory based toxicity testing. Correlation between DSB and ROS formation decreased in the order Al>Cd>Ni>Cr. The involvement of ROS is suggested in aluminium induced DSB. This thesis also provides evidence of possible P450-(CYP1A1) activity in sponge cells and concentration dependent increase in benzo[a]pyrene induced DSB. Using hydrofluoric acid (HF) and Aqua-Regia digestion methods, metals in sponge tissues, sediment and water column obtained along the coast of the Niger Delta were extracted. Chemical analysis showed consistent detection of aluminium (0.22-0.7µg/mg), arsenic (0.002-0.0047µg/mg), cadmium (0.00002-0.00157µg/mg), copper (0.0023-0.027µg/mg) and lead (0.00025-0.0027µg/mg) by both inductively coupled plasma (ICP) mass spectrometry (MS) and optical emission spectrometry (OES) in all samples (. Up to 100-fold metal bioaccumulation in sponge tissues higher than accumulation in sediment and water justifies sponges as excellent bioindicator of metal pollution. A positive correlation between aluminium level in sponge tissue and DSB was again established.

DEDICATION

This thesis is dedicated to God Almighty, My Lord and Saviour Jesus Christ and The Holy Spirit

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LIST OF ABBREVIATIONS

AH	Aryl Hydrocarbon
Al	Aluminium
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
As	Arsenic
B[a]P	Benzo[a] Pyrene
BPDE	B[a]P -7,8-dihydrodiol-9,10 epoxide
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CMFSW	Calcium magnesium free sea water
CMFSW+E	Calcium magnesium free sea water containing EDTA
Co	Cobalt
Cr	Chromium
Cu	Copper
CYP1A1	Cytochrome P450, family -1 subfamily –A polypeptide -1
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
DPR	Department of Petroleum Resources
DSB	Double strand break
EDTA	Ethylenediaminetetraacetatic acid
EPA	Environmental Protection Agency
ERA	Environmental Risk Assessment
EU MSFD	European Union Marine Strategy Framework Directives

FBS	Foetal bovine serum
FCM	Flow cytometry
Fe	Iron
FIAD	Feulgan image analysis desitometry
FPG	Formamidopyrimidine DNA glycosylase
GSH	Glutathione
GST	Glutathione-S- transferase
H_2DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HClO4	Perchloric acid
HF	Hydrofluoric acid
Hg	Mercury
HNO_3	Nitric acid
Hsp70	Heat shock protein 70
IARC	International Agency for Research on Cancer
ICP –OES	Inductively coupled plasma optical emission spectrometry
ICP-AAS	Inductively coupled plasma atomic absorption spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
KCl	Potassium chloride
LMPA	Low melting point agarose
LSM	Laser scanning microscope
MDA	Malondialdehyde
Mn	Manganese
MTT	3-(4, 5-dimethylethiazole-2-yl)-2,7-diphenyletetrazoliumbromide
$Na_2Cr_2O_7$	Sodium dichromate (VI)

Na_2EDTA	Disodium ethylenediaminetetraacetate dihydrate
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$,	Sodium hydrogen carbonate
NCBI	National centre for biotechnology information
Ni	Nickel
$NiCl_2$	Nickel chloride
NMPA	Normal melting point agarose
NSW	Natural sea water
O_2^-	Superoxide
OH^-	Hydroxyl ion
PAHS	polycyclic aromatic hydrocarbons
Pb	Lead
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PPB	Part per billion
PPM	Part per million
PSG	Penicillin, streptomycin, glutamine
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCGE	Single cell gel electrophoresis
SEM	Standard error of mean
SQG	Sediment quality guidelines
SSB	Single strand break

SSF	Strand scission factor
SSW	Synthetic sea water
TAE	Tris Base glacial, acetic acid and EDTA
TBB	Tenby Bay
UHQ water	Ultra-pure high quality water
UNEP	United Nations Environmental Protection
USEPA	United States Environmental Protection Agency
UV	Ultra violet
WHO	World Health Organization

Chapter 1 – General Introduction

1.1 Rationale

The aquatic ecosystem has in recent years since the birth of industrialization and mechanised agriculture become the sink for many environmental xenobiotics, both from anthropogenic sources and natural. Commonly reported pollutants in the aquatic media include: heavy metals, PCBs, and PAHs which have, overtime become of monitoring priority to environmental interest groups (Gentric et al. 2016; Moriarty 1988; Stohs and Bagchi 1995). Many environmental xenobiotics are persistent and non-biodegradable in nature with high propensity for bioaccumulation in the environment and in living systems; hence they are extremely toxic to both animals and humans owing to their ability to overwhelm and seriously compromise intrinsic cellular macromolecules and metabolic pathways. Interactions of xenobiotic with living systems are implicated with the alteration of genome integrity and are known precursors of many human disease conditions (Lee et al. 1999; Martins and Costa 2014; Roberts et al. 2008; Tubbs and Nussenzweig 2017; Van der Oost et al. 2003).

In the aquatic environment, toxic pollutants accumulate in sediment and in water column while some are stored up in benthic sessile organism and are eventually biomagnified along the ecological food chain. Most heavy metals have no known biological function; hence, their presence in the natural environment is usually deleterious to all life forms including humans (via consumption of sea foods). As a result, they need to be regularly and adequately monitored to ensure that they do not exceed the maximum tolerable level (Rainbow 1993; Roberts et al. 2008). To effectively measure the real impact and the risks of chemicals released into the aquatic environment, it is important to deploy a wide range of sessile benthic indigenous organisms because they provide information on both total bio-accumulated and

bioavailable pollutants in the medium. This is particularly useful in the assessment of risks associated with each pollutant and it also provides information needed for the enactment of environmental protection policies.

This study present for the first time the application of the comet assay, and reactive oxygen species assay to investigate the genotoxic and oxidative stress impacts of environmentally relevant xenobiotics in laboratory cultures of sponge cells. This thesis also relates DNA damage in field sponge samples from potentially polluted sites to pollutant levels in sponge tissues, sediment and water column. As sessile benthic, and multicellular metazoans sponges are able to accumulate a wide range of pollutants even when the concentrations are extremely low, sponges feed by pumping and filtering large amounts of water through their tissue (Patel et al. 1985). In the process particulate matters are trapped alongside other environmentally relevant pollutants which could be retained for an extended period of time (Pan et al. 2011; Patel et al. 1985). Thus, sea sponges are ready tools for profiling the health status of their aquatic environment and therefore constitute excellent biomonitors and bio-indicators of pollutants (De Mestre et al. 2012; Pan et al. 2011; Patel et al. 1985). Given their simple body form they also benefit from little or no inter-individual variability which is a common problem limiting the application of most invertebrate in mechanistic studies (De Mestre et al. 2012). These features therefore make sea sponges an ideal system for aquatic biomonitoring and genotoxicity measurement with the comet assay a useful tool for environmental risk assessment in the aquatic media.

1.2 'Heavy Metals' or 'Trace metals'

The term 'heavy metal' and 'trace metals' are used interchangeably to mean metals that are toxic at certain threshold (Deb and Fukushima 1999). They occur in small amount relative to their immediate environment or matrix in which they are found. In biological systems for example, their concentration is about 0.01% of the total body mass of the organism (Rainbow 2017). To eliminate the ambiguity associated with earlier definition of heavy metals "as metals with specific gravity more than four or five"(Parker 1984), it was generally accepted to group metals into three classes based on chemical and biological properties (Deb and Fukushima 1999; Nieboer and Richardson 1980; Rainbow 1985). Metals are generally grouped as Class A, Class B and Borderline metals (Table 1.1). Another criterion for metal classification was whether or not it was essential or non-essential for biological processes. Both borderline metals and metal in group B are heavy metals, some are essential at certain biological concentrations, beyond which they become toxic, others are non-essential and are toxic even in trace amount. Metal toxicity in the aquatic ecosystem, is influenced by a number of factors including availability, speciation and geochemical parameters (Abdullah 2007; Deb and Fukushima 1999; Rainbow 1985). Metal availability in the aquatic medium is dependent on the solubility of their oxides from natural sources and the speciation or chemical forms, which is influenced by physical and chemical parameters. Titanium for example, is rarely available in the aquatic system because of the poor solubility of its hydroxide in solution; hence it is a potential toxin (Rainbow 1985). Essential metals are moderately available; however, anthropogenic activities are known to elevate the levels to non-biological concentration which eventually becomes toxic to aquatic organisms and humans via trophic

transfer (Rainbow 1985). So comparatively, essential class B heavy metals at high concentration are more toxic than the rarely occurring metals. Borderline metals, are mainly non-essential metals and are readily available both by natural occurrence and man-made activities. Owing to their strong affinity for essential metal binding sites, they are highly toxic in both trace and elevated amount in the aquatic medium (Rainbow 2017; Rainbow and Luoma 2011; Rainbow 1985).

According to Shesterin (2010), an upset in some abiotic parameters or water qualities such as oxygen content, temperature, pH and hardness of water would enhance metal toxicity in the aquatic medium. For example, oxygen level depletion, increased water temperature, drop in pH level and decreased water hardness are factors that enhance metal speciation in the aquatic ecosystem and their resultant availability and toxicity in biota (Deb and Fukushima 1999).

Table 1.1.1 Metal classification based on affinity for oxygen, sulfur and non-metals in general; Adapted based on Rainbow (1985) and (Rainbow 2017).

Class A metals (Strong affinity for oxygen)	**Class B metals (Strong affinity for Sulphur)**	**Borderline metals (Unrestricted affinity for all non-metals and biomolecules)**
Lithium (Li)	Copper I (Cu+)	Titanium (Ti)
Beryllium (Be)	Rhodium (Rh)	Vanadium (V)
Sodium (Na)	Palladium (Pd)	Chromium (Cr)
Magnesium (Mg)	Silver (Ag)	Manganese (Mn)
Aluminium (Al)	Iridium (Ir)	Iron (Fe)
Potassium (K)	Platinum (Pt)	Cobalt (Co)
Calcium (Ca)	Gold (Au)	Nickel (Ni)
Scandium (Sc)	Mercury (Hg)	Copper II (Cu2+)
Rubidium (Rb)	Thallium (Tl)	Zinc (Zn)
Strontium (Sr)	Lead IV (Pb4+)	Gallium (Ga)
Yttrium (Y)		Arsenic (As)
Caesium (Cs)		Cadmium (Cd)
Barium (Ba)		Indium (In)
Francium (Fr)		Tin (Sn
Radium (Ra)		Antimony (Sb)
Bismuth (Bi)		Lead II (Pb2+)
Lanthanum (La)		
+ the Lanthanides		
Actinium (Ac)		
+ the Actinides		

1.3 Heavy metal pollution in aquatic ecosystem

Heavy metals are natural constituents of the aquatic media. At very low concentration some heavy metals such as zinc (approx.30mg/g), copper (approx.1.2mg/g) and iron (0.61-6.3mg/day) are useful for metabolic activities (Abbaspour et al. 2014; FAO 2001; Osredkar and Sustar 2011); however, at higher concentrations they become toxic and harmful to plants, animals and humans (FAO 2001). In contrast to organic xenobiotic, heavy metal pollutants are non-biodegradable and are not subject to metabolic breakdown, hence they cannot be removed from the aquatic ecosystem by natural means as they are trapped in sediment core and bioaccumulated in benthic organisms (Förstner and Wittmann 2012). Aquatic environment are particularly vulnerable to heavy metal pollution compared to the terrestrial ecosystem. The overall biomass in the aquatic system is limited to the species in the trophic levels, this then means that pollutants are compulsorily transferred from one level to the other in the aquatic environment (Förstner and Wittmann 2012).

Amongst pollutants in the aquatic ecosystem, heavy metals for example Cd, Hg, As, Pb, Ag, Cu and Zn present the greatest environmental risks because of their high toxicity profile, and the ease with which they bio-accumulate in lower organism in very high concentrations (Ahmed et al. 2013; Ahmed et al. 2010; Sarkar et al. 2015). Overtime, these heavy metals are bio-transformed and stored up in non-toxic forms in their host aquatic species, but eventually they bio magnify via trophic transfer along the ecological food chain (Shaw and Chadwick 1998). This is the major route of human exposure to non-industrial environmental xenobiotics (Schillack and Buisson 2005).

Considering the growing concern regarding increased pollution in coastal marine environment and the need for the development of reliable biomarkers required as early warning system in ERA (Beiras and Albentosa 2004); this thesis sets out to investigate the occurrence and levels of established metallic genotoxicants (Al, As, Cd, Cr, Cu, Hg , Ni and Pb) in the Niger Delta coastal environmental, and also to assess their genotoxic effects on the marine sponge (*A. kalibiama* and *H perlevis*). DNA strand breaks as a biomarker of genotoxicity was compared with the level of metals in Sponge tissues, sediment and water column.

Also, using novel in vivo exposure sponge cell model, cultured aggregates of *H. perlevis* were exposed to non-cytotoxic concentrations of environmentally relevant chemicals (Benzo[a] Pyrene, Al, Cd, Cr, and Ni) to investigate their genotoxic impact using the alkaline comet assay and to assess the amenability of the procedure under laboratory conditions.

Table1.2 Heavy metal occurrence in the marine environment, effects on marine organisms & mechanism of toxicity

	Occurrence/ levels in marine environment	**Effects on Marine organisms**	**Mechanism of Action**	**Author**
Aluminium (Al)	• **79.46-96.54** µg/g of *Nacella concinna* The Antarctic Limpet obtained from King George Island along the coast of the Antarctic sea.	Oxidative stress markers: Increased SOD activity	Reactive Oxygen Species formation	(Norcross et al. 1996; Weihe et al. 2010)
Cadmium (Cd)	• **3.7 -6.2µg/g** *Nacella concinna* • **0.37- 4.50** µg/g Fish samples from North east Mediterranean sea • **0.7nmol/L** in water column obtained from the Wood Bay in the ROSS sea in the Antarctica • **50µg/g** dry weight in muscle tissues of the bivalve *Laternula elliptica* obtained from Collins Harbour in Antarctic sea.	Oxidative stress Indirect Oxidative effect resulting from inhibition of PaH metabolism	ROS Formation, Glutathione (GSH) depletion ROS formation	(Weihe et al. 2010) (Canli and Atli 2003) (Scarponi et al. 2000) (Ahn et al. 1996)
Chromium (Cr)	• **17.1µg/g** Sardina Fish liver obtained from North East Mediterranean sea. • **34.97-136.99 µg/g** sediment and 0.15-0.44 µg/L water	Oxidative Stress	DNA adducts and crosslinks formation	(Al-Yousuf et al. 2000) (Nussey 2000)

Table1.2 cont. Heavy metal occurrence in the marine environment, effects on marine organisms & mechanism of toxicity

	sampled across two years from Olifant Bay, South Africa. • **2.9µg/g** in digestive gland of *Laternula elliptica* obtained from Collins Harbour along King George Island along the coast of the Antarctic sea.			(Ahn et al. 1996) (Kumari et al. 2014)
Nickel (Ni)	• **21µg/g** in the Kidney of *Laternula elliptica* from Maxwell Bay, in King George Island, Antarctica • **26.9**mg/kg in sedment from sediment samples obtained from Admiralty bay , Antarctica • **1.77µg/g** *Bovatia gigantia* (benthic Amphipod) obtained from Admiralty Bay, Antarctica. • **1.6 µg/g** in *Amphiopus acutus* Benthic Ophiuroid, Antarctica	Oxidative Stress	DNAadducts formation DNA crosslinks and 8-OHdG formation	(Ahn et al. 1996) (Trevizani et al. 2016) (Majer et al. 2014) (Majer et al. 2014)

1.3.1 Cadmium in the Marine Environment

Cadmium (Cd) is example of a non-essential metal in living systems and it is an established carcinogenic genotoxicant in both humans and animals (Hartwig 1998; Hassoun and Stohs 1996; Sarkar et al. 2015). Cadmium has no known biological function; and though it is naturally occurring it is easily elevated by anthropogenic activities to levels that are detrimental to the ecosystem (Müller et al. 1998; Schröder et al. 1999). Several researchers have reported Cd genotoxicity in different aquatic organisms including sponges (Mueller 1998; Schröder et al. 1999), it is a persistent, naturally and rarely biodegradable (Clark 1997; Muller et al., 1998). The mechanism of action of Cd toxicity is very well studied in most model systems and includes inhibition of DNA-synthesis and DNA-repair enzymes, generation of reactive oxygen species (ROS), induction of DNA-strand breaks and depletion of protective factors such as the antioxidant glutathione (Giaginis et al. 2006; Hartwig 1994; Hartwig 1998; Hassoun and Stohs 1996; Roccheri et al. 2004; Shimizu et al. 1997; Steinert et al. 1998; Valverde 2001; Yang et al. 1996). In the pristine environment, however, cadmium concentration is reported to be less than $1ngL^{-1}$ in seawater and less than 1 mg kg^{-1} of sediment (Goh 2008a; Nriagu 1980) while cadmium levels as high as 94-148.5 µg/L (Perera et al. 2016) in water and 0.78-2.16 mg/kg of sediment obtained from Forcados River in the Niger Delta (Iwegbue et al. 2018). In many waters of the world, Cadmium is elevated via natural sea rise and upwelling and tends to accumulate in benthic organisms (Perera et al. 2016).

This thesis also measured and developed methods to assess the genotoxic effects of other environmentally relevant heavy metals (Aluminium, Chromium, and Nickel). Cadmium,

Aluminium, Chromium, Nickel and Benzo[a]pyrene are priority carcinogens belonging to IARC class I genotoxicants and are readily available as pollutants in coastal marine environments (Sarkar et al. 2015)

1.3.2 Chromium in the Marine ecosystem

Chromium is applied in industrial processes such as production of pigment dyes, textile industries, synthesis of organic reagents and compounds such as benzoic acids, saccharine and as low-carbon stainless steel alloys (Nriagu and Nieboer 1988; Qixing 1999). Contribution to the aquatic environment are both from natural and anthropogenic sources; natural sources being mainly chromite ore (Nriagu and Nieboer 1988). There are several anthropogenic sources of chromium contribution to the aquatic medium, sources such as combustion of coal, oil and gas from both residential and industrial sources. Other anthropogenic sources are those from industrial waste water from paint, pigment, leather and electroplating industries (Wise Sr et al. 2009). Human exposure is primarily through cigarette, exhaust fumes from automobile, and steel welding (Nriagu and Nieboer 1988). Chromium exist in several forms in nature, toxicity therefore is dependent on the form an organism is exposed to; most stable forms are Cr(III) and Cr(VI). Cr(VI) compounds permeates anionic channels and interacts with cellular GSH and ascorbate forming DNA-adduct and DNA crosslinks of Cr(III) (Henkler et al. 2010). Chromium toxicity is also linked to solubility of its compounds in water, example of soluble chromium compounds are potassium chromate, sodium chromate, calcium dichromate and sodium dichromate (Nriagu and Nieboer 1988). Hexavalent chromium (Cr (VI)) is an established human carcinogen and it is a class I IARC priority metal, it is implicated with both lung cancer and breast cancer and although Cr (III) is not a human carcinogen, it is actively involved in Cr(VI) toxicity (Henkler et al. 2010; IARC 1990). Data on chromium

pollution in the aquatic media is limited mainly because of Cr (VI) which is the most abundant form of chromium in the marine environment is rapidly reduced to Cr (III) and Cr (IV). These two forms of chromium are not easily absorbed in aquatic biota and as a result not directly measurable, hence most aquatic pollution studies mainly report on the total accumulated chromium, which in many cases are below detection limit in biota (Wise Sr et al. 2009). However, Wise Sr et al. (2009) reports recorded high (7.1µg/g of skin tissue) chromium level in the North Atlantic right whale skin (*Eubalaena glacialis*). According to this group, this level is more than 20 times the levels in human skin without occupational exposure and it is only comparable to levels previously reported in the skin of workers who were reported to have died from chromium-induced lung cancer following industrial chromium exposure. At low pH, Cr (III) complexes with cellular biomolecules such as glutathione and amino acid residue and interferes with their antioxidative activities (Henkler et al. 2010). There is also strong evidence of chromium affinity for nucleic acids (Nriagu and Nieboer 1988). Other biomarkers of chromium interaction with cellular macromolecules, includes DNA-Crosslinks, DNA-strand breaks and there has also been report of Cr (III) metallothionein oxidation in humans and prawn (Nriagu and Nieboer 1988; Qixing 1999). Higher levels of chromium have also been reported in the American alligator *Alligator mississipiensis*; Wise et al. (2016) and Campbell et al. (2010) measured up to 30µg/g of chromium in *A mississipiensis* liver tissue. This is also in agreement with the report of Tan et al. (2010) who observed both cytotoxic and clastogenic effects in sea turtles (*Chelonia mydas*) following laboratory exposures to similar concentration (2.7µM) as those recorded in field samples.

1.3.3 Nickel in the Marine ecosystem

Although nickel is essential for some metabolic activities in living organisms (essential nutrient in plants and some microorganism) at low concentration and also applicable in certain industrial processes, at high concentration however is an established carcinogen (IARC 1990). In the aquatic system, Nickel pollution is prevalent especially in coastal environment, and has been enlisted as a priority metal pollutant in the aquatic media by the EU water frame work directive (Dallas et al. 2013). However, data on Nickel mechanistic effects on aquatic biota is limited and the actual mechanism of toxicity still a subject of debate (Henkler et al. 2010; Stohs and Bagchi 1995). Nickel induced DNA crosslinks is reported in both vertebrate and invertebrate system (Chakrabarti et al. 2001). Nickel toxicity follow different mechanistic pathways from those of other carcinogens, first accumulation of Nickel particles enters living system via phagocytosis and bounds intracellular biomolecules such as amino acids, peptides and other intrinsic defence molecules (Schillack and Buisson 2005). This compromises the antioxidant potential of these molecules leading to the production of large amount of reactive oxygen species and their attendant hazardous cellular effects (Henkler et al. 2010; Schillack and Buisson 2005). There has also been a report of a synergistic effect of Nickel toxicity and toxicity of other environmentally relevant carcinogens such as X-rays, UV-radiations and Benzo[a] Pyrene (Henkler et al. 2010; Schillack and Buisson 2005; Stohs and Bagchi 1995). Thus, it can be concluded that there are multiple pathways to Nickel toxicity (Schillack and Buisson 2005). Assessment of Nickel genotoxicity using the enzyme modified comet assay in the marine mussel; *Mytilus galloprovinciallis* was reported that exposure to 0–3200 μgL^{-1} Nickel for 5days showed no significant difference in level of DNA strand breaks between concentrations less than 1800 μgL^{-1} and control, suggesting that DNA strand break may not be the likely mechanistic route for Nickel toxicity (Dallas et al. 2013). In *Daphnia magna,*

Nickel - exposure of 1.76 - 17.6 mgL^{-1} produced large amount of reactive oxygen species, which suggests that ROS production is a major pathway in Nickel toxicity. Other pathway such as inhibition of DNA repair enzymes inhibition has also been suggested (Xie et al. 2007). Oxidative stress effects such as DNA-adducts, DNA crosslink and 8-OHdG formation were also reported in some other marine invertebrates (Table 1.2). In *Laternula elliptica* from Maxwell Bay King George Island Antarctica, following exposure to 21µg/g Nickel (Ahn et al. 1996), *Bovatia gigantia* from Admiralty Bay, Antarctica (Majer et al. 2014) and in *Amphiopus acutus* also from the Antarctica. Levels up to 26.9mg/kg were also reported in sediment samples from Admiralty bay in Antarctica.

1.3.4 Aluminium in Marine Environment

Aluminium is the most abundant element in the earth crust, and has no known biological function. However, it is an established neurotoxicant in its trivalent oxidation state, with exposure resulting in neurodegenerative diseases like Parkinson's disease and presenile dementia (Alheimer's disease) (Krewski et al. 2007). Aluminium toxicity is linked to its ability to induce DNA strand breaks, chromosomal aberrations, DNA crosslinks, sister chromatid fragments and inhibition of DNA repair enzymes (Lankoff et al. 2006; US-Department 1999). Human exposure is mainly through air, water and food commonly through aluminium cookware with aluminium industry workers at the highest risks. In the aquatic ecosystem, Aluminium toxicity is associated with acidic pH and accumulates in environmental and biological matrixes (Rosseland et al. 1990). Effluxes from terrestrial aluminium are a major contribution to aquatic aluminium; from rock weathering, aluminium smelting industries, industrial waste disposal and surface water runoff. Aluminium is also released into the environment from bauxite processing wastes and from water treatment

aluminium coagulants (US-Department 1999). Bioaccumulation of Aluminium is reported in number of aquatic invertebrates, in macroinvertebrates, 140.6-385.7mg/kg dry weight (Oberholster et al. 2012), and up to 309.4-981.50µgAl/g dry weight in the crayfish; *Procambarus clarkii* at an acidic pH (US-Department 1999). Aluminium concentration is higher in sub-littoral zone than in the inter-tidal zones in the marine environment; this is likely due to direct washing off from rocks and the earth crust. Aluminium concentration and effects in coastal environment was measured in sublittoral limpet (*Nacella concinna*). Weihe et al. (2010) and Ahn et al. (1996) reported marked oxidative stress response following increased production of ROS (Table 1.2) at Aluminium concentration of 96.54µg/g intertidally and 79.46µg/g in sub-littoral zone.

1.4 Reactive Oxygen Species (ROS) and metal toxicity

Reactive oxygen species are toxic end products of cellular oxidative processes with varying toxicity effects depending on target cellular molecule, specific species formed, its source and final location within the living system (Irani 2000). Reactive oxygen species formation is mainly induced by heavy metals; especially those of the transition metal group which are continually released into the environment through anthropogenic means. Deleterious effects result from their ability to by-pass intrinsic anti-oxidative systems, displacement of essential metabolic metals and eventually overwhelming the natural defence mechanisms. Commonly reported ROS species are O_2^- (superoxide), H_2O_2 (hydrogen peroxide), and OH^- (hydroxyl ion). Reactive oxygen radicals interact with cellular molecules and overwhelm the natural intracellular defences, resulting in the induction of tissue injury, DNA strand breaks, inhibition of DNA repair enzymes and cellular oxidative stress. These pathways are directly involved in the development of several neoplastic disease conditions in humans. Thus, tissue

injury induction and cellular oxidative stress are the mechanistic routes of metal toxicity and the associated deleterious health impacts. Reactive oxygen species interact with cellular proteins, DNA and lipids causing oxidative stress seen as single and double strand DNA damages, lipid peroxidations, cytotoxicity, genotoxicity, mutagenicity, neurotoxicity and sulfhydrl group depletion and alteration of calcium homeostatic pathways (Ercal et al. 2001; Stohs and Bagchi 1995). Heavy metals such as chromium, vanadium, copper and iron are redox active metals and mainly produce reactive oxygen species through Fenton-like reactions and redox cycling in cell membrane. While other redox-inactive metals such as cadmium, nickel, mercury, lead produce reactive oxygen species by depleting cellular sulfhydryl antioxidants and displacement of essential metals from their binding sites (Stohs and Bagchi 1995).

1.5 Fate of metals/Ionic pollutants in living System

Heavy metals in the aquatic media exist either in the dissolved state or as particulate matters incorporated with food materials and because they are not biodegradable, they are easily trapped in sediment, released into surrounding water and are bioaccumulated in aquatic organisms (De Mestre et al. 2012; Rainbow and Luoma 2011). Dissolved metals in marine environment are taken up by biota either directly though the hydrophilic (diffusion) route; which involves passive adsorption of metal ions via cell membrane channel or by incorporation from particulate matter during feeding through an endocytosis routes (Bryan and Darracott 1979). Ionic radicals and metals permeate water channels on cell membrane surfaces which are controlled by amino acids bases. Cell membrane water channels are highly specific for each ion, therefore an alteration in one amino acid base pair will result in a change to the ionic channels in another (Langston and Bebianno 1998). Heavy metals in the aquatic

media are transported into living systems through these water channels by displacing metabolically essential ions. Toxicity therefore results as cells are deprived of essential ions while toxic metals with deleterious effects interact with biomolecules in the system [5]. Uptake of particulate matters in the aquatic ecosystem by endocytosis is an important process for sedentary filter feeding aquatic invertebrates because it predisposes them to metal toxicity; for example sea sponges whose main source of food is in particulate matters such as phytoplankton and bacteria which are taken up via water filtration (Langston and Bebianno 1998).

Trace metal accumulation in aquatic organisms is from the surrounding water column, ecological food chain, particulate matter uptake, and via sediment remobilization (Rainbow 2002; Roberts et al. 2008; Van der Oost et al. 2003). However, in invertebrate organisms, different bioaccumulation patterns have been reported even between organisms of the same taxonomic phyla (Bryan and Darracott 1979). Accumulated metals are either stored up within the organism or excreted through normal metabolic processes (Figure 1.1). Storage of metal pollutants are enhanced by metal binding proteins such as Metallothionein, and they can be induced both in field and laboratory exposed organism. These proteins are among the most specific biomarkers of metal pollution deployed in aquatic monitoring and have been reported in sea sponges (Berthet et al. 2005b). High level of metals were measured in sea sponges as well as metal specific metallothionein classes suggesting a detoxification potential in sponges and their usefulness as sentinel organism for environmental biomonitoring in the aquatic ecosystem (Berthet et al. 2005a). Metals are also stored as mineral pellets or organic precipitates which are then incorporated unto cytoplasmic lysosomes (Berthet et al. 2005b; Marigómez et al. 2002). Although, heavy metals bio accumulates in sessile biogenic epifuana at high concentrations (Wise Sr et al. 2009), toxicity however, only results from the uptake

amount, that is the amount that is metabolically available to the organism. Toxicity of xenobiotics to aquatic invertebrates results when the rate of uptake exceeds the rate in which they are biotransformed or biomagnified which means that bioaccumulated metal loads may not necessary result in toxicity (Figure 1.1).Thus, toxicity assessment should be based on maximum concentrations of bioavailable metals rather than the concentrations of total accumulated metal within the animal tissues and organs (Rainbow 2002). This then justifies the increased demand for reliable biomarker data for ERA and laboratory based toxicity testing which enhances prompt and efficient determination of bioavailable and toxic concentrations of pollutants (De Mestre et al. 2012; Martins and Costa 2014; Moriarty 1988; Van der Oost et al. 2003).

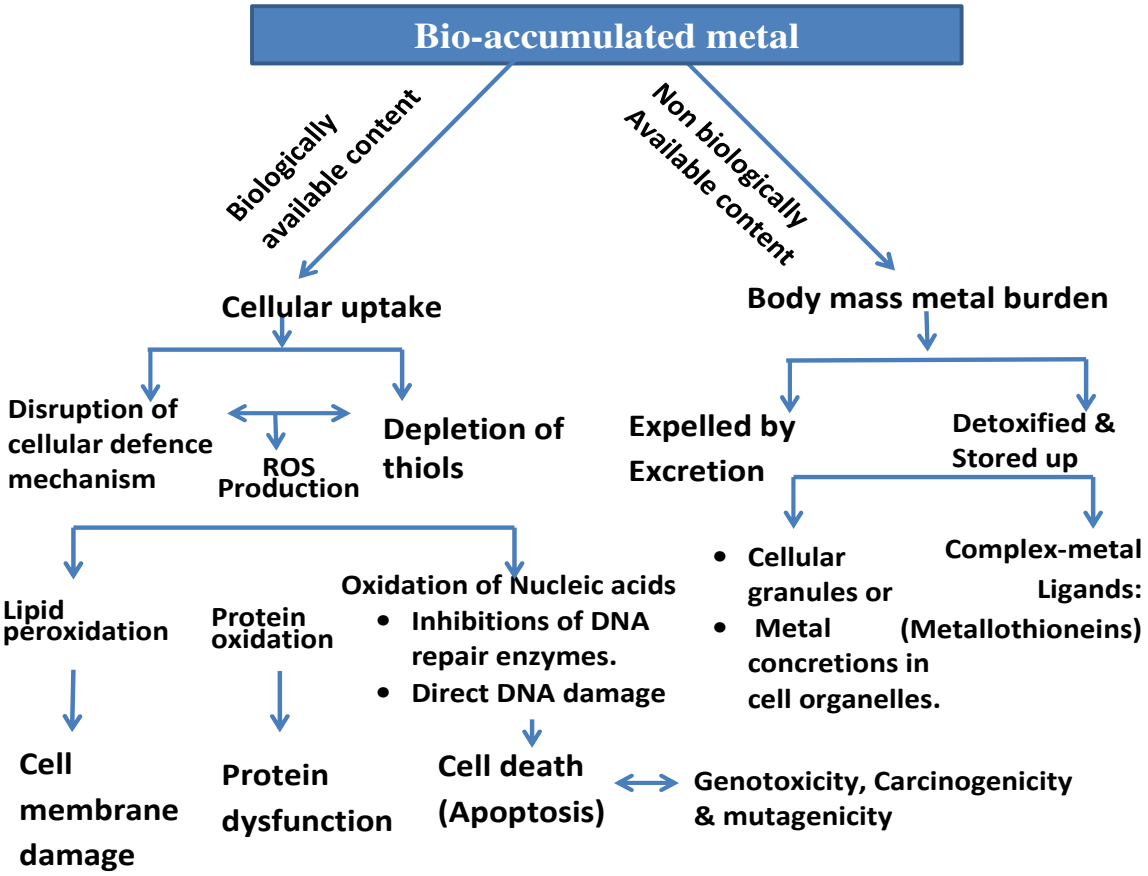

Figure 1.1 Schematic representation of heavy metal interactions with living system (adopted with modifications from Ercal et al. (2001))

1.6 Organic pollution in the aquatic ecosystem

Organic chemicals readily accumulate in aquatic biota from both natural and anthropogenic sources (D'adamo et al. 1997). Organic chemicals are mainly introduced into the environment by industrial process that utilise them as base materials and from end users (Richards and Shieh 1986). Other sources of organic pollutants in the environment is through indiscriminate waste disposal and effluents from industrial plants, from biotic and abiotic sources, direct waste discharges, underground seepage of sewages, clinical discharges and via the food chain (Livingstone 1998; Richards and Shieh 1986). Commonly reported organic pollutants in the

aquatic ecosystem are poly-chlorinated biphenyls (PCBs), Poly aromatic/aliphatic hydrocarbons (PAHs), and are rapidly absorbed, and bioaccumulate in living system (D'adamo et al. 1997; Gentric et al. 2016; Schröder et al. 2006). They are of interest because of their persistence in the environment and toxicity profile in both animals and humans (D'adamo et al. 1997; Goksøyr and Förlin 1992; Van der Oost et al. 2003). For biomonitoring and environmental risk assessment, the presence of their metabolites in body fluid and tissues of aquatic animals are extensively utilised as biomarkers of organic pollution (Bryan and Darracott 1979; Gentric et al. 2016). However, the fate of organic pollutants differ in aquatic organisms as does their toxicity, this is due to differences in the rate of biotransformation and detoxification unique to individual organism's metabolism (Livingstone 1998). For example, higher biotransformation rate was recorded in the fish *Carassius auratus* which was well correlated with the amount of P450 (CYP1A1) expression (Livingstone 1998). In this study exposure to parent concentration of 10nmol/g B[a]P resulted in an increased biotransformation rate in the order Fish > crustacean > molluscs, with values ranging from;19.3 ± 6.7, 4.8 ± 6.6 and 2.1 ± 0.2, nmol/g B[a]P respectively (Livingstone 1998). And also, occurrence of organic in environmental media is also influenced by factors such as toxicity, physico-chemical parameters, persistence and mobility (Shaw and Chadwick 1998)

Organic pollutants because of their lipophilic and highly hydrophobic nature are persistent in the environment and are not easy to excrete. This also enhances organic xenobiotic toxicity hence the need to biomonitor their presence and levels in the environment (D'adamo et al. 1997; van der Merwe et al. 2010; Van der Oost et al. 2003). In the aquatic system, they are easily accumulated in both sediment and biota and either stored up in living systems in inert non-toxic form, but become toxic in accidental releases into the environment (Bo et al. 2014; Thompson et al. 2010). Alternatively, lipophilic xenobiotics are biotransformed into non-

toxic substances by hepatic enzymes converting them into polar compounds that are easily excreted and subsequently detoxified via the urinary system (Boelsterli 2007; Shaw and Chadwick 1998). Aquatic sentinels are potent biomonitoring and bioindicators of aquatic xenobiotic pollution (Varanasi 1989)

1.6.1 Organic chemical metabolism

Metabolism of organic xenobiotic (PCBs and PAHs) involves several enzymes of the P450-CYP monooxygenase Phase I and Phase II conjugate enzymes (Bo et al. 2014). Metabolism of organic chemicals in many cases also results in the activation of more toxic metabolites, which in many cases are the precursors to organic chemical toxicity. Main enzymes induced by environmental xenobiotic are P450-CYP1A1 family, epoxide hydroxylase and Glutathion-S-transferase (Mitchelmore et al. 1998a; Routti et al. 2008; Thompson et al. 2010). Engagement with biological processes such as synthesis and inactivation of steroid hormones and lipid metabolism also predisposes hepatic enzymes toxic organic chemicals in living system (Goksøyr and Förlin 1992). Thus, metabolic enzymes are induced by both natural physiological processes and by environmental xenobiotics (Boelsterli 2007).

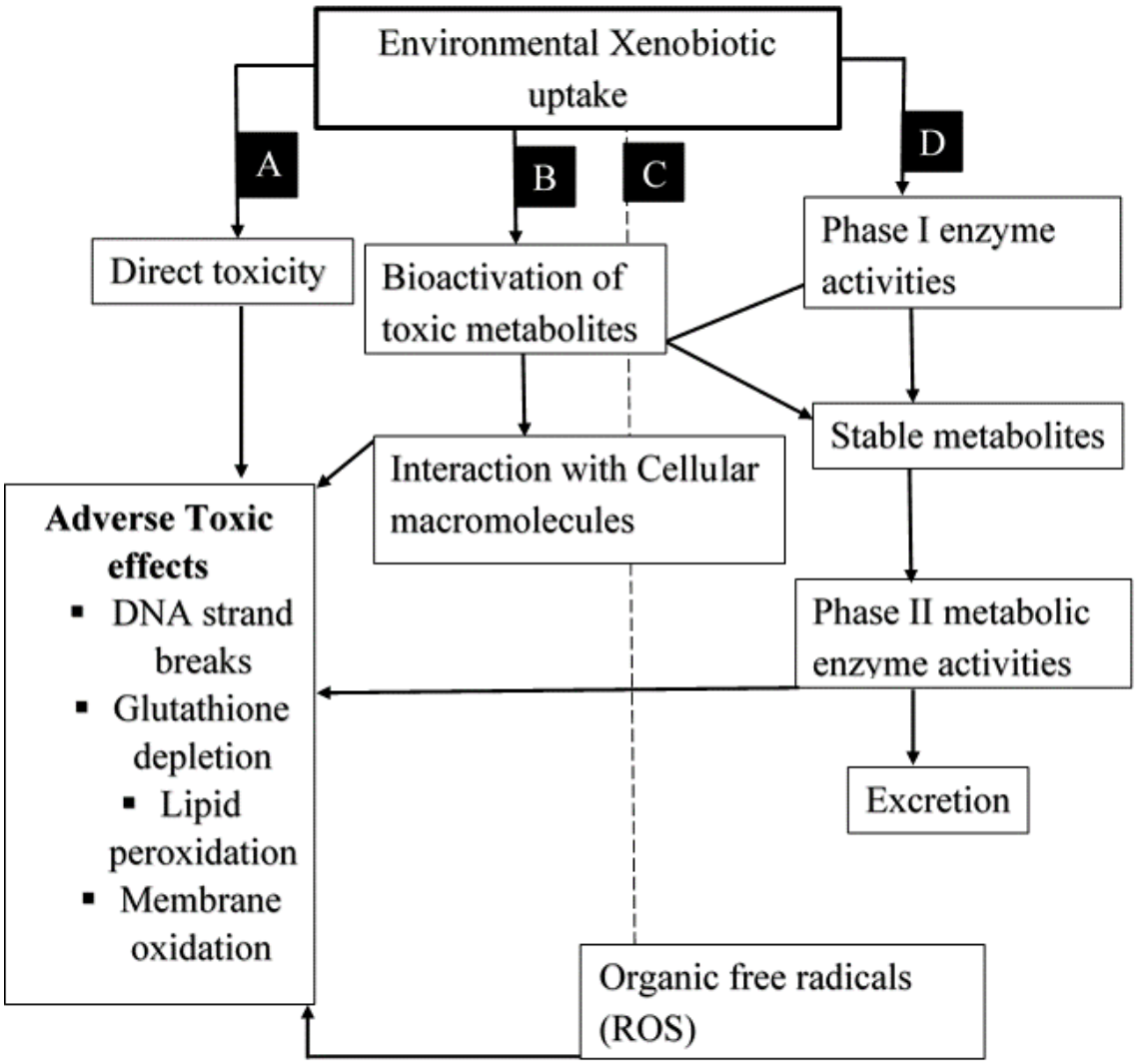

Figure 1.2 Simplified pathways of xenobiotic metabolism a), b), & c) biotransformation and activation pathways d) Detoxification pathway. Adapted with modifications from Van der Oost et al. (2003) & Livingstone (1991)

1.6.2 Biotransformation of PCBs

Biotransformation of PCBs involves the formation of hydroxylated and methyl-sulfurnated PCB complexes which are precursors of PCB induced toxicity (Boas et al. 2006; Lans et al. 1993; Letcher et al. 2000; Routti et al. 2008). An example is seen in Brouwer and Van den Berg (1986) in which exposure of female Sprague rats to 15mg/kg trichlorobensene resulted in marked tyrosine and vitamin A depletion induced by meta-para-OH –PCB. Routti et al. (2008) also reported significant activity of both hydroxylated and methyle sulphurnyl PCB metabolites in the plasma and liver of the Atlantic Seal (*Halichoerus grypus*).

1.6.3 Biotransformation of PAHs

PAHs are important organic pollutants in the environment; present both in the terrestrial and in the marine environment (D'adamo et al. 1997). Hydrocarbons sources are either from by-product of petroleum (Petrolytic) or from incomplete complete combustion of organic chemicals (pyrolytic) (Bakke et al. 2013; Rand 1995). PAHs are complex xenobiotics usually involved in triggering multiple adverse effect pathways with resultant multiple biological effects in living systems. Their activation in living cells often results in the induction of various genes responsible for several cellular responses including the activation of Aryl Hydrocarbon (AH) receptor (Henkler et al. 2010; Wang et al. 2012). A good example which has been extensively studied for its genotoxic potential is B[a] P (Bo et al. 2014; Sarkar et al. 2015; Thompson et al. 2010). It is formed as a by-product of incomplete organic matter combustion and main route of human exposure is via inhalation, skin adsorption, and ingestion (Bo et al. 2014). B[a]P is a priority pollutant in the marine environment and are grouped as class I IARC human carcinogens (Bo et al. 2014; Thompson et al. 2010; Zahn et al. 1982).

1.6.4 Benzo [a] Pyrene metabolism

B[a]P is a model organic pollutant in the aquatic system and it is metabolised in living systems by P450-CYP enzymes of Phase I and Phase II pathways resulting in the activation of the ultimate 7,8-dihydrodiol-9,10 epoxide (BPDE) carcinogen (Bo et al. 2014; Mitchelmore et al. 1998a) . The mechanism of B[a]P toxicity (genotoxic and carcinogenic) involves an active covalent bonding with genomic DNA (Hartwig 1998; Thompson et al. 2010). Mainly, on activation B[a]P undergoes redox cycling (Figure 1.3) which results in the production of subsets of metabolites such as B[a]P 1,6-hydroquinone, 1,6-semiquinone and 1,6-quinone which are all actively involved in the induction of reactive oxygen radicals, which then interacts with the DNA, resulting in DNA damage (Boelsterli 2007). Toxicity of B[a]P is linked to the interaction of its metabolites with intracellular macromolecules such as genomic DNA resulting in DNA-adducts formation and induction of oxidative stress through production of large amount of reactive oxygen species (Bo et al. 2014; Henkler et al. 2010). Other toxicity endpoints reported following B[a]P exposure are embryo toxicity and immunogenicity (Bo et al. 2014). Thompson et al. (2010), also exposed Zebra fish (*Danio rerio)* to environmental concentrationsof B[a]P (0.4µM and 8 µM) and observed significant increase in Cyp1A1 activity. Bo et al. (2014) also reported significant DNA damage following treatment of *Pagus major* (red sea bream fish) with 1, 4 and 8µg/L B[a]P for 96 hours resulting in significant DNA damage (Bo et al. 2014). In invertebrates, exposure of the sea sponge *Tethya lyncurium* to B[A]P (2×10^{-8} -1×10^{-11}) produced significant increase in ornithine decarboxylase (ODC) activity. Taban et al. (2004) also reported significant DNA damage in mussels

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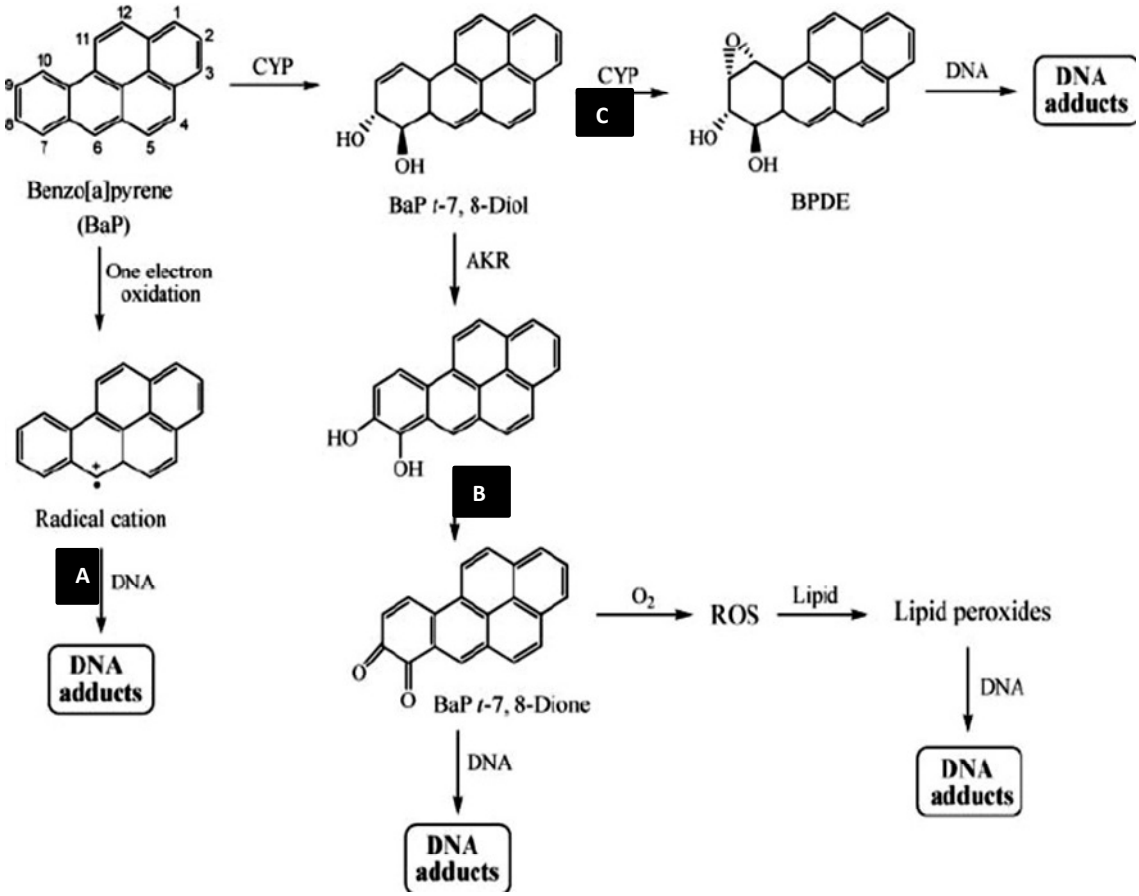


Figure 1.3 Benzo[a]pyrene Biotransformation pathways leading to ultimate carcinogenicity a) Induction of radical cations pathway, b) Production of B[a]P quinones c) Formation of Diol Epoxides and the toxic BAPDE mutagen. Reproduced with permission from Fu et al. (2012) .

1.6.5 Phase I metabolism

Phase I metabolism is usually the 'first line of action' in organic chemical metabolism, and it involves oxidizing lipophilic chemicals to a polar compound which is water soluble and easy to excrete (Boelsterli 2007; Goksøyr and Förlin 1992). Usually the resulting soluble compounds or substances are subjected to further metabolism before it is finally detoxified via Phase II conjugation enzyme pathway, converting them to polar compounds, are eventually excreted (Shaw and Chadwick 1998). A typical example is the metabolism of paracetamol (acetaminophen), in the liver it is taken up and conjugated by sulfate and glucuronide groups and converted to water soluble compounds which is then excreted via urine. Also, benzene is easily metabolised to phenols via this pathway (Boelsterli 2007; Shaw and Chadwick 1998).

However, the processes involved in organic chemical metabolism also results in the activation of toxic metabolites in some cases (Routti et al. 2008; Thompson et al. 2010). Bioassays for measurement of Phase I monooxygenase enzyme activities are EROD (7-ethoxyresorrufin O-deethylase) assay, BROD (benzyloxyresorufin-0-dealkylase) assay, PROD (pentoxyresorufin-0-dealkylase) assay and MROD (methyloxyresorufin-0-demethylase) assay (Routti et al. 2008).

1.6.6 Phase II metabolism

GST (Glutathione-S- transferase) and uridine diphosphate glucuronyl transferase are examples of Phase II enzyme activities, (Thompson et al. 2010). In phase II metabolic pathways, endogenous molecules of sulphate and glucuronide groups conjugate xenobiotic metabolites creating a metabolic 'handles' (hydroxyl and carboxyl groups) which are conjugated by glucuronic acids (Livingstone 1998; Shaw and Chadwick 1998). The 'handles' are easily released from the conjugate complex via the action of glucuronidase bacteria in the

environment. This releases the toxic metabolite back into the environment resulting in an increase toxicity of the metabolite (Shaw and Chadwick 1998).

1.7 Environmental Monitoring of aquatic pollutants

Commonly utilised matrixes in coastal environment for xenobiotic monitoring are seawater, sediment and biota (Cebrian et al. 2007; Phillips and Rainbow 1993). Environmental pollutants can be quantified in the water column of any given aquatic system; however, pollutant concentrations in water column are usually very low (Søndergaard et al. 2015). For most analytical equipment water samples from marine environment must be diluted further to comply with the maximum permissible saline content. This makes quantitation of the actual pollutant concentrations very challenging (Søndergaard et al. 2015).

On the other hand, total environmental pollutants and their historical exposure levels over time can be characterized from sediment core allowing for measurement of the total level of pollutants in the natural environment (Cebrian et al. 2007). Information on total bioavailable pollutants is however needed for the assessment of the actual risks posed by different environmental xenobiotics to indigenous species, which cannot be obtained from the measurement of pollutant levels in sediment. This therefore requires the deployment of aquatic biota to measure both pollutant concentrations in the natural environment and their bioavailable contents. Commonly employed biota in the assessment of both total pollutant accumulation, and their total bioavailable contents are fish, gastropods, clam, mussels, oysters, snails (Roberts et al. 2008; Valverde and Rojas 2009; Van der Oost et al. 2003). Among these, fish are most popular in its use as a bio-accumulator and in the measurement of bioavailable pollutants. This is because fish are widely distributed and are able to accumulate varying environmental pollutants, and are major energy distributor from one trophic level to the other on the ecological food chain (Powers 1989; Van der Oost et al. 2003). Benthic

invertebrate biotas, like mussels and sea sponges have also started to gain significant recognition as bio-monitors and bio-indicators of pollutants in the aquatic environment. They are also used to study biochemical processes in higher organisms (Cebrian et al. 2007; Martins and Costa 2014). Because sessile benthic epifuana are unable to escape pollution sources in their aquatic ecosysytem, they develop adaptive mechanism for tolerating constant exposure to environmental xenobiotics. Hence measurement of both biomarkers of exposure and effect is a useful tool in environmental biomonitoring and risk assessment of the aquatic ecosystem. In this way, sessile invertebrates are considered reliable bio-monitors in the aquatic eco-system and can provide reliable information of the health status of an environment (Cebrian et al. 2007; Martins and Costa 2014; Rainbow 2017; Schulz et al. 2013; Suter II 2016).

The use of model organisms (vertebrates and invertebrates) for mechanistic studies dates back a long time in scientific research and was almost (until very recently) the only established method of investigating the impacts of environmental xenobiotics (Martins and Costa 2014; Moriarty 1988). However, these methods mainly relied on laboratory exposures of either whole organisms or cultured cells under controlled laboratory condition to study the impacts of environmental pollution. However, to monitor the aquatic environment for pollution level and their effects on biogenic fauna, resident sentinel species would be required rather than laboratory exposures of model systems (Martins and Costa 2014). Lower invertebrate species can be used to study complex biochemical processes obtainable in higher metazoans. For example, the mechanism of action of endocrine disruptors that are investigated in sea sponges (Hill et al. 2002). Despite the potential advantages of sessile sentinel invertebrates in the prediction of pollution levels and impacts in the aquatic ecosystem, these organisms are still underutilised in monitoring investigations; main reason being the lack of complete genomic

annotation of most sessile invertebrates and characteristic inter-species variability (Cebrian and Uriz 2007b; Cebrian et al. 2007). The limitation of inter-species variability notwithstanding, sessile biogenic invertebrate organisms are gaining significant research attention in environmental risks assessment (ERA) and aquatic biomonitoring. As bio-monitors and bio-indicators they help provide a better 'picture' of the extent and the impact of pollutants. They have also helped to enhance the understanding of pollutants molecular effects on higher metazoans.

Pollution in the marine environment is a global issue and has in recent times received tremendous research attention (Berthet et al. 2005a; Goldberg 1975; Moriarty 1988; Sarkar et al. 2015; Schulz et al. 2013). Coastal environments are particularly susceptible to pollution due to anthropogenic activities, such as dredging, waste disposal, oil exploration, and mining. Indiscriminate disposal of wastes such as industrial wastes, oil and gas spillages, household generated wastes, plastics, and agricultural wastes with concomitant release of noxious chemicals are primarily implicated as sources of coastal pollution (Shesterin 2010). Among pollutants released into the coastal ecosystem following human overexploitation of the natural resources in the coastal ecosystem; heavy metals, hydrocarbons and organochlorinated compounds are of global monitoring priority because of their high toxicity profile. Heavy metal contribution in the aquatic ecosystem are from both natural sources and anthropogenic sources such as rocks and industrial waste disposals, hydrocarbon load in coastal environment are mainly those of petrogenic and pyrolytic sources while contribution from fertilizer products and pesticides constitute the main source of organochlorine in the coastal environment. Heavy metals bioaccumulate very easily in sessile epifauna at a high concentrations, some of which are stored up in non-toxic forms, others are expelled via excretory process (Ferrante et al. 2018; Rainbow 2017). Stored metals in sessile invertebrates

are however transferred from one trophic level to the other following predation by higher animal. Lipophilic pollutants are trapped in sediment following discharge of chemical wastes into the marine environment, these chemicals bioaccummulate in marine organisms and are sometime detoxified and stored up in inert form (Kotelevtsev et al. 2009). Lower marine organisms many times serve as food for the higher animals (fish and other sea food) which eventually takes up the stored up pollutants which are then biomagnified by trophic transfer along the food chain (Wang 2002). For example methyl mercury in fish and sea foods from mercury contaminated environment concentrate in humans following consumption (Díez 2008); Oken and Bellinger (2008) also reported neurodegenerative disorders in both foetuses, neonates and late infant development in babies. These babies were those whose mothers were previously reported to have elevated concentration of methylemercury, following high consumption of fish and sea foods from the environment. Hydrophobic pollutants such as pesticides, organochlorines and hydrocarbons on the other hand, are biotransformed to form intermediate products via metabolic pathways which are very toxic (Shaw and Chadwick 1998). These metabolites interact with cellular macromolecules to form complexes which undermines the antioxidant function of the molecules and results in adverse molecular effects and related health complications (De Flora et al. 1995; Shaw and Chadwick 1998). On beaches, littoral and pelagic zones, contamination from plastics for instance is a major issue as indigenous vertebrate and invertebrates species are in danger of being entrapped, suffocated. Ingestion of plastic materials by these organisms also means that toxic chemicals from plastic materials would be transferred along the food chain. Other implications of plastic contamination in the aquatic system is that they harbour invasive species resulting in loss of habitat (Schulz et al. 2013). Empirical data on the distribution pattern of plastics in tropical waters, the Mediterranean, Arctic and North-Eastern Atlantic gave room for the

OSPAR convention (convention for marine environment protection of the North-East Atlantic). The OSPAR convention ensures strict adherence of its member countries to the directives the EU MSFD (Marine Strategy Framework Directive) in North- Eastern waters including the arctic (Dameron et al. 2007; Martinez et al. 2009; Ryan et al. 2009; Schulz et al. 2013).

To adequately monitor and protect the environment, there is need to establish a "cause-effect" relationship between exposure to toxic environmental chemicals and adverse health effects observed in life forms including plants, animals and humans. ERA is a scientific process of establishing 'cause –effect' relationship between environmental pollutants and adverse health effects. Given the limited resources, risk assessment are performed to precisely identify potential toxic compounds, human exposure routes, frequency and chances of being exposed, concentration dependent effects, duration or time dependent effects, vulnerable group and mechanistic pathways of each identified toxic compounds (Martins and Costa 2014; Suter II 2016; Van der Oost et al. 2003). Thus Environmental risk assessment in the aquatic media seeks for the establishment of biomarkers ("cause–effect relationships) of both effects and exposure using resident organisms (Van der Oost et al. 2003). In this regards, monitoring efforts are made to study the harmful effects observed today on both animals and humans following exposure to environmental pollutant (Ferrante et al. 2017). A number of marine monitoring programmes aimed at studying the effects on aquatic biota, for example the 'mussel watch' were successfully launched in the 1970's in North America to monitor pollutions from metals, organics and other environmental pollutants using bivalves and other gastropod (De Mestre et al. 2012; Goldberg 1975; Goldberg et al. 1978; Mahaut et al. 2013). This programme has also been extensively applied in the French coast to assess compliance to environmental guidelines for the North East Atlantic (Commission 2000; Mahaut et al. 2013)

. Following the success of the mussel watch programme, application of other benthic organisms for biomonitoring assessments were suggested, including for example, the 'sponge watch' introduced by (Olesen and Weeks 1994). However, for complete assessment of the different species in the marine environment , (Phillips and Rainbow 1993) suggested the use of 'batteries' of species, that is, more than one organism each from different benthic levels in the system for biomonitoring exercises, this he suggested would provide a more robust data for ERA in the aquatic ecosystem.

1.8 Genotoxicity assessment in the marine environment

For years, assessing the genotoxic effects of pollutant especially in coastal environment has been major target for ERA and has in recent times gained wider application in sentinel invertebrates and non-model organisms in ecotoxicology field (Martins and Costa 2014).

Genotoxicity endpoints are recurrent and reliable biomarker in ERA because they provide information on both biomarkers of exposure to environmental xenobiotic and biomarkers of their specific effect in living system, hence genotoxicity endpoints or biomarkers are widely utilised for monitoring exposure and effects from environmental pollutants (Martins and Costa 2014; Moriarty 1988). Information on the levels of toxic chemical in the environment is important as it helps with the understanding of the health status of an ecosystem.

In the aquatic ecosystem, especially coastal environment, pollution is mainly contributed by anthropogenic activities, mainly in the industrialised environments (Dixon et al. 2002; Steinberg et al. 1994). In order to understand and validate the exact impact of human pollution contribution to the environment therefore, the relationship between chemical contributions and resulting adverse health effects in living systems ('cause-effect' relationship) or biomarkers must be established (Regoli et al. 1998). Biomarkers are an important tool in

ERA, because they serve as early warning system for safeguarding against more advanced toxic effects on the environment, animals and humans. At the molecular level, the DNA is the main target organ for many environmental xenobiotics which directly interact with and undermine the integrity of the molecule (Tubbs and Nussenzweig 2017).

Compromised DNA structure, either as strand breaks, base deletions, cross linkages, or mispairing are the primary precursor to most neoplastic disease conditions (Nacci et al. 1996; Reinecke and Reinecke 2004) and they constitute important genotoxicity endpoints for environmental biomonitoring and risk assessment (Martins and Costa 2014). In the aquatic environment, assessment of genotoxicity involves the determination of genotoxic impact on indigent biota following exposure to environmental pollutants, and this information is important in understanding the mechanism of action of toxic agents in higher members of the ecological food chain (Dixon et al. 2002). Thus, the use of benthic indigenous species is particularly encouraged for pollution biomonitoring because they are able to bioaccumulate pollutants from both the suspension phase and as particulate matters in their immediate environment and also help to provide information on both the levels of pollutants in the environment and the bioavailable content of these pollutants (Rainbow 2017; Roberts et al. 2008).

Genotoxicity in the aquatic medium is usually measured using either of or all of the three toxicity endpoint determination stages: Embryo or early life stage effects; in-vitro genotoxicity testing with model species and in-situ genotoxicity evaluation in target organisms (Hose 1994). Each assessment stage is uniquely applicable in ERA, for example adult marine organisms typically partition pollutants in their target organs and this makes pollutants availability subject to biomagnification and might be unavailable for assessment, however, assessment of genotoxic effects in embryos and larva is much more easier and

reliable and this is because of their small size, which enhances even distribution of pollutants throughout the system (Lee et al. 2000; Lee et al. 1998; Roccheri et al. 2004). In-vitro assessment is useful because test model can serve as surrogate for higher target species, helping to understand the mechanism of various toxicity endpoint which could be applied in target organisms. In-situ evaluation provides information on the exact influence on target species (Çavaş and Ergene-Gözükara 2005; Hose 1994). Hose (1994), Longwell et al. (1992) and McIntyre et al. (1980) demonstrated the application of these endpoint in the marine environment. Using samples collected from marine locations in the New York Bight apex along the Atlantic coast of United States, McIntyre et al. (1980) and Longwell et al. (1992) measured mortality, mitotic aberrations and malformation in the embryo and larvae of the Atlantic Mackerel (*Scomber scombrus)*. Results of all three toxicity endpoints were well correlated with pollutant levels measured in water column and phytoplankton (Hose 1994). Common pollutants reported in the New York Bight Apex are PCBs, PaHs and Heavy metals (Burnett and Schaeffer 1980). The same group also investigated using micro nucleus assay the genotoxic impact in vitro in fish samples collected from the same test site and control obtained from a near pristine site along the Atlantic coast, differences in chromatid aberrations between test samples and control was statistically significant (Burnett and Schaeffer 1980). In other examples, Hose et al. (1996) and Norcross et al. (1996) also assessed the genotoxic impact of the Exon Mobil Valdez oil spill on the embryo hatched from an indigenous pacific herring (*Clupa pallasi*) egg. They also recorded marked genetic aberrations and early life stage malfunctions which was also well correlated with levels of heavy metal, PCBs and PAHs.

Generally, ERA involves the determination of when contamination becomes pollution; that is, when environmental contamination becomes high enough to elicit adverse genotoxic effects

on ecological habitats or living systems (Chapman 2007; Martins and Costa 2014). Different biomarkers (toxicity endpoints) and bioassays have been deployed in the assessment of genotoxic impacts of environmental xenobiotic; examples of biomarkers deployed in measuring the effects of environmental xenobiotic include aberrations in amino acid bases, DNA strand breaks, DNA-DNA crosslinks, and DNA-Protein crosslinks (Lee and Steinert 2003a). Bioassays such as the alkaline comet assay, micronuclei assay, sister chromatid exchange assay, and alkaline elusion assays are commonly utilised in ERA.

The comet assay, also known as the alkaline single cell gel electrophoresis (SCGE) assay measures DNA strand breaks migration and alkaline labile sites in individual eukaryotic cells (Koppen et al. 2017; Lee et al. 2000). It is an important tool in environmental bio-monitoring and in effect focused research to measure genotoxicity (Lee et al. 2000; Lee and Steinert 2003b; Mitchelmore and Chipman 1998). Main advantages of the assay include increased sensitivity to environmental stressors, ability to detect specific classes of DNA damage e.g. oxidative stress lesion and bulky DNA adducts, application in quantifying stress level in individual cells with the potential for studying heterogeneity of responses (Coughlan et al. 2002b; Lee and Steinert 2003a; Taban et al. 2004). The comet assay is a reliable assay, straight forward, visual, economical and sensitive to genotoxicants and requires relatively small amount of single cells (Lee and Steinert 2003a). A number of studies have shown the comet assay to be more sensitive to genotoxicants than other genotoxicity assessment assays such as the alkaline elusion assay and the micronuclei assay (Leroy 1996; Meier et al. 2002; Mitchelmore and Chipman 1998; Nacci et al. 1996; Petras et al. 1995). Examples of assessment of DNA strand breaks in invertebrates and application in ERA include the embryos of the marine shrimp *Palaemonetes pugio* exposed to Chromium, hydrogen peroxide and B[a]P in-vitro (Hook and Lee 2004). Also, *Amphipod*s exposed to polluted sediment to

assess metal bioavailability and toxicity as a result of ocean acidification (Roberts et al. 2013) and also in Table 1.3.

Table 1.1.3 **Comet assay application in aquatic invertebrates**

Aquatic species	Chemical Exposure/ Genotoxicant	Gentoxicity endpoint	Significant Dose dependent response	Reference
Oyster (*Crassostrea virginica*)	Benzo[a]pyrene ; Diethylenitrosamine; 2- aminofluorene	DNA Tail length	Yes	Cotelle and Ferard (1999)
Mussel (*M. edulis*)	Hydrogen peroxide ; Dimethylnitrosamine;	DNA Tail length	Yes	Wilson et al. (1998)
Mollusks (*Patunopecten yessoensis* & *Tylotiella. japonica*)	1-methyl-3-nitro-1-nitrosoguanidine ; Benzo[a]pyrene ;	DNA Tail length	Yes	Sasaki et al. (1997)
Mollusks (*M. edulis* & *M. arenaria*)	Petroleum (crude oil) fractions	DNA Tail length	Yes	Hamoutene et al. (2002)
Polychaete (*N. virens*)	Methanesulfonic acid ethyl ester ; Benzo[a]pyrene	DNA Tail length, DNA Tail moment	Yes	De Boeck and Kirsch-Volders (1997)
Blue crab (*C. sapidus*)	Menadione and 4-Nitroquinoline 1-oxide	DNA Tail moment	Yes	Lee et al. (1999)
Grass shrimp(*P. pugio*)	Menadione , Chromium and Mercury	DNA Tail moment	Yes	Lee et al. (2000)
Zebra mussel	Pentachlorphenol	DNA Tail length	Yes	Pavlica et al. (2001a)

Table 1.1.4 **Comet assay application in aquatic invertebrates cont'd**

(*D. polymorpha*)				
Polychaetes (*Hediste diversicolor*)	Silver nano-particle	% DNA Tail intensity	Yes	(Buffet et al. 2014)
Echinoderm (*Asterias rubens)*	Methanesulfonic acid methyl ester and cyclophosphamide	% DNA Tail intensity	Yes	(Canty et al. 2009)
Sea Anemone (*Anthopleura elegantissima*)	B[a]P and H_2O_2	Tail moment, Tail length and % DNA Tail intensity	Yes	(Mitchelmore and Hyatt 2004)
Mud whelk (*Nassarius jacksonianus)*	Hydrogen peroxide	DNA Tail Length	Yes	
Mussel (*M. edulis*)	Hydrogen peroxide , Copper , B[A]P	DNA Tail Length	Yes	(Lee et al. 1998)
Rubbed mussel (*Aulacomya atra*)	Hydrogen peroxide	DNA Tail Length	Yes	(Lee et al. 1998)
Manila clam (*Tapes semidecussatus*)	Contaminated sediment	Tail moment	Yes	(Coughlan et al. 2002a)
Sea sponge (*Hymeniacidon perlevis*	Cd, Cr, Ni and Al	% DNA Tail intensity	Yes	(Akpiri et al. 2017)

1.9 Overview of the comet assay

The comet assay was first developed by Ostling and Johanson (1984) as the 'neutral assay' to measure nuclear abnormalities in single eukaryotic cells. In this study single mammalian cells, previously irradiated were suspended in an agarose gel obtained from patients undergoing chemotherapy; these were lysed in strong salt solution and thereafter electrophoresed in an electric field. Negatively charged DNA molecules were observed to migrate toward the anode in the presence of electric current, while broken strands migrated further towards the anode still attached to the nuclear core and forming a loop in the form of a 'comet'. They also observed that the extent to which broken strands migrated were proportional to the amount of radiation individual cells were exposed to (Fairbairn et al. 1995). The micro electrophoretic neutral assay introduced by Ostling and Johanson was, however, only able to measure DNA double strand breaks, therefore in the late 80's to early 90's the assay was modified to also detect single strand breaks in individual eukaryotic cells and for improved sensitivity (Olive et al. 1990; Singh et al. 1988). The detection of single strand breaks (SSBs) in the supercoiled DNA double helix of eukaryotic cells is particularly important because SSBs is a major form of nuclear abnormality which has been linked with serious disease conditions as it undermines genome integrity (Griffin 1996). DNA single strand breaks are only detectable in protocols involving the use of strong alkaline solution like the alkaline sedimentation assays, alkaline elution assays, and the alkaline unwinding assays). The tightly packed, high molecular weight DNA double helix is made to naturally fit in within the nucleus of eukaryotic cells; this structure is however, compromised in the presence of SSBs because a relaxed or sagging effect is introduced to the compact structure thereby reducing functionality of this important macromolecule (McKelvey-Martin et al. 1993). If not

detected and removed promptly, SSBs can be converted to double strand breaks and other forms of aberrations that are linked to most neoplastic diseases (Tubbs and Nussenzweig).

In principle, loose and broken negatively charged DNA fragments embedded in a gel are unwound in high alkaline solution, these are stretched out from the nuclear head and move towards the anode when electricity is applied in an electrophoretic set up forming a cloud of DNA tail known as 'comet tail'. DNA damage is visualised using DNA-intercalating fluorescence dyes (e.g. sybr gold, ethidium bromide, gel red and quantified as length and intensity of genomic DNA in the 'tail' (extended loop) and head (nuclear core) by fluorescence microscopy (McKelvey-Martin et al. 1993) ; genotoxicity (DNA damage) are quantified by the comet IV software and expressed as percentage tail intensity (Coughlan et al.,2002) (Figure 1.4 and 1.5)

Outline of comet assay method

1) Slide preparation
2) Lysis
   - FPG -modification
   - Electrophoresis
   - Neutralization
   - Staining

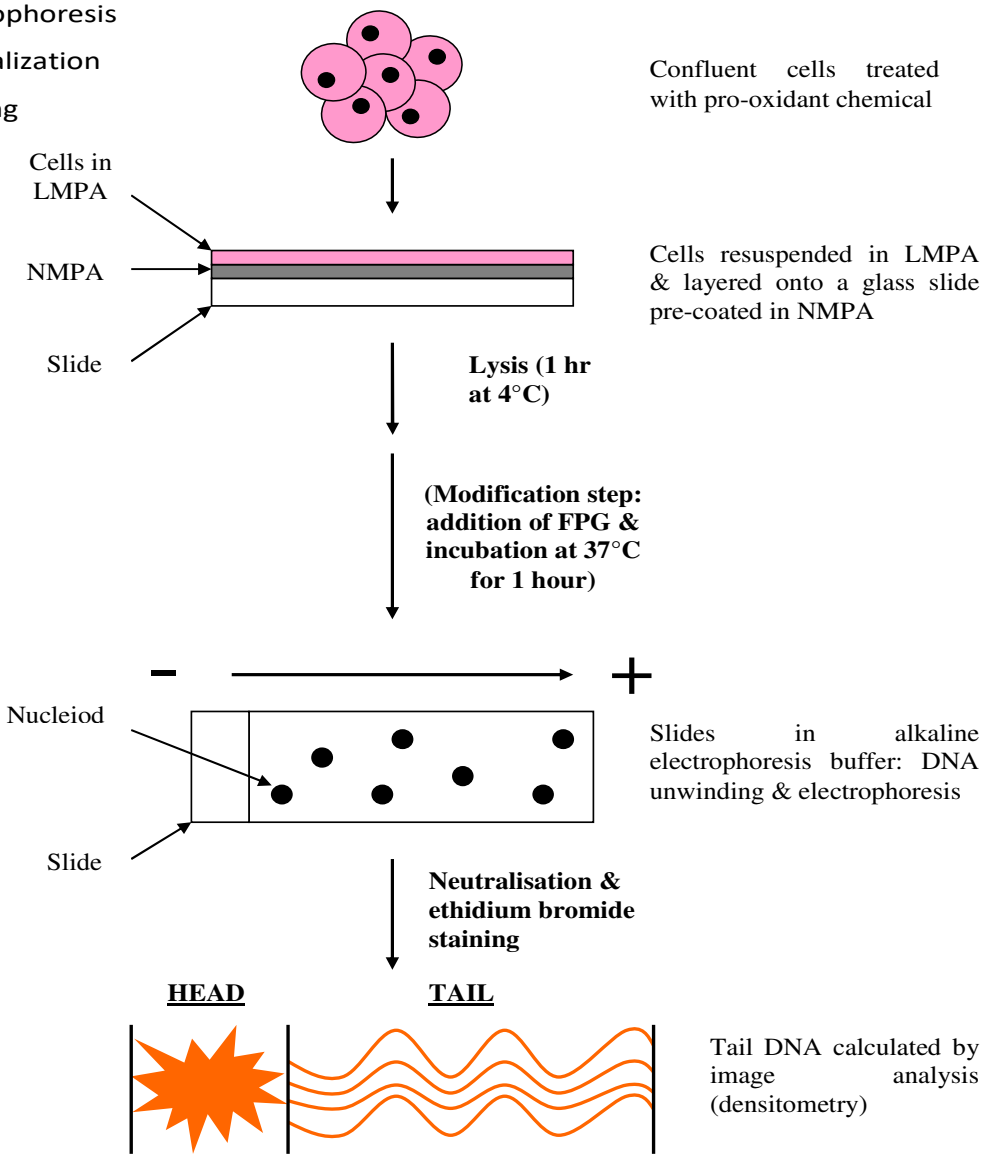


Figure 1.4 Schematic representation of the comet assay protocol (Reproduced from Daniel Smart, PhD thesis 2006)

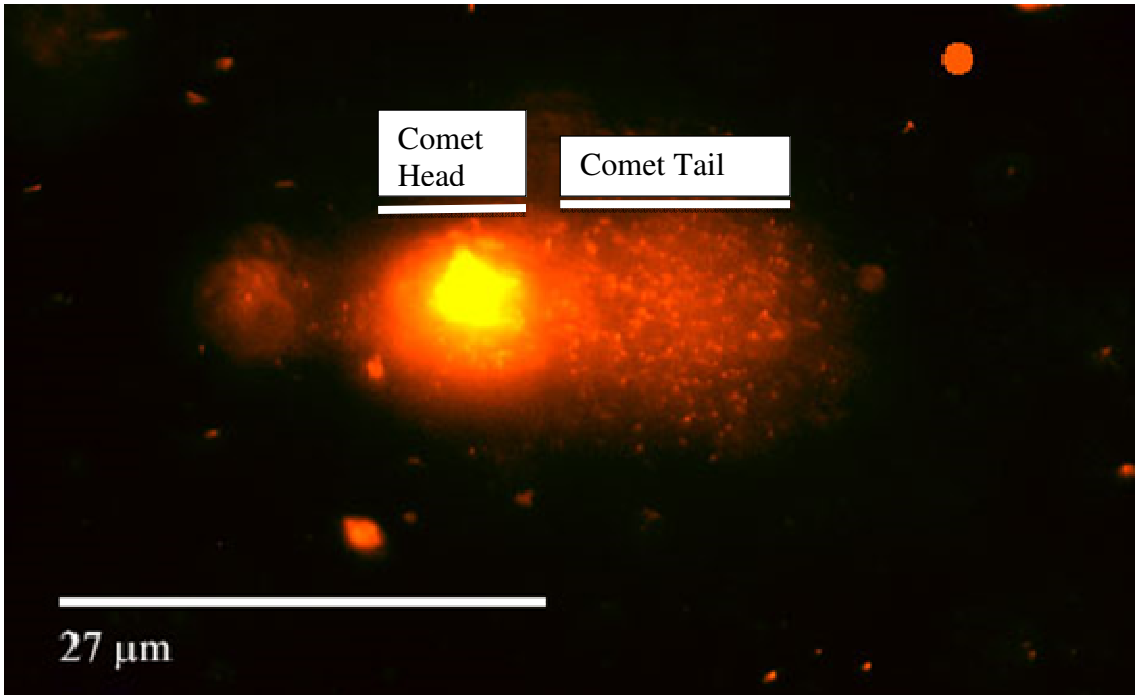


Figure 1.5 Comet head and tail measurement by the Comet IV software

1.9.1 The comet assay and application in ERA

The comet assay technique has been employed for the measurement of DNA strand breaks in many eukaryotic cells; it is a straightforward, visual, sensitive and reliable bioassay that is able to quantify DNA-strand breaks in individual cell nuclei (McKelvey-Martin et al. 1993). The comet assay protocol makes use of a strong alkaline solution and alkaline unwinding in combination to unwind broken or damaged single strands from the supercoiled DNA double helix. Although different studies (summarised in Table 1.3) have demonstrated the measurement of DNA damage using the comet assay technique in a number of aquatic invertebrates (Coughlan et al. 2002a; Lee and Steinert 2003b; Nacci et al. 1996; Pavlica et al. 2001b; Rank and Jensen 2003; Taban et al. 2004), to the best of our knowledge there are no studies until now on the potential application of the alkaline comet assay to measure DNA-strand breaks in marine sponge cells; neither using single sponge cell suspension nor cells from sponge cell aggregates.

The comet assay is applied in effect-focused research to measure DNA strand breaks as biomarker of effects from exposure to environmental pollutants (Collins et al. 1997; Coughlan et al. 2002a; Martins and Costa 2014). Although Chapman et al. (2013) and other studies have observed some limitations of the comet assay; such as challenge with result interpretation needed for research planning as it does not provide information on specific source toxicants. The assay however, despite this specificity restraint, has a number of advantages; in addition to the advantages earlier mentioned.

The comet assay has an increased sensitivity to environmental stressors, and the potential to detect specific classes of DNA damage e.g. oxidative stress lesion, bulky DNA adducts; etc. The comet assay quantifies the level of stress in individual cells which makes it possible to observe and study heterogeneity of individual response to known genotoxicants.

Pollution in coastal ecosystems results from myriads of toxicants of varying environmental sources; thus for ERA, the consideration of whether environmental stressors have adverse or deleterious effects on aquatic biota and on human health by extension is of a higher priority than specificity to toxicants source. Hence, the comet assay, which measures DNA damage as a biomarker of effects is significantly important in environmental risk assessment (Martins and Costa 2014).

1.10 DNA damage as Biomarker of Genotoxicity in Environmental Monitoring and Risk Assessment

Detection of DNA damage in eukaryotic cells remains one of the most reliable biomarkers of genotoxicity applicable in environmental toxicology (Martins and Costa 2014) with DNA single and double strand breaks being the most commonly reported (Bernstein and Bernstein 1991; Steinert et al. 1998). DNA is critical for the maintenance of genetic stability in species because it is the template for genetic replication and transcription (Tubbs and Nussenzweig ; Tubbs and Nussenzweig 2017), hence abnormality in the structure would result in serious consequences on both development and general functionality of the entire organism. This therefore justifies the focus of ecotoxicological research on environmental bio-monitoring of DNA damaging agents, their genotoxic effects on indigent organisms and possible risks from exposure (Berthet et al. 2005b; Mueller 1998; Schröder et al. 2006). Induction of DNA strand breaks by xenobiotics follows three main mechanistic pathways; first is the direct interaction of ionizing and oxidizing chemical agents with cellular molecules resulting in cell death such

as necrosis and apoptosis. The second mechanism involves the activities of reactive oxygen radicals, overwhelming and depleting cellular antioxidant defence systems and the third involves interference with and impairment of DNA repair enzymes.

Most chemical methods of analysing environmental samples for pollutants are sometimes unable to measure the bio-accumulated levels of xenobiotics in both geochemical and biological matrices as they occur in low amount and are therefore below detection limits (Faranda et al. 2012; Søndergaard et al. 2015). Hence, environmental bio-monitoring studies especially in the aquatic ecosystem rely heavily on biomarker data from indigenous biotas because they are able to show responses to pollution that might not be detectable by analytical methods (Berthet et al. 2005a; Müller et al. 1998; Schröder et al. 2006). Genotoxicity is a commonly utilised biomarker for biomonitoring the effects genotoxic pollutants on aquatic organisms and it has been deployed in ERA (Martins and Costa 2014). According to Chapman (2007), and Martins and Costa (2014) environmental contamination does not necessarily mean environmental pollution; contamination in the environment therefore, only becomes pollution if it is high enough to cause deleterious effects on ecological habitats or living systems. A commonly reported adverse effect of environmental pollutants is their ability to compromise genomic DNA integrity via intrinsic and extrinsic interactions; examples includes single or double strand breaks, formation of alkali-labile sites, cross linkages between DNA bases and protein; DNA sequence/base alterations, DNA adducts, and inhibition of DNA repair genes. Thus, emphasis in ecogenotoxicology research is currently focused on assessing the genotoxic effects of pollutant especially in coastal environment on indigenous species, which can be extrapolated on other organisms (Martins and Costa 2014; Moriarty 1988; Rainbow 2017; Van der Oost et al. 2003). For example Costa et al. (2012) exposed fish both in situ and ex situ in a 'batteries' of assay to determine suitable biomarkers

for ERA. Samples were exposed to organic and metal contaminated sediment; with approximately 1.5µg/g PAHs, 0.71µg/g mercury and 43.8µg/g lead; result showed that DNA strand break assessed by the alkaline comet assay was the most consistent biomarker compared to micronuclei (Costa et al. 2012).

.The effects of heavy metals and organics was also investigated in a biomonitoring study among school children living close to a mining industry (Wilhelm et al. 2007), the study showed positive correlations between metabolites of B[A]P, lead in ambient air and blood and DNA strand breaks (Wilhelm et al. 2007). Other examples are found in an extensive review of human biomonitoring studies using the comet assay among occupational workers predisposed to genotoxic pollutants (Valverde and Rojas 2009). They reported in increased DNA damage in workers exposed to different environmental genotoxicants such as aluminium, cadmium, chromium, nickel and lead in welding fumes (Botta et al. 2006). Also, exposure to benzene in elevator manufacturers, radiation, cigarette and polluted air in other workers were all reported to produced increased DNA strand breaks (Cavallo et al. 2002; Lam et al. 2002).

Because environmental xenobiotic are easily bioaccumulated in aquatic biota and humans through consumption of fish and other sea food, aquatic organisms are increasingly being deployed as tools for the establishment of quick, efficient and reliable biomonitoring system in the aquatic media. They are also useful to ensure adequate monitoring of coastal waters given recurrent pollution from anthropogenic activities in this environment (Batista et al. 2013; Gentric et al. 2016; Martins and Costa 2014).

1.11 Invertebrate organism in aquatic pollution monitoring

Sessile marine invertebrates are commonly deployed in bio-monitoring studies because of their inability to escape sources of pollution in the aquatic media. According to the UNEP/WHO report (pour la Mediterranee 1995), the aquatic system is contaminated with different pollutants, ranging from genotoxic, mutagenic, carcinogenic and teratogenic substances, however sessile biogenic epifaunas which are continually exposed to these xenobiotics have developed protective systems (biotransformation and detoxification) for resisting their effects (De Flora et al. 1995; De Mestre et al. 2012). Despite continuous exposure to environmental xenobiotics, sessile biogenic epifauna do not develop neoplastic diseases, hence, sponges and other benthic invertebrates are widely researched for their potential as environmental monitors and possible anti-cancer factors (De Flora et al. 1995). Compared to mussel, sea sponges have been reported to be more sensitive to organicpollutants like PAHs (Batista et al. 2013) and are therefore better suited for monitoring organic and persistent pollutants. Other advantages of sea sponges over mussel is their ability to bio-accumulate both petrogenic/pyrolytic and pyrogenic pollutants (Batista et al. 2013). In order to enact good environmental protection policies, therefore, adequate understanding of what the real risks of exposures to pollutants are is imperative; this understanding however, cannot be fully obtained from laboratory cell cultures of model organisms. Hence the increasing trend in the application of non-model benthic invertebrates in both effect-based research and general ecogenotoxicology research (De Mestre et al. 2012; Martins and Costa 2014). An important feature of invertebrates that has also helped to increase the application in biomonitoring studies is their ability to bioaccumaulate wide range of pollutants especially in their aquatic environment ecosystems. Mussels and oysters for example, have been particularly very useful in this regard and have been extensively deployed in monitoring both

organic and heavy metal pollution in coastal aquatic systems (Taban et al. 2004). However they are not able to bio indicate the levels of pollutants in water columns because gastropods and bivalves such as mussels and oysters, are mostly intertidal, (Batista et al. 2013; Gentric et al. 2016). Sea sponges on the other hand owing to their extensive water filtration and filter feeding potential are able to accumulate very efficiently both particulate and suspended pollutant in the water column and have also been reported to indicate levels of PAHs in their local environment (Batista et al. 2013).

Sea sponges however though cosmopolitan with an all year availability and ability to bioaccumulate a broad range of environmental xenobiotic, have received very little attention with respect to pollution biomonitoring (Cebrian et al. 2007). They occur both intertidally and in depth of up to 600-700m. Like bivalves, sponges have large deposits of lipids, which is particularly useful for biomonitoring of lipophilic pollutant. According to the Denton et al. (2006), 16 USEPA priority PAHs were similarly accumulated in both sponges and bivalves, however, Mahaut et al. (2013) reported higher levels of phananthrene bioaccumulation in the sea sponge *Hymenaicidon pelervis* than in the blue mussel *Mytilus edulis* by the same group.

1.11.1 Sea sponges as Bio-monitors of Aquatic pollution

Sea sponges are sedentary and cosmopolitan in almost all regions of the globe (Van Soest et al. 2012) and have in recent ecotoxicological studies gained significant attention. The main attractions to sea sponges results from their ability to pump large volume of water containing particulate matters, ability to serve as host to a wide range of microorganisms especially bacteria and very importantly their ability to accumulate varying range of environmental xenobiotic and the mechanisms to retain them for an extended period of time without any

direct adverse effect to them(De Mestre et al. 2012; Ferrante et al. 2017; Perez et al. 2003) (Berthet et al. 2005b; Cebrian et al. 2007).

Sponges bioaccumulate pollutants both in the suspension phase and in the dissolve form, with strong affinity for metal pollutants (Philp 1997). Other important features, that has increased research interest in sea sponges is the fact that, because they lack complex organ system, inter species variation to the level of accumulated metals is almost eliminated (De Mestre et al. 2012; Roberts et al. 2008). Also, being local in almost every marine environment means the problem of disease organism translocation as common with most bivalves is also eliminated (De Mestre et al. 2012).

Their relative abundance in both intertidal and sublittoral zones of the aquatic ecosystem and their availability all year round are all important biomonitoring features which have also contributed to making them choice sessile epifauna in aquatic environmental monitoring and in ERA (Cebrian et al. 2007; De Mestre et al. 2012). Compared to mussels and fish which are commonly utilised for biomonitoring in the aquatic system (Hamoutene et al. 2002; Taban et al. 2004; Van der Oost et al. 2003), sponges tend to preferentially bioaccumulate persistent organic chemical pollutants, including high molecular weight PAHs (Batista et al. 2013; De Mestre et al. 2012).

Table 1.1.5 Applications of sponges as biomonitors of environmental pollution

Sponge species	**Pollutants monitored or Indicated**	**Author**
Hymeniacidon perlevis	Heavy metal and organic chemical pollution (Copper, Zinc and Fluoranthene)	(Mahaut et al. 2013)
Hymeniacidon perlevis	Poly Aromatic Hydrocarbons	(Batista et al. 2013)
Chrondrosia reniformis *Phorbas tenacior* *Dysidea avara*	Heavy metal pollution (Copper and Lead)	(Cebrian et al. 2007)
Hymeniacidon perlevis	Bacteria accumulation (Potential for bioremediation	(Longo et al. 2010)
Chondrilla nucula	Heavy metal accumulation (Cadmium, Lead and Copper)	(Ferrante et al. 2018)
Chondrilla nucula	Accumulation large amount of bacteria (*Escherichia coli*): Potential for bioremediation tool.	(Milanese et al. 2003)
Spongia officinalis	PCB accumulation	(Perez et al. 2003)
Halichondria panacea pallas	Metals (Copper, Zinc and Cadmium)	(Hansen et al. 1995)
Clathria venosa	PAHs PCBs, metals	(Alcolado 2007)
Scopalina ruetzleri *Halichondria panacea*	• Urban pollution • Cadmium pollution	(Olesen and Weeks 1994)
Haliclona tenuiramosa	Metal pollution (As, Cd, Co, Cu, Fe, Mn, and Ni)	(Rao et al. 2009)
Spongia officinal	Metal pollution (Expression of Metallothioneins as indicator of heavy metal pollution)	(Berthet et al. 2005a)
Amphimenidon spp	Metal pollution	(Pan et al. 2011)
Mycale sp & Suberites diversicolor	Metal pollution monitoring	(De Mestre et al. 2012)
Scalarispongia scalaris	Halogenated organic compounds	(Melcher et al. 2007)
Tedania charcoti & Prostylyssa foetisda	Metal pollution	(Roberts et al. 2008)
Amorphinopsis atlantica	Inhibition of ABTS centre dot+ (Free radical for measuring the antioxidant activity of natural products)	(Montaño-Castañeda and Santafé-Patiño 2011)

As filter feeders they help to clean up the aquatic system by filtering off dirt especially bacteria, as a result, sponges are able to trap over 90% of bacteria within their tissue making them a rich source of bioactive compounds with various therapeutic applications (Koziol et al. 1997b; Müller et al. 2000b; van der Merwe et al. 2010). A good example is Norharman (a beta-carboline alkaloid) isolated from *Hymeniacidon perleve,* which is known to have cytotoxic effects on both the HeLa cervical-cancer cell line and the BGC-823 stomach-cancer cell line (Zheng et al. 2006). Although, there are not many mechanistic studies with non-model invertebrates organisms, reason being the challenges associated in processing single cells (as there are no commercial cell lines), lack of genomic annotation for most invertebrate species and high inter-species variation in commonly utilised invertebrates sentinels like fish and mussels (De Mestre et al. 2012; Martins and Costa 2014; Reinecke and Reinecke 2004; Rinkevich 2005). Even more challenging is the attempt to perform sensitive genotoxicity assays like the alkaline comet assay in non-model invertebrate organisms, being originally developed for use in vertebrates cells (Martins and Costa 2014; Reinecke and Reinecke 2004). In spite of these setbacks, a number of studies (Table 1.4) on genotoxic impacts of environmental pollutants in sponges have been reported (Mukherjee et al. 2016; Ugarković et al. 1991).

Although sessile aquatic invertebrates are continously exposed to pollutants in the aquatic system, they do not suffer from neoplastic diseases like other advanced species higher up in the ecological food chain, this feature makes sessile aquatic benthos especially sea sponges of high research interest, for their anti-carcinogenic, anti-mutagenic and general therapeutic potentials (De Flora et al. 1995; De Mestre et al. 2012; Ferrante et al. 2018). In this respect, a ‘sponge watch’ programme similar to the ‘mussel watch’ programme has been proposed as a biomonitoring and risk assessment project in aquatic environment(Goksøyr and Förlin 1992;

Hansen et al. 1995). Table 1.5 show sponge species previously utilised for aquatic pollution biomonitoring and genotoxicity responses in sponges.

Table 1.1.6 Genotoxicity assays and endpoints in sponge species

Sponge Species	Genotoxicant	Genotoxicity assay	Key finding	Authors
Eunapuis fragilis & Heteromyenia sp	Ethylbenzene (1.5mg/L) Nonylephenol (22 and 11mg/L) Bisphenol (16mg/L)	Cell proliferation assay (Assessment of Endocrine disrupting effect	Significant reduction in cell growth	(Hill et al. 2002)
Suberites domicunla	1mg/L PCBs (six days incubation)	Microplate (Fast micro method) assay	• Time dependent DNA strand breaks. • DNA cross linkages. • Heat shock protein 70 expression	(Müller et al. 1999)
Suberites domicunla	2 days exposure to Cadmium 0.1-1mg/L	Fluorometric microplate (Fast micro method) assay	• Time dependent increase in cadmium bioaccumulation 16.6 fold, 20.4 fold from an initial treatment with 1mg/L • Dose dependent increase in DNA strand breaks, measured as Scission strand factor (SSF)	(Schröder et al. 1999)
Microciona prolifera	H_2O_2 (100µM) and Bleach (20mM)	Sponge aggregation assay	pH mediated aggregation response	(Philp 1997)
Haliclona sp	Cu^{2+} (100, 500 & 1700 µg/L)_ and	Sponge Aggregation assay	Metal induced reduction in sponge cell aggregation	(Goh 2008b)

Table 1. 5 cont'd. Genotoxicity assays and endpoints in sponge species**.**

	Cd^{2+} (100, 500 & 1500 µg/L)_			
Baikalospongia Intermedia *Lubomirskia fusifera* *Lubomirskia abietina*	Temperature < 10°C Pb (40-400µg/L), Cu (40-400µg/L), Zn (10-100µg/L) & organics	Fluorometric (Fast microplate method) assay Gel electrophoresis Western Blotting	• Time dependent heat shock protein 70 expression. • Increased DNA strand breaks.	(Schröder et al. 2006)
Suberites domicunla	In vivo exposure to 5mg/L cadmium	Fluorometric microplate (Fast micro method) assay	• Concentration dependent DNA strand breaks. • HSP73 expression.	(Mueller 1998)
Geodia Cydonium	Lauryl-sodium dodecyl sulphate detergent & Centrimonium bromide detergent.	Ras gene activation assay	• Deactivation of sponge aggregation factor. • Inhibition of Ras gene expression	(Ugarković et al. 1991)
Eunapius carteri	Sodium bicarbonate	Cell aggregation assay & DCF-DA assay	• Increased ROS formation after 48 hours treatment. • Inhibition of ROS, GSH, superoxide dismutase following prolonged exposure of up 8 days	(Mukherjee et al. 2016)
Geodia Cydonium	Exposure to 7°C Temperature	Northern Blot	DnaJ-like protein expression	(Koziol et al. 1997b)

1.12 General Biology of Sea sponges

Marine sponges are sessile multicellular invertebrates belonging to the phylum porifera in the animal kingdom; they are eukaryotic animals and mostly found in marine environment with a few freshwater species. In both marine and fresh water environment, sponges are found attached to sea caves, boulders, rock pools, mangrove stomps and on any hard surface, with different species occurring sub-tidally, inter-tidally or very deep in the sea. As important members of the benthic ecosystem, sponges make up large biomass of the coral reef community with approximately 98% abundance in marine environments and are mostly filter feeder with the ability to pump large volume of water (Stevely and Sweat 2015). Approximately a gallon size sponge is estimated to pump about 100-1200 ml/hour/kg sponge and able to filter and refill a residential swimming pool a day (Stevely and Sweat 2015). Although grouped as primitive multicellular metazoans, they are however, endowed with enormous physiological and molecular sophistication. Sea sponges constitute rich sources of important therapeutic metabolites; undergo complex cell-cell network and interaction; and are usually in a symbiotic association with different members of aquatic ecosystem (Lugg and Sullivan 2001). Taxonomically Sponges are classified into three general groups; the Demospongiae, Hexactinellida and Calcarea based on cellular features, types of skeleton and development pattern. Demonspongiae are cosmopolitan while calcarean tend to diminish with depth while hexactinellids are mostly found in ocean depths of polar waters (Picton et al. 2007; Van Soest et al. 2012). They assume different forms in their natural environment (Standing or Encrusting), different colours and textures in their habitat (Sub-tidal or Inter-tidal), and exist as individuals rather than as colonies, sponges are also without defined sides (front, back, dorsal, or ventral view).

More than 15,000 species have been described and about 7000 species have been identified scientifically, systemic identification is based on comparison of morphological features against a set of keys and descriptions enlisted in Systema porifera (Boury-Esnault and Rützler 1997; Picton et al. 2007). Of all described sponge species, only one species (*Amphimenidon queenslandica*: native to the Australian coast) has its genome fully sequenced. The process of sponge nomenclature and taxonomy is complex and often times misleading because of the close phenotypic ties between species, the sponge barcoding project (Vargas et al. 2012) therefore is meant to serve as an access point for sponge DNA data signature sequences and provide information for rapid and reliable sponge morphological taxonomy (Srivastava et al. 2010). Molecularly , sponges serve as tools for understanding the evolutionary relationship among other animal taxa (BioMEDIA-ASSOCIATES 2003) and as filter feeders, they help to clean up the aquatic system by filtering off dirt especially bacteria, as a result, sponges are able to trap over 90% of bacteria within their tissue making them rich sources of bioactive compounds with various therapeutic applications (Ferrante et al. 2018; Müller et al. 2000a; Sipkema et al. 2003a). Because sponges are immobile and 'static ' in their natural environment, they serve as host to other biota in the aquatic medium, examples are fish, algae, crustaceans, bacteria, mollusc and even other sponges; this predisposes them to predation. As a defence mechanism therefore, sponges produce different toxic substances to ward off predators; these substances though extremely toxic to other living organisms are however important source of bioactive compounds with vast therapeutic application (Anjum et al. 2016; Queensland-museum 2010-2018; Sipkema et al. 2005). Some of the therapeutic applications of sponge-derived bioactive compounds are anti-carcinogenic, anti-inflammatory, inti-malaria and as pain killers (Queensland-museum 2010-2018). Production of these bioactive compounds is greatly enhanced by the association with other microorganisms whose toxins are sources for sponge metabolic activities (Longo et al. 2010; Queensland-museum

2010-2018; Sipkema et al. 2003a). Apart from therapeutic relevance, sponges possess specific biochemical characteristics which are important endpoints for sponge taxonomy and phylogenetic application (Thomas et al. 2010). Recently, the number of studies utilising sponges for environmental studies has risen tremendously due to its applicability in almost all fields of life science (Batista et al. 2013; Cebrian et al. 2007; Ferrante et al. 2018) and more importantly, they are cultured in the laboratory under controlled conditions and are known to possess vital biochemical pathways present in more complex metazoans. Hill et al. (2002) reported sponge response to endocrine disrupters (ED) following investigation of the effect of water pollution, thus they suggested that sponges can be deployed as model organisms for investigation of endocrine disrupting chemicals and other xenobiotics. An important physiological feature of the sponge is the re-aggregation of its dissociated cells in to primorphs (functional sponge organism with differentiated cells). This is employed in the development of sponge cell cultures to study important mechanistic and environmental processes in higher metazoans (Munro et al 1999 In Pomponi, 2006).

1.12.1 Sponge Anatomy and physiology

Sponges are made of very simple body forms consisting of epithelial cells, they are without organ systems and occur in different brightly coloured forms (Figure 1.6). Morphologically sponges are made of pores (osculum) through which large volume of water are drawn in and out by special collar cells (choanoctyes) lining the internal body surface. Most sponges secrete inorganic microscopic spicules (made of calcium carbonate or silica) and organic fibres (Spongin); both of which function as skeletal support for the animal and are mostly the basis for systematic sponge taxonomy (Simpson 2012). In addition to the two specialized epithelial sponge cells: Pinacocytes and Choanocytes; there are up to sixteen other sponge

cells identified on the basis of the amount and function of cytoplasmic organelles (Simpson 2012).

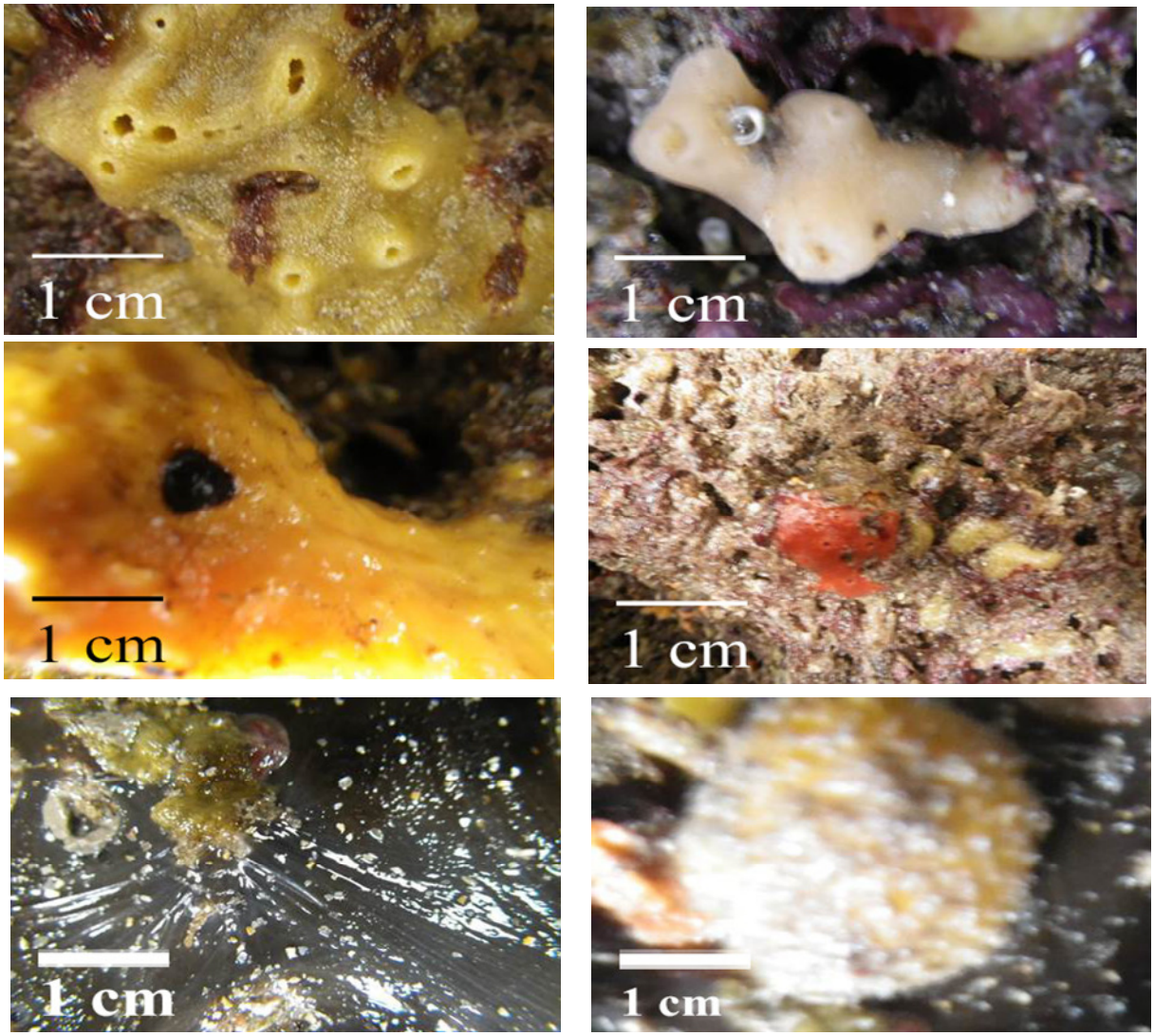


Figure 1.6. Demonspongia Sponges insitu; showing different species distinguished by bright colours on exposed rock pools at low tide.

There are different sponge cell types (choanoctyes, sclerocytes, pinacocytes, and the archaeocytes) (Richelle-Maurer 2001). All except one (the archaeocyte) have terminal cell division and proliferation in culture. The archaeocyte, are sponge totipotent stem cells with the ability to divide and proliferate unlimitedly in culture medium, they are also able to produce and replenish other sponge cell types and are therefore responsible for sponge cell differentiation and aggregation (Sun et al. 2007; van der Merwe et al. 2010). They are also responsible for sponge cell attachment and aggregation (Pomponi 2006a) hence, archoecytes are considered the future for the establishment of long term sponge cell line needed for sustainable production of sponge bioactive metabolites and for environmental biomonitoring of xenobiotics (Pomponi 2006a; Zhang et al. 2003).

Till date there are still no commercially available sponge cell lines, which is a major setback to the production of large scale sponge bioactive compounds and their resultant therapeutic products. However, recent advancement in sponge cell cultures and consequent development of the primorphs system (functional sponge aggregates) using cryopreserved single sponge cell suspension, promises a potential future for sustainable and large scale production of sponge metabolites (Custodio et al. 1998a; Mussino et al. 2013). Improvement in media optimization and the need for careful selection of sponge totipotent cells for sponge cell aggregate formation (primorphs) is however a major requirement for any meaningful progress. Sponge cell aggregates (primorphs), is also proposed as a suitable system for environmental biomonitoring of xenobiotics (Pomponi 2006b).

1.12.2 General Biology of the study sponge species: Hymeniacidon perlevis & *Amorphinopsis kalibiama*

Hymeniacidon perlevis (Montagu, 1818): Study species for method development and laboratory genotoxcity experiments

Figure 1.7 *Hymeniacidon perlevis* on exposed rock pool at low in Tenby Bay Castle beach, March 2015.

Kingdom: Animalia

Phylum: Porifera

Class:Demospongiae

Order: Suberitida

Family: Halichondridae

Genus: *Hymeniacidon*

Species: *Hymeniacidon perlevis : Hymenaicidon perleve*(Montagu,1814) as it was formally known is now generally accepted as *Hymeniacidon perlevis,* following systematic and molecular revision of the taxa as a female species (Erpenbeck and Van Soest 2002).

H. perlevis, is an intertidal species widely found in the on British Isles, Mediterranean and the Arctic sea; with records of appearance in Belgium and France (Mahaut et al. 2013; Picton et al. 2007). Structurally, it is a 'firm-to-touch' species with varying body forms and colours depending on exposure; it mostly exists as cushion, thin sheets, massive-finger-like forms in shelters under rock boulders, crevices, and on rock pools. *H. perlevis* is mostly found in association with other aquatic benthos like sea anemones, seaweeds and other sessile shellfishes possibly to avoid predation. On the surface of the animal are several tiny, almost invisible oscular chimneys through which large volume of water is pumped in and out of the animal (Picton et al. 2007). Depending on exposure, *H. perlevis* assumes different colours ranging from very bright orange to blood red and sometimes yellowish-orange to pinkish-red (Figure.1.7). Other identifying features includes a unique sweetish-spongy smell, fleshy appearance, compressible and rarely branching structure (Picton et al. 2007).

Internally, the endosomal and ectosomal skeleton of *H. perlevis* are typically those of a Halichondroid sponge with confused network of spicules, mainly tangential to the surface. There are no microscleres, spicules are made of only styles without oxeas, it is an all year round species and relatively easy to collect at low tide. *H. perlevis* is an established sponge species with complete systematic and molecular description (Mahaut et al. 2013; Sipkema et al. 2003a; Sun et al. 2007) . They have also been reported as a model for environmental biomonitoring being able to better accumulate certain in-situ pollutants than bivalves for example the blue mussel: *M. edulis* (Mahaut et al. 2013). Also, *Hymeniacidon* has been reported to be more sensitive in the detection of petrogenic and pyrogenic xenobiotics than the brown mussel: *P. perna* (Batista et al. 2013).

1.12.3 *Amorphinopsisi kalibiama* (Carter, 1887): Study species employed in field investigations

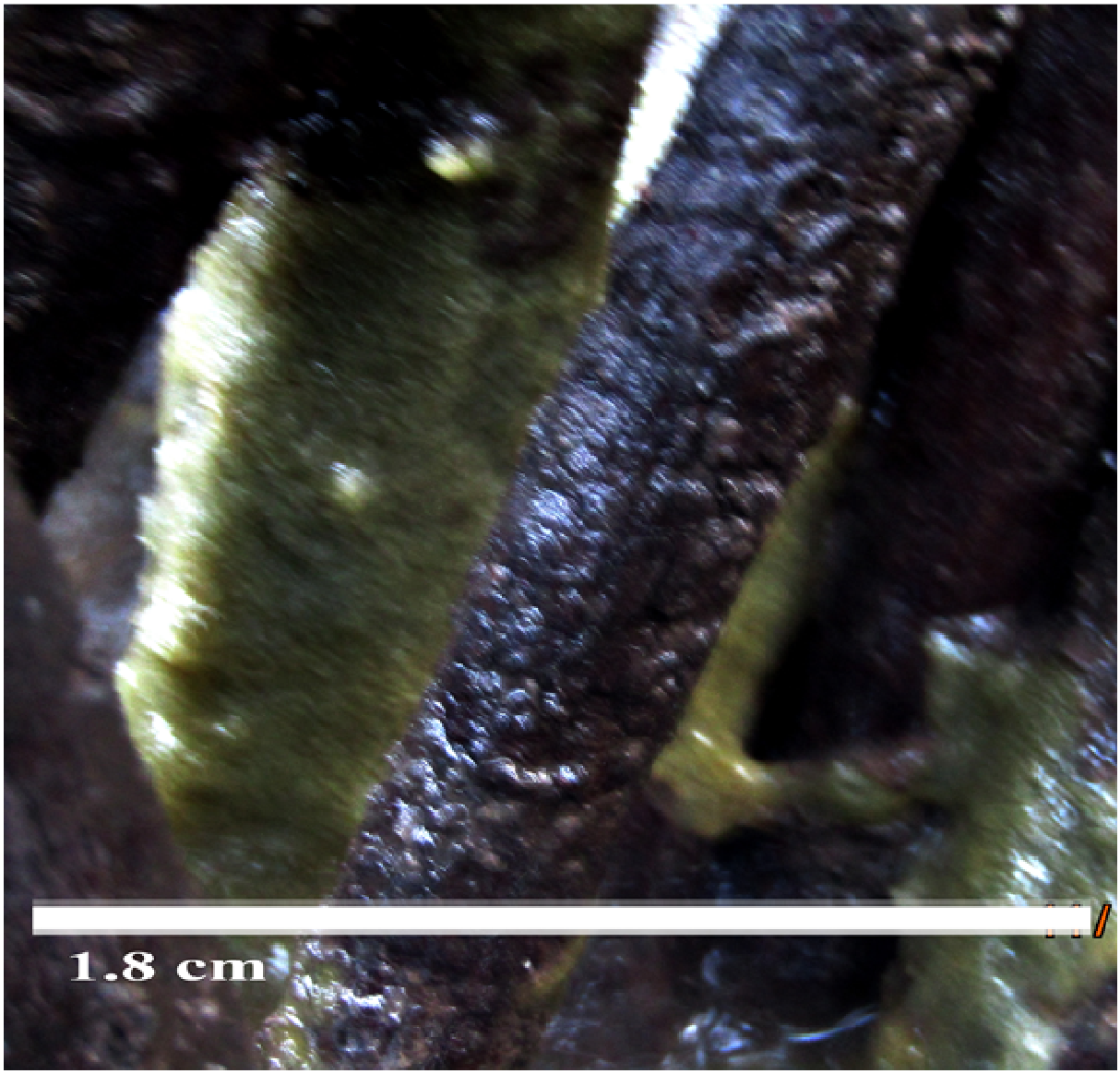


Figure 1.8 Amorphinopsis kalibiama wrapped around mangrove stomp in Pokokri creek, Nembe /Brass Sea in Bayelsa state, Niger delta Nigeria. Sample was collected at low tide in December, 2015

Kingdom: Animalia

Phylum: Porifera

Class: Demospongiae

Order: Suberitida

Family: Halichondridae Gray, 1867

Genus: *Amorphinopsis*

Species name: *Amorphinopsis kalibiama* (species name unofficially assigned but in manuscript preparation. Species name would be used subsequently in this thesis)

A.kalibiama is an encrusting mass with occasional short projections, rough skin surface and compressible; mostly found wrapped around mangrove roots in shallow coastal waters, yellow-olive in colour and sometimes light grey to dull lime. The type species of the genus *Amorphinopsis* Carter, 1887, *A. excavans* Carter, 1887 was originally described as being an excavating sponge, but was later reassigned to the family Halichondriidae (Erpenbeck and Van Soest 2002). There were 23 species at one point but subsequently 6 of these were reassigned to other genera, leaving 17 species currently in the genus. Morphologically, the genus is structurally firm to touch, and has hard consistency (Erpenbeck and Van Soest 2002). The Genus *Amorphinopsis* is however, one of the problematic poriferan genera with a history of complex taxonomy, hence the reassigning of some of the species to different genera (Hymeniacidon, Halichondria, and Prostylissa). Some species of *Amorphinopsis* are difficult to tell apart because of inadequate description, thus, systematic taxonomy would need to be supported with molecular phylogeny for reliable speciation in this group and in all of poriferan genera (Van Soest et al. 2012). Because of the surfix –"opsis" *Amorphinopsis* is accepted as 'female' (Carvalho et al. 2004). This is species of *A kalibiaba* is collected and identified for the first time in West Africa and has been recognised as a newly described species by relevant authorities (Manuscript in draft preparation).

1.13 Sponge cell Culture for Primorphs formation

Sponge cells unlike cells of other metazoan are of mixed population and in many cases sponge cell suspensions also include cells of other epibiotic organisms and therefore require extra purification steps in order to obtain a homogeneous sponge cell population in suspension (Cai et al. 2013). Sponges serve as host to a number of endosymbiotic microorganisms, mainly cyanobacteria and protozoa (which are inadvertently isolated alongside single sponge cells) as such it is challenging to obtain a purely axenic sponge cell

suspension. And also, because sponges are multicellular without an organ-sytem structure, there is no particular section of the sponge tissue where aseptic inoculum can be obtained, extra steps are therefore required to eliminate as much as possible associated symbionts and non-symbionts in sponge cell culture (Cai et al. 2013; Cai and Zhang 2014; Rinkevich 2005). Current trend in sponge cell culture studies are now geared towards improving the 'primorph' (functional sponge cell aggrgates with water channels) sytem, sponge media optimization and selection of immortal sponge cells. The primorph system is articularly important in sponge cell research as it serves to further purify the sponge single cell suspension, and as a model for environemental studies.

1.13.1 Development of functional sponge cell aggregates: "Primorphs"

Primorphs are compact spherical-round shaped functional sponge cell aggregates with organised outer layer and internal network of cells (Le Pennec et al. 2003; Pomponi 2006a). An important physiological feature of the sponge is the re-aggregation of its dissociated cells into functional aggregates controlled by macromolecules (de Caralt et al. 2007; Grasela et al. 2012; Pomponi and Willoughby 1994). Usually this involves three main processes; first primary aggregate formation in the presence of calcium, secondary sponge cell aggregates formation in the presence of an aggregation factor equipped with an extensive plasticity and thirdly the formation of functional aggregates with osculant channels for water inlets and outlets (Müller and Müller 1980). The primorph system is the potential future for sustainable production of sponge bioactive compounds, and for continues production sponge therapeutic products and as ready tools for environmental biomonitoring (Pomponi 2006b; Pomponi and Willoughby 2000).

The system is a novel model developed for sponge cell cultures, to enable the high throughput investigations such as cell apoptosis and cell regeneration, production of bioactive compounds and biomonitoring of environmental contaminants (Müller et al. 1999).

At the cell level, sponge cell culture is mainly established with either, single sponge cell suspension or sponge cell aggregates obtined from single cell suspension (de Caralt et al. 2007). Because there are no commercially available sponge cell lines, cryopreservation of both sponge single cells and sponge cell aggregates seems to have gained the most research attention in sponge studies nowadays, although a few in situ studies still exist (De Caralt et al. 2010). For, successful sponge cell culture therefore, priority must be given to cell growth, cell viability and microbial contamination control. According to Pomponi and Willoughby (2000) cell culture and cryopreservation allows for the production of large quantity, quality and uniform cell population, this is particularly important for molecular studies. They also outlined the following steps and methods as key requirments for sponge cell culture: sponge cell dissociation, cryopreservation, culture media development and optimization, control of microbial contamination, and cell type verification and selection.

1.13.2 Cryopreservation of cells

Cryopreservation of vertebrates and invertebrates animal cells have in recent time gained significant attention in publications (Mussino et al. 2013; Pomponi 2006a). This is because, cryopreservation gives the opportunity for the production of more homogeneous and high quality cells for physiological and biochemical analysis. Some authors have however, argued that for non model systems like sea sponges, it would be preferable to culture cells dissociated from freshly collected sponge tissues as this would enhanced cell viability. This is however not practicable given the difficulty in the achievement of prolonged sponge cell

culture and the challenge with frequent field sampling (Rinkevich 2005). Furthermore, it is uneconomical and time consuming to embark on a field sample collection each time cultures are to be made; thus most recent studies utilizing vertebrate and invertebrate systems are now in favour of cryopreserved single cells , which has also seen the increased trend in the application of cryopreserved sponge cells for sponge cell cultures(Mussino et al. 2013; Pomponi 2006a). Current areas of sponge cell culture with research opportunities according to (de Caralt et al. 2007; Rinkevich 2005) are in the investigation of the biochemical mechanism involved in sponge cell growth, steps to microbial contamination reduction or elimination with minimal or no adverse effects on the cells and sustainable generation and selection of sponge totipotent stem cells.

1.13.3 Media development and and optimization

In sponge cell culture, the priority is to achieve as much as possible a combination of cell growth, cell viability and microbial contamination control; and these are greatly influenced by the culture media. Hence for successful sponge cell culture, an optimised culture media with the right conditions must be established (Grasela et al. 2012; Pomponi 2006a). For example Pomponi (2006b) reported increased cell growth following the use of animal culture basal medium supplemented with amino acid, vitamins, salt, glucose, organic supplements, hormones and growth factor. There has also been report of increased cell division stimulation in *Hymeniacidon helicophila* culture following media supplement with phytohemagglutinin.

For cell viability, a number of cell manipulation steps have been suggested (Zhang et al. 2004). Zhao et al. (2005) also reported increased cell viability when culture media were supplemented with a combination of pyruvate, vitamin C and sodium chloride (NaCl). Insulin and the agglutinin. *Ulex europaeus* are also useful for the enhancing cell viability in

culture media (Pomponi and Willoughby 2000). Another major requirement for succcesful sponge cell culture is the elimination of microbial contamination, thus the use of antibiotics cocktails (e,g Penicillin, Streptomycin, Ampicillin, Gentamycin etc.) in culture media is the most efficient method for contamination control in sponge culture. Some physical steps such as cleaning sponge tissues of all debries before prepping for culture and slow speed centrifugation of cell suspension (Pomponi 2006a) are proposed. (Rinkevich 2005) also reported that dissociation of cultured sponge aggregates into single cell suspension as an extra purification step which would greatly increase the chance of obtaining an axenic cell population.

1.13.4 Sponge cell dissociation

A number of methods have been employed to obtain sponge single cells by different studies; chiefly among these are chemical, physical and enzymatic methods (Cai et al. 2013; Pomponi 2006a) . Of all three methods the physical method is popularly reported; sponge tissues are cut into tiny cubes, briefly soaked in EDTA containing solution and then dissociated into single cells by squeezing through micropore nylon mesh or fine seive (Cai et al. 2013; Cai and Zhang 2014; Mussino et al. 2013). Sponge single cells are thereafter harvested as pellets following slow speed centrifugation. The slow speed centrifuge (usually between 300- 500 x g) is useful for ensuring the selective pelleting of denser cells in the freshly squeezed suspension which are mainly sponge cells. This step selects against less dense endosymbiontic cyanobacteria which are left in the supernatant and are discarded afterwards (Rinkevich 2005). To obtain monodispersed sponge single cells, the use of calcium magnesium free sea water supplemented with chelators for example, EDTA has been suggested (Custodio et al. 1998a). This is also needed to dissociate sponge cell aggregates into single cell suspension. However, great care must be taken in the choice of EDTA

concentration and the length of time sponge cells are left in EDTA containing solution, because EDTA and trypsin are known to have an inhibitory effect on sponge DNA synthesis and cell viability are also reportedly reduced following prolonged suspension for longer than 20 minutes in solutions containing chelators (Pomponi 2006a).

1.14 Experimental Design and the development of an "In vivo" exposure model

All investigations in this thesis, a method of disaggregating sponge cell aggregates, previously cultured from cryopreserved single sponge cells was developed. For investigations with *H perlevis,* aggregates were incubated as demonstrated in figure 1.9 with environmentally relevant model chemicals in the laboratory and thereafter investigated for response. Investigation of genotoxic impacts of field pollutants, was with mechanically dissociated aggregates of *Amorphinopsis kalibima* previously cultured from cryopreserved single cells. This experimental design was used for all other experiments in this thesis.

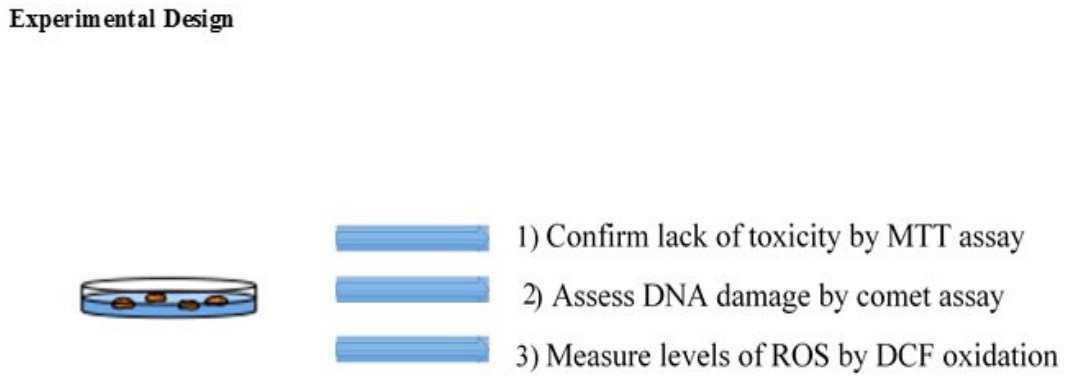


Figure 1.9 **Laboratory investigation of sponge cell cultures were exposed for 12 hours to non-cytotoxic concentrations of Cd, Cr, Ni, Al (0.1-0.4mg/L) and B[a]P (0-10** μM) as assessed by MTT viability assay. Field sponge cells were cultured without treatment and same assays were performed.

1.15 Overview of Niger Delta and chemical monitoring of pollution

The Niger Delta is Africa's largest delta, and third largest delta in the world next to American's Mississippi and Brazilian Pantanal (EKPO et al. 2012). It is a region characterized by vast expanse of mangrove and coastal forests which serve as rich sources for different plant products, fish, shellfish and timber; it is made up of 9 political states of Abia, Akwa Ibom, Bayelsa, Cross River, Delta, Edo, Imo, Ondo and Rivers State (Figure 1.10)

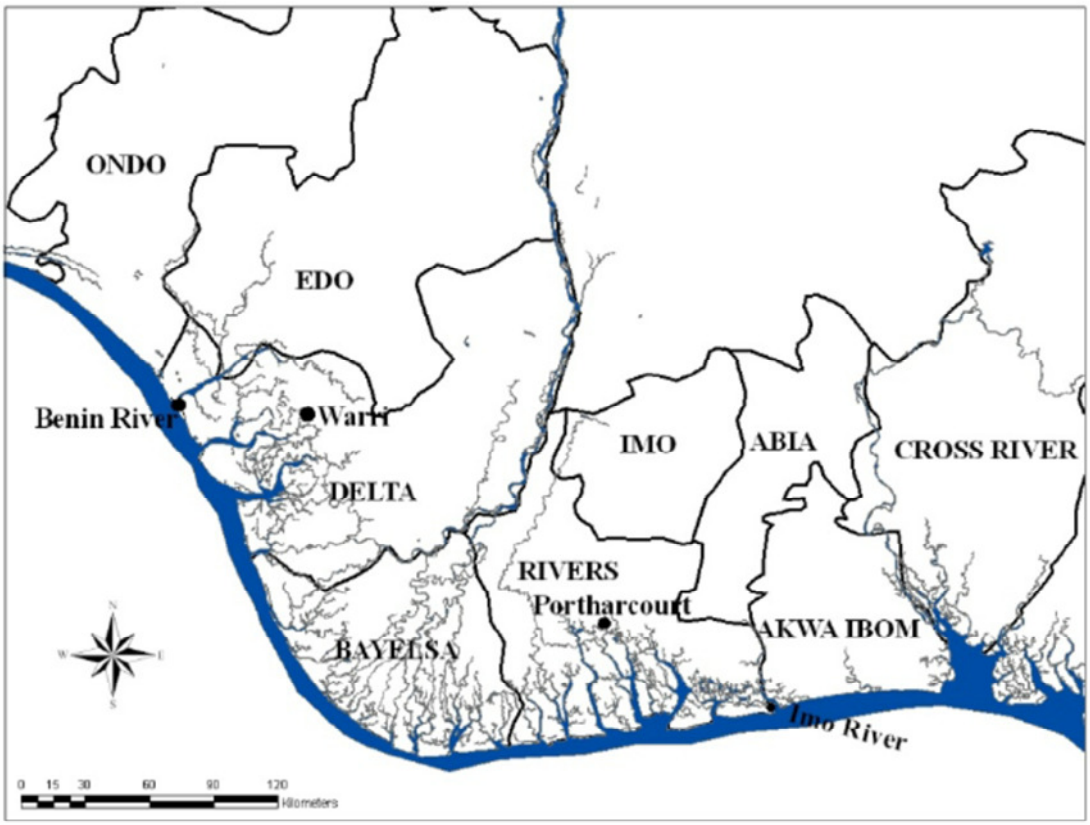


Figure 1.10 Niger Delta map showing the 9 political states. Reproduced from (Adegoke et al. 2010)

Because there are no established public sewerages in the Niger Delta, wastes are disposed of into drainages and the river, which eventually make their way into the creeks and are trapped in environmental matrices (Sediment, water column and biota) (Ajao and Anurigwo 2002). Pollution sources in this region are mainly associated with oil and gas exploration, agriculture and metal smelting activities, with most contributions being attributed to the oil and gas industries (Ajao and Anurigwo 2002; Iwegbue et al. 2018). Commonly reported pollutants in the region are hydrocarbons (Anyakora and Coker 2006; Olajire et al. 2005; Sette et al. 2013), heavy metals (Chindah 1998; Iwegbue et al. 2018; Obunwo and Cookey ; Olowoyo 2011; Otitoju and Otitoju 2013) and organochlorine (Chindah 1998; Ezemonye 2005; Oribhabor 2016; Sojinu et al. 2012) which are summarised in Table 1.6 for metals. In Forcados River (Niger Delta) for example, significant levels of Al(9560–25,000 mg/kg), Fe (9350–18,400mg/kg), Pb(11.5–72.0mg/kg), Cu(4.48–13.7mg/kg), Cd(0.78–2.16mg/kg), Cr(21.9–49.6mg/kg), Ni(4.75–11.7mg/kg), and Co(5.03–11.7mg/kg) were recently reported in sediment (Iwegbue et al. 2018). Along Bonny estuaries, Onojake et al. (2015) and Babatunde et al. (2013) have also reported Zn, Fe, Ni and Cr in Sediment, Biota and water column. Most report on metal pollution in the Niger Delta region are however, based on only data obtained from sediment, water column and biota mainly crustaceans and fish (Izah and Angaye 2016; Onojake et al. 2017). This is also in line with most ERA studies; whereby predictions and measurements of metal levels are directly compared with set regulatory standard, and this information is used to assess potential risks to the environment(Van der Oost et al. 2003). However, information on only metal and pollutants levels in the environment are no longer sufficient for assessing the risks associated with exposure to exogenous and toxic environmental chemicals (De Mestre et al. 2012; Lee et al. 1999; Martins and Costa 2014; Phillips and Rainbow 1993; Rainbow 2007; WHO 1993) .

Table 1.7 Heavy metal levels in Environmental Matrixes in the Niger Delta.

Matrix	Metals (mg kg−1 in sediment and biota ; mg/L in water)													Authors
	Cd	Pb	Cr	Ni	Cu	Co	Ba	Mn	Zn	Fe	Al	As	Hg	
Sediment (Forcados River)	0.78–2.16	11.5–72.0	21.9–49.6	4.75–11.7	4.48–13.7	5.03–11.7	2.04–3.34	153–545	11.7–35.1	9350–18,400	9560–25,000	NM	NM	(Iwegbue et al. 2018)
Sediment														(Fatoba et al. 2016)
• Kokori creek	NM	4.9-6.1	4.9-11.2	0.45-3.9	NM	NM	NM	NM	23.6-69	NM	NM	NM	NM	
• Kolo creek	NM	0.02-5.05	0.00-1.85	5.0-11.9	NM	NM	NM	NM	24.6-65	NM	NM	NM	NM	
Moss (Polytrichum juniperinum) and Lichen (Pamelia caperata)	0.001-0.092	0.001-17.380	0.004-8.793	1.425-21.730	2.350-110.760	0.989-1.950	NM	NM	23.5-130.6	NM	NM	NM	NM	(Ite et al. 2014)
Sediment (five sites in Benin river)														(Ogbeibu et al. 2014)
S1	NM	0.00-0.29	0.03-0.47	0.03-0.19	0.01-0.47	NM	NM	NM	0.22-3.71	NM	NM	NM	NM	
S2	NM	0.001-0.31	0.04-0.47	0.05-0.25	0.01-0.50	NM	NM	NM	0.32-3.94	NM	NM	NM	NM	
S3	NM	0.01-0.33	0.05-0.49	0.06-0.39	0.01-0.52	NM	NM	NM	0.32-4.14	NM	NM	NM	NM	
S4	NM	0.01-1.91	0.05-2.87	0.13-1.23	0.01-3.06	NM	NM	NM	0.34-10.90	NM	NM	NM	NM	
S5	NM	0.02-2.05	0.11-3.07	0.13-1.23	0.00-3.27	NM	NM	NM	1.61-11.66	NM	NM	NM	NM	

*NM: Not Measured

Table 1.6 cont'd Heavy metal levels in Environmental Matrixes in the Niger Delta.

Oron River (Akwa-ibom State														
Tympanatus fuscastus	**0.27**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**		
	NM	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**64.2**	**(Otitoju and Otitoju 2013)**
	NM	**NM**	**NM**	**0.011**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**	**NM**		
Metals in Water from Warri River	**0.00-0.05**	**0.00-0.001**	**0.00-0.06**	**0.00-0.32**	**0.00-0.26**	**NM**	**NM**	**0.002-0.68**	**0.00-0.63**	**0.03-5.02**	**NM**	**NM**	**NM**	**(Wogu and Okaka 2011)**
Metals in Sediment														**(Ekwere et al. 2013)**
• **Cross River sediment**	**1.85**	**56.51**	**0.97**	**7.03**	**NM**	**NM**	**NM**	**NM**	**108.77**	**NM**	**NM**	**2.05**	**NM**	
	1.78	**53.12**	**0.82**	**5.44**	**NM**	**NM**	**NM**	**NM**	**90.55**	**NM**	**NM**	**2.56**	**NM**	
• **Qualboe River Sediment**	**1.40**	**18.81**	**40.06**	**15.73**	**NM**	**NM**	**NM**	**NM**	**65.90**	**NM**	**NM**	**6.88**	**NM**	
• **Imo River Sediment**														

In this study, metal levels in sponge, sediment and water samples obtained from 6 sites in the Niger Delta and a control site in Badagry, Lagos state were independently analysed in three laboratories. DNA strand breaks as a biomarker of effects from metal exposure was measured in sponge cells from all sample locations using the comet assay technique. DNA strand breaks were correlated with metal levels in all matrices.

Although the comet assay has been used extensively in aquatic biomonitoring using both model and non-model organism (Table 1.1) here the application of the comet assay to study DNA strand breaks in the sea sponge *Amorphinopsis kalibiama* as a biomarker of metal toxicity is presented.

1.16 Aims and Objectives

1.16.1 Application of Comet assay in sponge cells to environmental regulations

The overall aim of this thesis was to deploy DNA strand breaks in marine sponges using the comet assay technique as a reliable biomarker for environmental biomonitoring of xenobiotics in the aquatic environment and as a potential tool in ERA.

Practical steps to achieving this would be to identify with interest groups both locally and internationally. For example, attendance at OECD expert meetings, networking and seeking collaborations with regulated assay validation groups. Other means of translating these findings to impact would be to submit draft proposal to environmental interest groups and local regulators in Nigeria, on the potential of this system to be adopted as part of test system and environmental biomonitoring tool and risk assessment. Possible interest groups are Oxford Policy Management (OPM) under the Facility for Oil Sector Transformation (FOSTER 2) project, National Environmental Standard and Regulation Enforcement Agency (NESRA), Niger Delta, Development Commission (NDDC) and The Department for Petroleum Resources (DPR). Considering that biomarkers are key components in ERA and are mostly relied on as evidence based tools for environmental regulations, the development of comet assay in sea sponges, which are important sentinel invertebrates is a major contribution to ecotoxicology .

Specific objectives of this project was to use the sea sponges *Hymeniacidon perlevis* (laboratory exposures) and *Amorphinopsis kalibiama* (field samples):

- To assess the amenability of the comet assay techniques on cryo-preserved single sponge cells.
- To test metal induced DNA strand breaks and reactive oxygen species formation in cryopreserved single sponge cells exposed to environmentally relevant heavy metals and investigate P450 protein metabolism in cultured sponge tissues; then test the genotoxic effects of B[a]P in laboratory sponge models.
- To measure levels of heavy metals in water samples, sediment, and sea sponges from polluted sites in the Niger Delta and then compare DNA strand breaks in untreated sponge cells form all Niger delta.
- To identify the UK and Nigerian sponge species used in this study and contribute to the sponge "barcoding" project (Vargas et al. 2012; Wörheide et al. 2007) and the NCBI GenBank.

Chapter 2 –

Materials and Method

2.1 Chemicals

All chemicals were purchased from Life technologies (Fisher Scientific) and Sigma Aldrich unless otherwise stated and were all of the highest available quality and purity.

2.2 Sponge collection and preservation

Sponges were collected for method development and subsequent experimental studies from two clean sites in Pembrokeshire, South Wales, UK: Manorbier Bay (**Latitude:** 51.6554° N, **Longitude:** 4.7983° W) and Tenby Bay Castle beach (**Latitude:** 51° 40' 22.04" N; **Longitude:** -4° 42' 16.09" W) between October 2014 and October, 2016 (Figure. 2.1 A) on exposed rock pools at low tide and seven contaminated sites from the Niger Delta, Nigeria between October 2015 and January, 2016 on exposed mangrove stomp at low tide (Figure 2.1 B). These were immediately transported back to the laboratory in aerated sea water and processed into single cells (Figures.2.2 & 2.3) and cryopreserved in vapour phase liquid nitrogen following previously described protocol with modifications (Mussino et al. 2013).

2.3 Single sponge Isolation and Cryopreservation

For single sponge cell isolation, EDTA-ethylene diamine tetra acetic acid containing calcium magnesium free sea water (CMFSW+E) and calcium magnesium free sea water (CMFSW) were prepared according to Cold Spring Harbor Laboratory Protocols (2009) recipe in Cold Spring Harbor laboratory protocols for CMFSW-E (450 mM NaCl, 9 mM KCl, 37 mM Na_2SO_4, 2.2 mM $NaHCO_3$, 10 mM Tris-HCl pH 8.0, 20 mM Na_2EDTA) and CMFSW (450 mM NaCl, 9 mM KCl, 37 mM Na_2SO_4, 2.2 mM $NaHCO_3$, 10 mM Tris-HCL pH 8.0). Sponge tissues were thereafter carefully cleaned of all debris and dirt, washed three times in filtered natural sea water and chopped into cubes (approx.1cm^3) with a sterile scalpel. Sponge cubes were transferred into 50 ml falcon tubes containing 40 mL CMFSW+E at a ratio of 1:5 (sponge tissue to CMFSW+E). The tubes were then placed on a rotor shaker (Rotator Labnet Orbit 1900) and allowed to shake gently at 40 rpm, first for 20 minutes at room temperature and then 60 minutes after discarding the initial CMFSW+E solution and refilling with fresh 40 mL solution. Using a 250 µm nylon mesh, single sponge cells were collected by squeezing the CMFSW+E soaked tissues into a 50 mL falcon tube and pellets obtained at 300 x g, after 7 minutes and the supernatant discarded. The resulting single sponge cell pellets were then washed three times with CMFSW and re-suspended in 1mL freezing media made of sponge media (made from 16.5 g instant ocean sea salt in 500 ml Ultra High Quality water -

according the manufacturers instruction described at http://www.instantocean.com), 0.2% RPMI (Roswell Park Memorial Institute medium), 1mg/mL PSG (penicillin, streptomycin, glutamine) solution, 0.1% v/v Pluronic® F-68, 10% v/v DMSO-dimethylsulfoxide (cryoprotectant), and 10% FBS (Foetal bovine serum) modified for sponge cells. 1 mL freezing re-suspended single cells were then aliquoted into corning cryogenic vial and stored in vapour phase liquid Nitrogen until needed.

Figure 2.1 Sponge sample collection at low tide. A: *H. perlevis* in situ on exposed rock pool in Tenby Bay castle beach, collected in October 2014. B) *A. kalibiama* in-situ on exposed mangrove stomp in Pokoikiri creek in Nembe/Brass Sea Bayelsa State Nigeria. Collected at low tide.

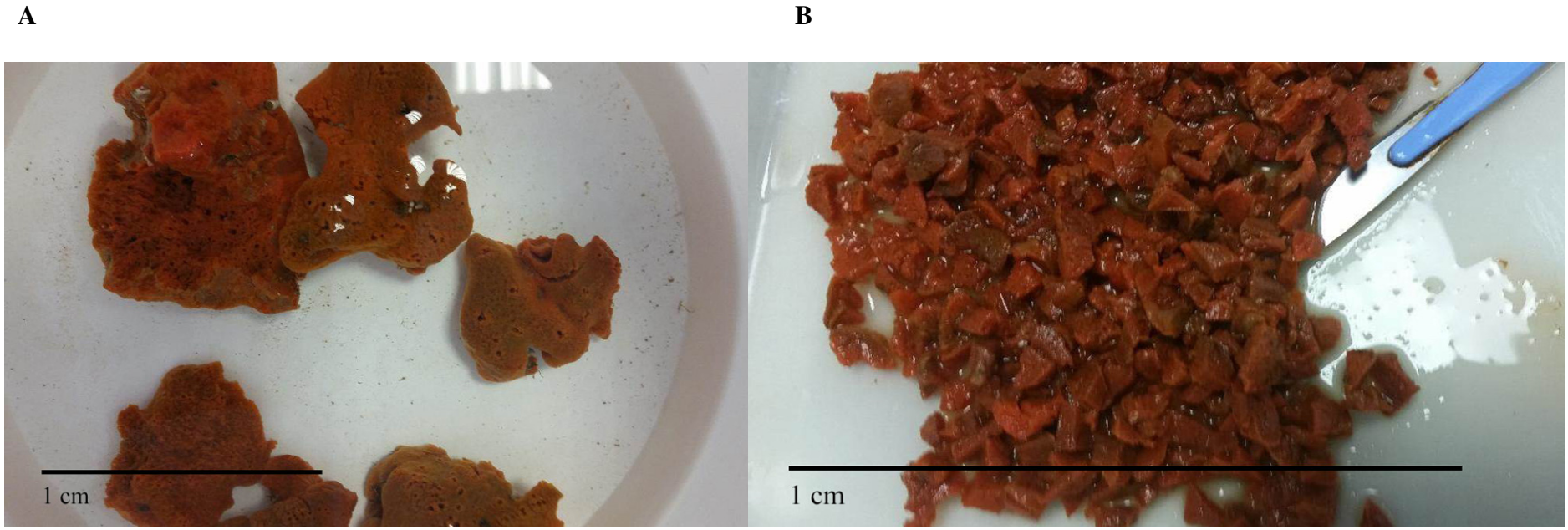


Figure 2.2 Freshly collected *H. perlevis* fragments A) Cleaned fragments B) Chopped cubes for single cell isolation. Fragments were mechanically cleaned using sterile scalpel to remove any debris and clinging epibionts like sea weeds and shell fishes before single cell extraction and subsequent culture for aggregate formation.

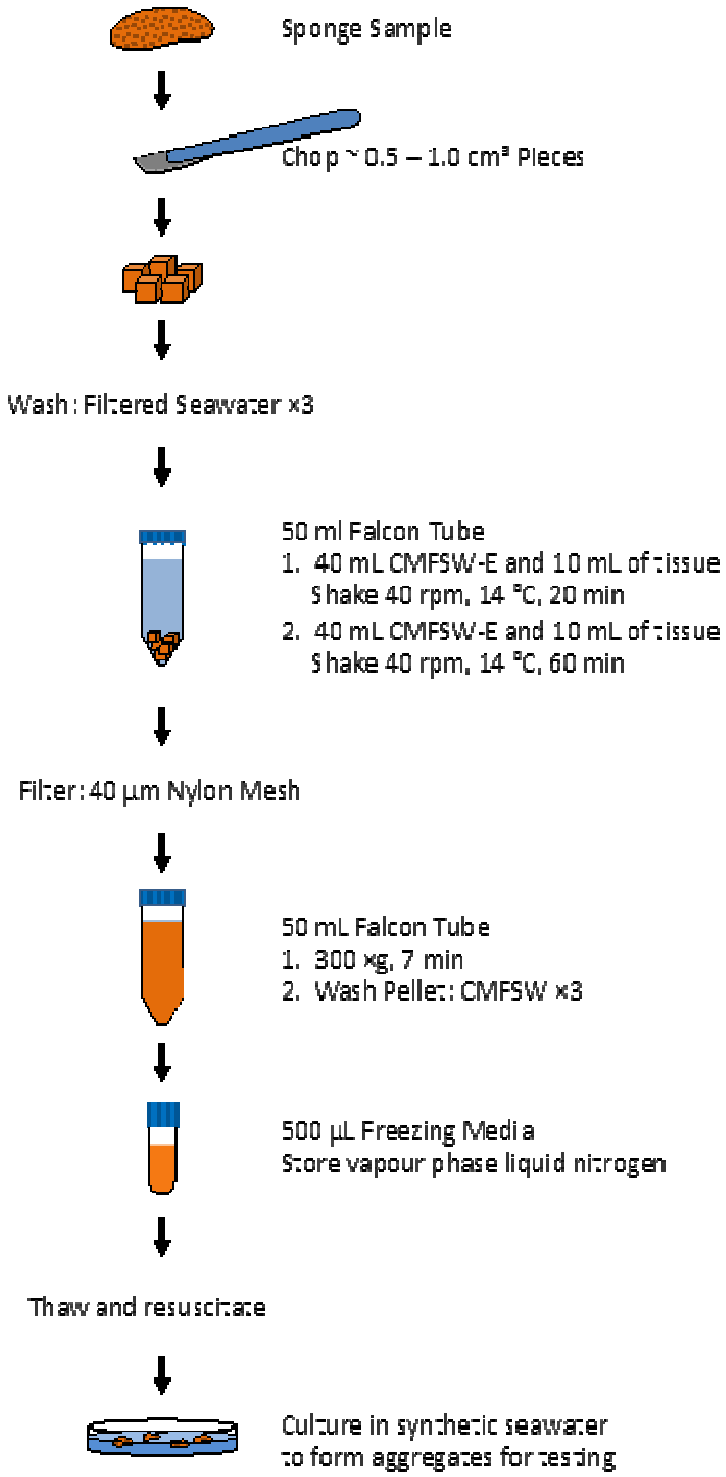


Figure 2.3 Sponge cell Isolation and Cryopreservation steps. Reproduced from Akpiri et al. (2017)

2.4 Systematic and Molecular speciation of *Hymeniacidon perlevis* and *Amorphinopsis kalibiama*.

Systematic identification of the type specimens were undertaken by two sponge taxonomist (Bernard Picton, National Museum, Northern Ireland and Dr Rob Van Soest, Naturalis Biodiversity Centre, The Netherlands) for *H. perleve* (UK species) and *Amorphinopsis kalibiama* (Niger Delta) respectively. Sponge sample identities were confirmed by molecular characterisation of both the cytochrome oxidase 1 gene and the 28S gene, using primers described previously in the sponge barcoding project (http://www.palaeontologie.geo.uni-muenchen.de/SBP/). Fragments of the type specimens are archived at the school of Biosciences, University of Birmingham teaching lab museum and at the Naturalis Biodiversity Centre, Marine Diversity Department, Leiden, The Netherlands.

2.4.1 Sponge genomic DNA extraction

Genomic DNA was extracted from 2 year old 70% ethanol preserved sponge tissues from Kalibiama creek in Niger Delta Nigeria and 3 months old 100% ethanol preserved Manorbier Bay samples using a DNeasy96 Qiagen kit (Thermo Fisher Scientific, UK). Briefly, 25mg of each sample was chopped into small fragments of about $1mm^3$ and placed in a 1.5 ml Eppendorf tube, 180µL ATL buffer and 20 µL Proteinase K buffer (30 mM Tris-Cl; pH 8.0; 30 mM EDTA (pH 8.0) ; 5% Tween 20; 0.5% Triton X-100; 800 mM GuHCl) were added and samples left to lyse on a heating block at 56°C, with periodic vortexing. Complete lysis was achieved after 3 hours and 45 min incubation. Samples were again vortexed and 200 µL AL buffer and 200 µL of 100% ethanol added and vortexed. The mixture was then added to a DNeasy mini spin column placed in a 2mL collection tube and centrifuged at 8000rpm for 1min, after discarding the flow through and replacing the collection tube, 500 µL of AW1 buffer was added and centrifuged for 1min at

8000rpm. The washing stage involved addition of 500 µL AW2 buffer after discarding the flow through above, centrifuged firstly at 8000rpm for 1min and then 14000rpm for another 3min. The flow through and the collection tube were both discarded at this stage. The column was placed on clean 1.5mL Eppendorf (lid labelled and detached) with the addition of 100 µL of AE (elution buffer: 10 mM Tris-Cl 0.5 mM EDTA; pH 9.0) and left to stand at room temperature for 1min, centrifuged for 1min afterwards at 8000rpm to eluate. The DNeasy mini spin column was finally discarded and the earlier detached lids replaced. The amount of genomic DNA was determined by measuring the A260/A280 and A260/A230 ratio in 2µL of sample extract with ND-1000 V3.8.1 NanoDrop and the integrity checked on a 1% agarose gel in x 1 TAE (40 mM Tris Base, 19 mM glacial acetic acid, and 1.3mM EDTA) with 0.98 µg/ml ethidium bromide stain in comparison with a 100bp genomic DNA ladder.

2.4.2 PCR DNA Amplification

To amplify the DNA extracts, a mixture of forward and reverse primers of both cytochrome C oxidase 1 gene and 2 sets of 28S genes were used. For Cytochrome C gene amplification, forward primer **dgLCO1490: GGT CAA CAA ATC ATA AAG AYA TYG G** with subsets (dgLCO1490**a**: GGT CAA CAA ATC ATA AAG ACA TCG G; dgLCO1490**b**: GGT CAA CAA ATC ATA AAG ATA TTG G; dgLCO1490**c**: GGT CAA CAA ATC ATA AAG ACA TTG G and dgLCO1490**d**: GGT CAA CAA ATC ATA AAG ATA TCG G) and reverse primer **dgHCO2198: TAA ACT TCA GGG TGA CCA AAR AAY CA** with subsets (dgHCO2198a:TAA ACT TCA GGG TGA CCA AAA AAT CA; dgHCO2198**b**:TAA ACT TCA GGG TGA CCA AAA AAC CA; dgHCO2198**c**:TAA ACT TCA GGG TGA CCA AAG AAT CA; and dgHCO2198**d**:TAA ACT TCA GGG TGA CCA AAG AAC CA) (Vargas et al. 2012) were utilised.

28S primers used were those of 28S-C2-fwd (GAA AAG AAC TTT GRA RAG AGA GT) and 28S-D2-rev (TCC GTG TTT CAA GAC GGG) (Erpenbeck et al. 2016; Vargas et al. 2012). Using *Taq* DNA polymerase (Thermo Fisher Scientific, UK) and the following PCR programmes; An initial 95^{o}C for 3 minutes denaturation step , 95^{o}C 30 seconds , 40^{o}C 30 seconds, 72^{o}C 1 minute , step 2 (95^{o}C 30 seconds) 35 times , and 72^{o}C for 5 minutes; *H perlevis* and *A kalibiama* DNA were amplified. Successful amplification was confirmed by running a 2µl aliquot of the PCR product on a 1% agarose gel and DNA bands visualized with UV light using ethidium bromide fluorescent dye. Visible bands were thereafter chopped out using sterile blade and approximately 10ng genomic DNA templates were purified using a QIA quick PCR Purification kit following the protocol provided by the manufacturer and thereafter submitted for sequencing at Birmingham genomics laboratory, University of Birmingham.

2.4.3 Phylogenetic analysis and Sequence alignment

Purified PCR amplicons were sequenced using Sanger dideoxy sequencing method with the same PCR primers and data checked against the NCBI GenBank Blast Algorithm tool. DNA sequence contigs were assembled and aligned and phylogenetic cladogram was traced using codoncode v.7.1.2. Data is deposited on the NCBI GenBank with the accession numbers MF685334, MG029167.1 and MF685333.1

2.5 Quantitative Analysis and Differentiation of endosymbionts and epibionts from Sponge cells.

To optimise the best protocol for obtaining sponge single cells and to differentiate between sponge cell nuclei and endosymbionts for correct scoring in the comet assay, Feulgan quantitative reaction using Schiff reagent staining was conducted. Single sponge cells were obtained from 1) re-suspended cryopreserved sponge single cells 2) ethanol preserved sponge tissue and 3) dissociated sponge aggregates. Feulgan reaction was performed according to the method described in Jeffery et al. (2013) and Hardie et al. (2002) with minor modifications. To quantify sponge cell population in cryopreserved single sponge cell suspension, 100µL aliquot of the 6mL stock suspension was added to microscope slides and left to air dry overnight. Approximately 0.5cm^3 of ethanol fixed tissue was chopped into cold PBS and left to hydrate for 20 minutes, afterwards it was held firmly against a slide and gently pressed across the slide to make a thin layer of cells. Cultured functional sponge cell aggregates-Primorphs (section 1.15.2 in chapter 1) were re-dissociated in single cell suspension with calcium magnesium free seawater containing EDTA (CMFSW-E); 100µL aliquot of the resultant cell suspension was added to a microscope slide and air dried overnight. All slides were fixed overnight in a mixture of methanol, formalin, and glacial acetic acid (85:10:5) after air drying. Slides were thereafter rinsed with warm water for 10minutes, hydrolysed for 2 hours in 5M HCl, and finally rinsed in 0.1M HCl before staining.

2.5.1 Schiff Staining (Feulgan raction) Differention of sponge cells from other symbionts

Feulgen reaction was performed following methods outlined in (Hardie et al. 2002). The reaction is an aldehyde specific reaction with an acid hydrolysis. In this reaction the Schiff reagent reacts with aldehydes in DNA to form a bright red product. One set of slides were stained with Schiff reagents. Briefly slides were placed in a Coplin jar containing Schiff reagent in a fume cupboard for 2hours. This was followed by 3x washing for 5 minutes with freshly prepared 0.5% sodium metabisulfite solution (10mL of 10% stock solution diluted 20x with distilled water) to decolourize and remove unbound stain), 10 minutes rinse in lukewarm water and another 3x rinse for 2minutes in distilled water. Slides were left to air dry and cells mounted using 19 mm circular cover slip and 100uL non-flouroscent hydro mount (Fisher Scientific), transparent nail varnish was used to seal up the coverslips which were left at 4^{o}C in the dark until analysed.

2.5.2 Image Analysis

Schiff stained images were acquired with the Zeiss LSM 710 Microscope using a 63x oil immersion objective and were analysed using imageJ software (1.48v, Java 1.6.0_20 [64bit]).

2.6 Viability assessment of cryopreserved sponge cells: Trypan Blue Exclusion Test

The trypan blue dye is an azo dye that typically stains dead cells, it is an established viability assessment dye in biological sciences (Hunger et al. 2005). Adopting the methods described by Mussino et al. (2013) with modifications, cryopreserved single sponge cell suspensions were assessed for viabilty using trypan blue exclusion assay.

2.6.1 Assay Procedure

Briefly, cryopreserved sponge cells were quickly thawed in a water bath at 37ºC and re-suspended in 5.5mL sponge media; sponge cell pellets were obtained by centrifuging cell suspension for 7 minutes at 300 x g. Pellets were then resuspended in 6mL (stock suspension volume used for all experiments in the thesis). Cell density was determined using a haemocytometer (Neubauer improved superior Marienfield, Germany) and viable sponge cells assessed using 1:10 dilution (30µL cell suspension mixed with 270µL 10 x Trypan Blue exclusion solutions) of resuspended cryopreserved sponge cells suspension. 100 µL of the resultant mixture was added to 5x5 hemacytometer chambers, allowing approximately 2 minutes incubation time.Viable cells were counted immediately in triplicates using 20x objective, average cell count per mililitre was then recorded.

2.7 Sponge cell culture for aggregate formation

All cell culture work was under aseptic conditions. All solutions and cell culture equipment used as well as sponge media were autoclaved before use.

2.7.1 Sponge Media development and optimization.

Sponge media was initially prepared with filtered natural sea water, 0.2% RPMI (0.2% Roswell Park Memorial Institute medium) and 1mg/mL of 10x penicillin-streptomycin-glutamine antibiotic cocktail (PSG) antibiotic, however natural sea water was subsequently replaced with 33mg/L instant ocean sea salt according to the manufacturer's specification.

To optimize the media for all experiments in this study, three sets of media with different constituents were investigated, following methods in literature (Pomponi and Willoughby 1994; Pomponi 2006b; Sipkema et al. 2003b)

1) Synthetic sea water (SSW), 2% PSG antibiotics and 0.2% RPMI
2) SSW , 2% amphotericin (10x diluted) and 0.2% RPMI
3) SSW, 2% PSG antibiotics, 0.2% RPMI and 0.1% pluronic F-68.

After initial optimization experiments, media 3 was utilised, as it was less prone to contamination and most suitable sponge cell culture.

2.7.2 Sponge cell Culture and cell density estimation

Procedures for aggregate formation utilised in all experiments in the entire projects, involved plating approximately 20 x 10^6 cells/ml in sterile T_{25} culture flasks which was then made up to a final volume of 6 mL with sponge media and left on a horizontal rotator shaker at 45 rpm at room temperature for up to 12 hours. The culture media was changed daily for the first three days, allowing aggregates to settle under gravity for approximately 5 minutes before carefully taking out 3 ml of the media and replacing with 3 ml of fresh media. In all cases sponge cells rapidly formed aggregates that maintained viability for more than 1 week in culture (as shown by MTT viability assay: Section 3.2.3 in chapter 3).

2.7.3 Effect of cell density on aggregate formation

Preliminary experiments show that cell density was a major determining factor for aggregates formation. To determine the number of cells needed for all assay in this thesis, cryopreserved single sponge cells were plated in sterile T_{25} flasks at different cell densities (10, 15 and 20 million cells) in a final volume of 6mL synthetic sea water (SSW). Culture flasks were then placed on a horizontal orbital shaker (Labnet Orbit 1900™ High capacity LabShaker) at 45rpm and incubated at room temperature; after 24hours in culture media, sponge cell aggregates were observed for both sponge species. Aggregates and possible primorph formation were monitored for up to 1 week using a Nikon eclipse TS100: ELWD 0.3/0D75 inverted microscope and camera.

2.7.4 *'In* vivo' exposure model:

Sponges are endowed with enormous amount of plasticity because of an aggregation factor on their cell surfaces (Müller et al. 1977); this alongside calcium and magnesium are responsible for the aggregation and re-aggregation of single sponge cells into functional aggregates. However, previous studies have shown that, with the aid of a chelator like EDTA, the aggregation factor is easily deactivated, setting individuals cells apart (Lavrov and Kosevich 2014). With this mechanism, a method for disaggregating sponge cell aggregates and primorphs into single cell suspensions was also developed. Using EDTA containing CMFSW cultured sponge cell aggregates were dissociated back into single cell suspension which was compatible with all assay in this thesis. Thus, this system (cultured sponge cell aggregates exposed to genotoxicants and dissociated into single cells for all bioassays) was used as an "in vivo" exposure model for all experiments including the comet assay and MTT assay for assessment of both biomarkers of effects and exposures to environmental xenobiotic.

2.8 Exposure of sponge cell model to known genotoxicants.

To test this model's suitability for assessing the effect of environmental xenobiotic on living systems especially in the aquatic media, sponge cell aggregates were initially treated with 0, 0.01, 0.1 and 1.0 mg/L $CdCl_2$ in a six well plate at room temperature, and 300µM hydrogen peroxide was used as positive control. This was a pilot study to measure cadmium induced DNA strand breaks in sponge cells and to determine the amenability of comet assay with the 'in vivo' exposure model.

Subsequently, approximately 20 million single sponge cells were cultured for aggregate formation and were thereafter exposed to varying concentrations of environmentally relevant

xenobiotics under laboratory condition for 12 hours. In a minimum of three experimental repeats each, aggregates were exposed to 0 mg/L, 0.1 mg/L, 0.3 mg/L and 0.4 mg/L Cadmium, Nickel and Chromium. Other exposures were 0 mg/L, 0.1 mg/L,0.2m/L, 0.3 mg/L and 0.4 mg/L Aluminium chloride and 2.5µM, 5 µM, and 10 µM Benzo[a] Pyrene with 1% DMSO as negative control. All experiments were conducted at room temperature in 12 well Corning Costar plates placed on a gentle horizontal shaker set at 45 rpm. Adopting the same protocol, untreated cryopreserved sponge cells from seven polluted sites in the Niger Delta, Nigeria were defrosted and cultured for 12 hours for aggregate formation prior to analysis.

2.8.1 Single Sponge cell prep from cryopreserved single cells for all assays

Cryopreserved single sponge cells were cultured in artificial sea water as previously described for aggregate formation; briefly, aggregates were removed from culture plates, washed with 1 x 5 ml fresh media and excess liquid was carefully removed by gently dabbing on dry paper towel. Next sponge cell aggregates were disaggregated into single cell suspension using CMFSW+E and completely dissociated sponge cell suspension were transferred into clean Eppendorf tubes and centrifuged at 8000 rpm (on a bench top Sanyo Gallen Kemp Micro centaur) for 7 minutes. The cell pellet was then re-suspended and used for downstream assays as described below.

2.9 3-(4, 5-dimethylethiazole-2-yl)-2,7-diphenyletetrazoliumbromide (MTT) viability assay of Xenobiotic treated sponge cell aggregates.

MTT is a water soluble tetrazolium salt, which is reduced to blue formazan by the enzyme mitochondrial succinate dehydrogenase within the mitochondria of living cell (Zhang et al. 2004). The blue formazan product is insoluble and forms an insoluble purple solution when dissolved in Dimethyl sulfoxide (DMSO). The purple formazan product is impermeable to the cell membrane so it accumulates in healthy cells and as a colorimetric assay, measurement of the intensity of the absorbance at wavelength range of 500-590nm gives the measure of cell viability. High absorbance intensity measure indicates more viable cells and low intensity is an indication of less number of viable cells.

Following 12 hours laboratory exposures of *H perlevis* sponge cell aggregates to different concentrations of benzo[a]pyrene, aluminium chloride, nickel chloride and sodium dichromate (VI), MTT viability assay was performed to investigate the cytotoxic effects of the test concentrations on sponge cell aggregates. Same protocol was adopted to assess the viability of cryopreserved untreated field samples of *A. kalibiama* sponge cells after 12 hours in culture. All exposures were done in triplicate for each compound.

2.9.1 MTT Assay procedures

Single sponge cell pellets from section 2.8.1 were washed 3 x 5mL with CMFSW (to remove the EDTA) and then re-suspended in 1mL 0.5 mg/mL MTT in sponge media. All cell suspension were then transferred into 6 well plates and incubated at 37^{o}C for three hours. After incubation, well plate contents were transferred into appropriately labelled Eppendorf tubes and centrifuged again at the same speed and time as before and the resulting pellets

were suspended in 100 µL DMSO. MTT reduction of the DMSO solubilized cells was then visualized at 570 nm absorption with infinite 200 Pro spectrophotometer against a 100 µl DMSO blank in 96 well corning transparent flat bottom plates.

2.10 Standard Alkaline comet assay protocol

The comet assay protocol involves five basic steps (slide preparation, lysis, electrophoresis, neutralization and staining) with an optional FPG-modification step to the lysis stage (Figure 1.4, Chapter 1). The comet assay can only be performed with single cell suspension; thus to process both plant and animal material for comet assay, they must be dissociated into first into single suspension. Single eukaryotic cells are mixed molten agarose (low melting point agarose) and are then added onto a pre-coated air-dried microscope slides (coated with a thin layer Normal melting point agarose gel). Thus, the cells are embedded (trapped) in a 'gel within a gel' and are lysed in a strong salt solution (lysis buffer) to get rid of both cytoplasmic and nuclear membrane and all other nuclear/cytoplasmic materials leaving just the DNA, proteins and RNA (Collins 2004). The electrophoresis circle involves the unwinding of broken or damaged DNA strand from the relaxed super coiled double helix and migration of same in the presence of electric field towards the anodes in a strong alkaline (pH ~ 13 or more) solution. Slides are neutralized by washing with neutralization buffer. In the migration stage, broken strands are stretched within the gel towards the anode forming a loop in the form of a comet, hence the name 'comet tail' and the assay comet assay.

The migrated comet tail remains attached to the nuclear core ('comet head') and can be visualized by staining with a fluorescent dye; DNA content in the nuclear core (comet head) and in the comet tail are analyzed by fluorescent microscope using the comet IV software. 50-100 fluorescent comets are scored and different parameters such as percentage tail intensity

and percentage head intensity, tail length, tail moment, head length, and tail migration are measured as genotoxicity endpoints (Bright et al. 2011; Duez et al. 2003).

Percentage tail intensity is however, currently adopted as the most suitable and appropriate endpoint for accessing genotoxicity by the comet assay. At the international workshop on genotoxicity testing (Burlinson et al. 2007), percentage tail intensity results from different laboratories and studies were compared and contrasted with other measurements and were found to be consistent and reproducible in all the results. Unlike other genotoxicity endpoints or parameters accessed by the comet assay; the results also showed that large amount of data can be generated by image analysis of percentage tail intensity using the comet IV software. Most importantly is the positive correlation between percentage tail intensity and DNA damage (Burlinson et al. 2007; Collins 2004; Hartmann et al. 2003). Measurements of Percentage tail intensity ranges from 0-100 %, with 0% representing undamaged nuclear DNA; without any migrated loop/tail and 85-100% measurements represents completely damaged nuclear DNA without any nuclear core, but only completely diffused and extended tail known as *'hedgehogs*' are observed.

Cells with only *'hedgehogs*' are considered non-viable or dead; and are therefore not accounted for in comet scoring for statistical analysis (Bright et al. 2011).

2.10.1 Alkaline comet assay with modification for use with sponge cells.

In this project , the standard protocol for comet assay as outlined by Singh et al. (1988) was adopted with modifications. The electrophoresis buffer was maintained at approximately pH 13 and chilled to almost freezing each time to retain a cold temperature throughout the electrophoresis process. DNA unwinding times between 20-45minutes and electrophoresis times between 20-40 minutes were investigated to obtain an optimal unwinding and

electrophoresis times amenable to sponge cells. Also exposure times between 1-12 hours to known genotoxicants and oxidizing agents were also investigated. Significant DNA tails were observed in all instances following increased time for all assay stages including exposure, unwinding time and electrophoresis. Beyond 30 minutes electrophoresis, cells exposed to high concentrations up to 1mg/L were however immeasurable with much diffused DNA tails which were mainly 'hedgehogs' (Figure 3.9, Chapter 3). This is in agreement with the report of Nacci et al. (1996); exposure of oysters (*C. virginica*) to 500µM H_2O_2 resulted in immeasurable DNA tails. Optimal conditions were 45 minutes DNA unwinding time, 30 minutes electrophoresis time and 12 hours exposure time point to xenobiotics.

2.10.2 Assay Procedure.

Briefly, Cell suspensions obtained as described in session 2.8.1 were pelleted on a bench top Sanyo Gallen Kemp Micro centaur centrifuge at 8000 rpm for 7 minutes; supernatants were discarded and pellets re-suspended in 100µL CMFSW without EDTA. 15µL of CMFSW suspended cells aliquots (in duplicates) were mixed with 150µl of 0.5% molten low melting point agarose (LMPA) in PBS and added to previously coated (with 0.5% w/v normal melting point agarose in phosphate buffered saline (PBS) microscope slides. These were covered with cover slips and placed on a cold metal block for a minimum of 20 minutes to allow the gel to set. After 20 minutes cover slips were carefully removed from the slides (gently slided off horizontally) and slides tranferred into previously chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% sodium N-lauryl sarcosinate, 10% dimethylsulfoxide, 1% Triton X-100, 10 mM Tris, adjusted to pH 10.0) in coplin jars for 1 hour. To achieve maximum cell lysis, coplin jars with lids containing slides and buffer were submerged in ice in an ice box, covered with aluminum foil and left in the cold room at 4°C in the dark. Afterwards, slides were transferred

into a horizontal electrophoresis tank model: GSA/VA FisherBiotech™ Horizontal Electrophoresis Systems, containing previously chilled (to freezing in -20°C freezer for maximum cooling) electrophoresis buffer (80 mL: 0.3 M NaOH, and 12 mL :1 mM EDTA, made up to a final volume of 2400 mL with UHQ water, pH >13.0). An unwinding time of 45 minutes was allowed without any power, for damaged/ relaxed DNA strands to unwind from the double super coil DNA helix; next 300 mA current at a voltage of 32V was applied to the electrophoresis setup for 30 minutes. Slides were thereafter neutralized with a 400 mM Tris HCL (neutralization) buffer at pH 7.5 by washing three times for 5 minutes; stained with 50µ sybr gold (Invitrogen) fluorescent dye (1 µl in 1000 µl neutralization buffer) and cover slipped. Slides were then left in a moist box in the dark in the cold room overnight. Images were visualized with fluorescent microscope using X 40 oil immersion objective and analysed with comet IV software. Statistically analysed median value of the percentage-mean-tail-intensity of 50 comet scores per slides were utilised as the genotoxicity end point (Duez et al. 2003).

2.11 DCFH-DA Assay for the measurement of Reactive Oxygen Species (ROS)

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) is an excellent fluorescent probe dye, for determining cellular antioxidant status. The principle involves the hydrolysis of the dye by cellular esterases to H_2DCF; which is non-fluorescent and impermeable to the cell membrane. In the presence of reactive oxygen radicals however, especially H_2O_2 (intracellularly produced in response to xenobiotic exposure or other oxidizing agents); it is oxidised to DCF which is a highly fluorescent, and can be measured between an excitation wavelength of 480 and emission wavelength of 520nm. The fluorescent intensity of DCF is proportional to the amount of ROS produced and it is equivalent to the level of oxidative stress induced by the exposure.(Carini et al. 2000; Mao et al. 2012)

2.11.1 Assay procedure

Following the steps in figure 2.4, sponge aggregates were incubated at room temperature for 12 hours in 6 well plates, with 0.4 mg/L, 0.3mg/L, 0.1mg/L and 0mg/L Cadmium. After incubation, aggregates were taken out of cadmium solution and re-suspended into single cells with CMFSW containing EDTA; 100µL of single sponge cells were then mixed with 1mL aliquot of 3µL 100µM 2,7, dichloro fluorescein diacetate cellular reactive oxygen species and 1mL RPMI mixture in 6 well plate. Plate was wrapped in aluminium foil to protect from light and incubated at room temperature on the bench for 1 hour, sponge cells were thereafter digested in 100µl of 10 mM NaOH and pelleted on a bench top centrifuge at 8000 rpm for 5 minutes; supernatants were carefully aliquoted into 96 well plates and fluorescence measured at 485 nm excitation, 535 nm emissions and 10 nm band width with 200 pro infinite spectrometer.

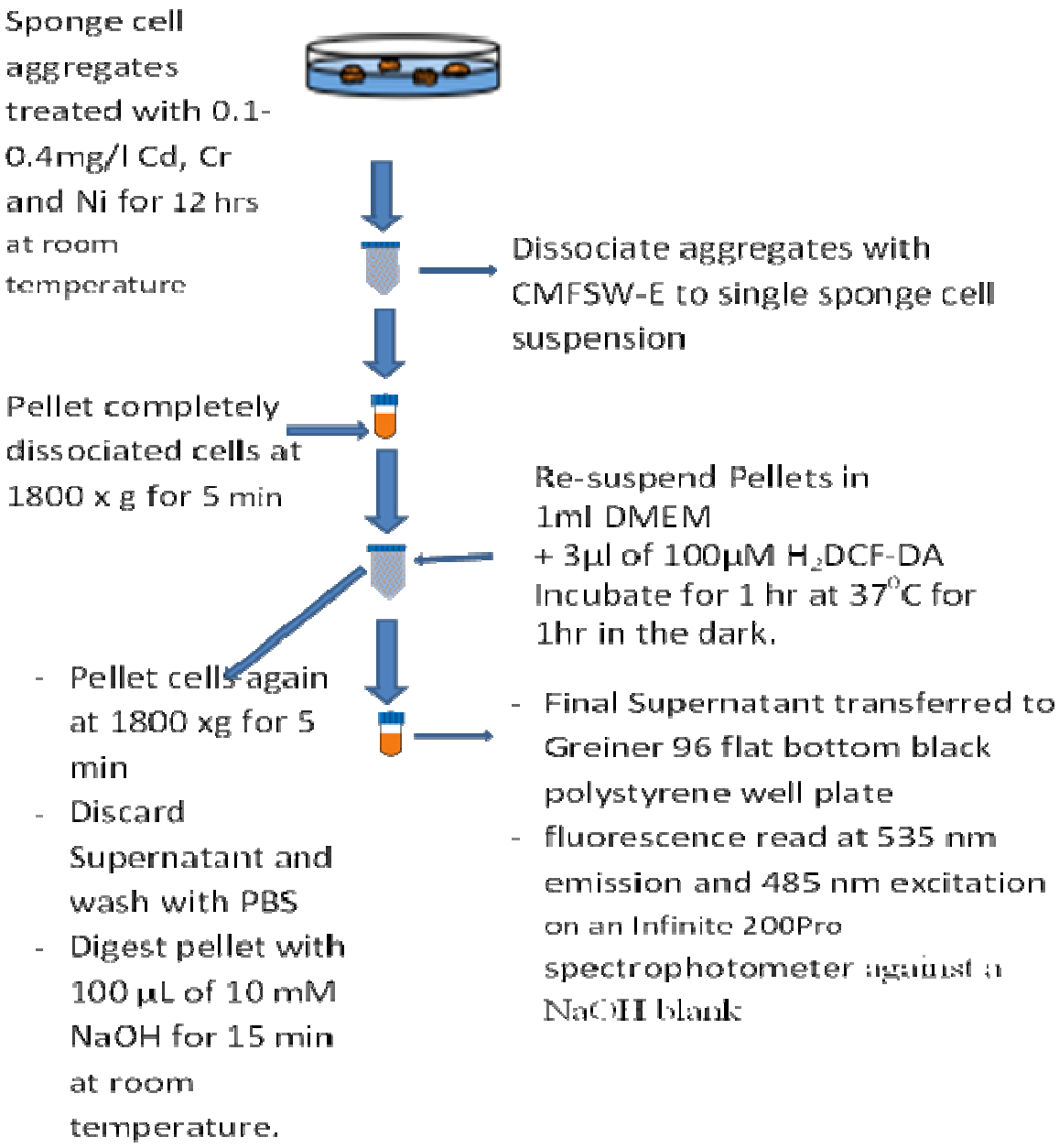


Figure 2.4 Assay procedure for the measurement of reactive oxygen species in sponge cells exposed to environmental xenobiotics

2.12 Inductively Coupled Plasma Mass Spectrometry (ICP-MS): Measurement of Cadmium, Nickel and Chromium uptake in sponge cell aggregates.

Metal bioaccumulation in sponge cell aggregates from previous exposures was assessed by ICP-MS following published protocols (Cebrian and Uriz 2007b; Kingston and Walter 1988). Aggregates were washed three times with distilled water after exposure and kept in the freezer until required. When required, aggregates were defrosted on the bench at room temperature and the weights measured with fine scale analytical balance (Metller Toledo ^{TM} NewClassic ME Analytical Balances). Aggregates were digested in accordance to set standards (Mangum 2009) with 2.5 ml 70% HNO_3, 500 µl 37% v/v HCL and 660 µl 37% v/v H_2O_2 in MARS-5 600115 Rev. 0 microwave CEM Corporation. Sample digestion was for 6 minutes process time and 10 minutes holding time both at 180^0C , P=1200 W. Uptake were measured against 6 standards (10 ppm, 5 ppm, 2.5 ppm, 1.5 ppm, 0.5 ppm and 0 ppm) prepared from a VWR 1000 mg/L stock solution with an ex-demo Perkin Elmer NexION 300X ICP-MS. Cadmium uptake was however measured with the Agilent 7500ce ICP-MS (inductively coupled plasma optical emission-mass spectrometer) operated in standard mode and ^{114}Cd isotope measured using ^{115}In as internal standard, with Calibration standards (0–100 µg/l).

2.13 Sponge, sediment and water samples collection from polluted sites and measurement of metal levels in field.

Water, sediment and sponge samples were collected from 6 sites in the Niger Delta and a control site in Badagry, Lagos between the periods of October 2015 and January, 2016. Location are in table 2.1 and Figure 2.5. Location A, topo creak in Badagry is an open beach, the soil from this beach is only silt and fine sand unlike those collected from the Niger Delta, that were organic rich mud which are mainly clay. Badagry was chosen to serve as the control site.

Table 2.0.1 Sampling locations along the Niger Delta coast, Nigeria

Locations	Location name	Geographical coordinates
L_A	**Topo-creek, Badagry, Lagos.**	**6°26'31.7''N 3°09'12.9''E**
L_B	**Kalio-Ama Creek, Okrika**	**$4^0$45′30.6″N $7^0$04′48.0″E**
L_C	**All-Marine Creek, Ologbbogbo**	**4°46'17''-- 4°46'02''N 7°01'18''-- 7°01'43'' E**
L_D	**Kalibiama creek, Bony Island**	**$4^0$44′26.8″N $7^0$00′10.9″E**
L_E	**Ebgomu River, Andoni**	**4°33'06'' N 7°23'50''E**
L_F	**Isaka creek, Eagle Island**	**4°43'58.9''N 7°00'22.8''E**
L_G	**Pokokri creek/ Nembe_Brass sea**	**4°44'18'' N 6°59'19'' E**

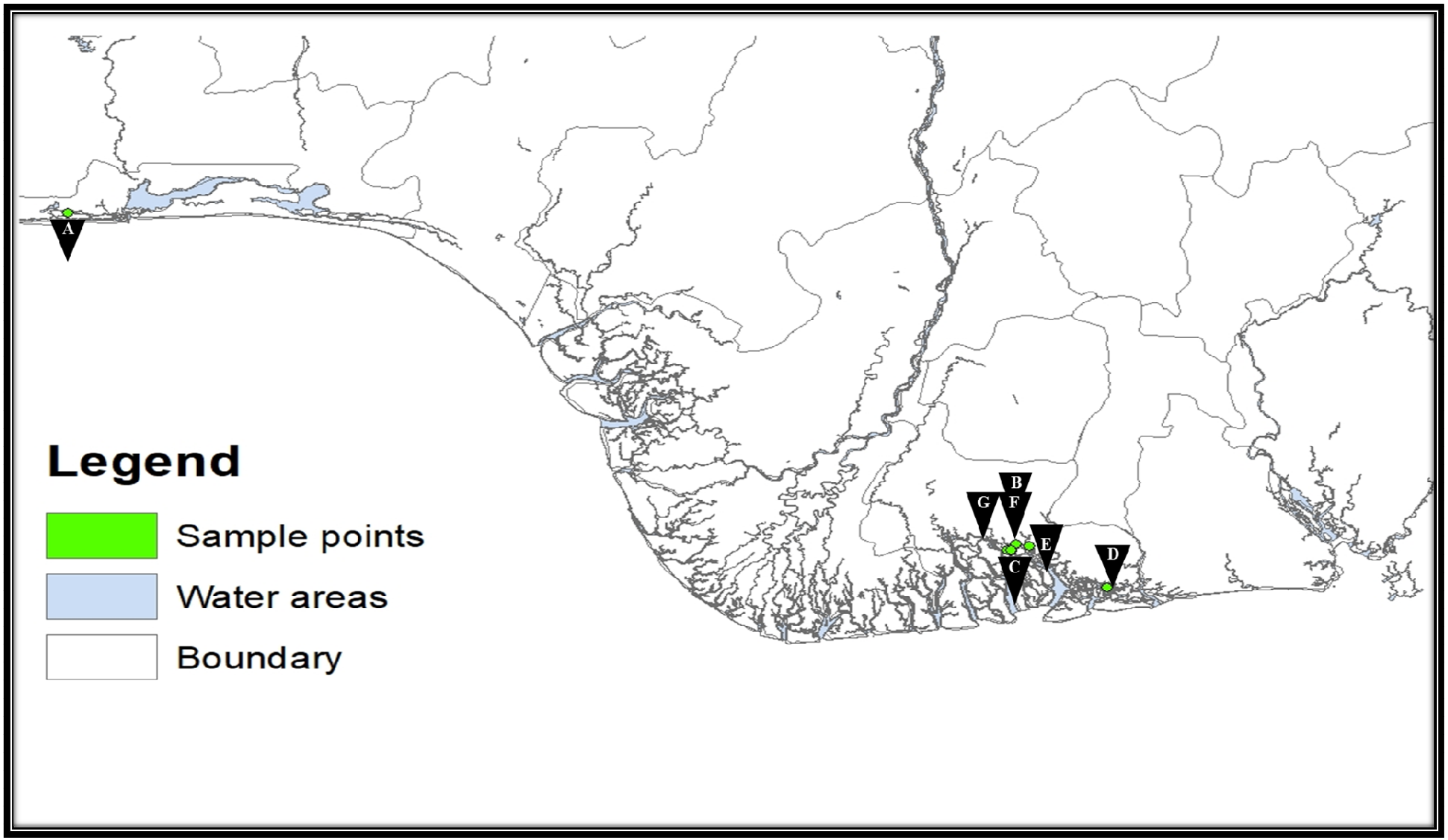


Figure 2.5 Map of Niger Delta showing study sites. Letter names in table 2.1 above

All samples were collected by hand at low tide between 0.1 - 0.3m. Sponge tissues were scraped off mangrove stomp (Figure 2.1b) using sterile scalpel, and were placed in seawater. Sediment and water samples were also collected from the same area as the sponge tissues. All samples were collected in triplicates, sediment samples were collected into transparent zip lock bags, and all samples transported back to the laboratory within 4 hours of collection in aerated sea water.

2.14 Statistical Analysis

IBM SPSS Version 22.0 and graph pad prism version 7 were utilised for all data analysis in this thesis. All data were normalised using Shapiro- Wilk's test and homogeneity of variance was determined using Lavene's test. Mean difference between samples was measured using one way and two way Analysis of Variance with Bonferroni multiple comparison correction. For non-normally distributed data, Kruskal-wallis non-parametric test was used to estimate mean difference between test samples and control. Reported values were mean ± SEM (standard error of mean) at P values of ≤ 0.05.

Chapter 3 Development of the Comet assay using cryopreserved *H. perlevis* sponge cells: Potential tool for Environmental Biomonitoring and Risks assessment

3.1 Introduction

3.1.1 Sessile invertebrates and Biomonitoring studies

The usefulness and application of sessile invertebrates in environmental biomonitoring, stems from the fact they are unable to escape pollution sources in their natural environment. They also have the potential to serve as sentinel species and act as surrogates to higher organisms. As a result of the continuous exposure to pollutants, sessile biogenic epifuana have evolved and developed defence and protective mechanisms such as biotransformation of pollutants to other chemical forms and detoxification (storage)(Berthet et al. 2005a) . In contrast to vertebrate sentinel species, whose major detoxification mechanisms are via the production of metallothioneins, invertebrates' biomonitoring species have different pollutant detoxification pathways. Sessile invertebrates accumulate a wide range of pollutants from the environment, which are either metabolised, detoxified or bio-transformed, hence they provide information on both bio-accumulated and bioavailable content of pollutants and their effects at the molecular level, thereby, constituting useful tools for profiling the health status of their immediate environment (De Mestre et al. 2012). Heavy metal pollutants, for example are removed by intra-cellular molecules such as metallothionein, lysosomes or they are precipitated into ionic or organic hard balls (Berthet et al. 2005b; Livingstone 1998). Induction of cellular GSH by sentinel invertebrate in response to environmental pollutant and conjugation of intermediate metabolic electrophiles of organic pollutants are the main mechanistic route for invertebrates pollutant detoxification (De Flora et al. 1995).

Sea sponges, which are sedentary and cosmopolitan in almost all regions of the globe, have in recent studies gained significant attention as biomonitoring tool (Perez et al. 2003); the

mechanism of biotransformation of pollutant in sponges is mainly via chemical detoxification (stored in nontoxic form) rather than xenobiotic metabolism. Multiple xenobiotic resistance (MXR) is another prominent defence mechanism against environmental pollutants in sea sponges (De Flora et al. 1995). Very high concentrations (>0.25mg/g dry sponge weight) of cadmium, mercury, copper, silver and zinc were reported in the mediteranian sea sponge; *Spongia officinalis* with significant positive correlation between metallothionein induction and the levels of mercury, copper and zinc (Berthet et al. 2005a). Also, (De Flora et al. 1995) reports three evidences of MXR in two sponge species; *Geodia* and *Tethya* S12 fractions, such as drastic reduction in the mutagenic potential of 4-nitroquinoline 1-oxide and $Na_2Cr_2O_7$, Depletion of GSH following exposure to buthionine sulfoxime, activation and storage of large amounrt of GSH in response.

Other important features of sea sponges which are very useful for environmental biomonitoring are their ability to pump large volumes of water containing particulate matters, serving as host to a wide range of microorganisms especially bacteria and most importantly their ability to accumulate varying range of environmental xenobiotic and the mechanisms to retain them for an extended period of time (Berthet et al. 2005b; Cebrian et al. 2007). Several studies have also reported the expression of biomarkers of stress such as heat shock proteins, DNA strand breaks, and DnaJ-like ptotein (Molecular chaperones and specific regulators of Hsp70) (Berthet et al. 2005b; Koziol et al. 1997a; Roberts et al. 2008). These stress response mechanisms and genes found in sponges, which have also been reported in higher organisms suggests that sea sponges like other sessile biogenic invertebrates serve as surrogates for higher metazoans and are therefore ready tools for indicating the health status of their immediate aquatic environment.

3.1.2 Sponge cell culture

Sponges have mixed cell population and in many cases sponge cell suspensions also include cells of other epibiotic organisms, this makes quantificication of single sponge cells in bioassays challenging as a result of background noise from non-sponge materials . They therefore require extra purification steps in order to obtain a homogeneous sponge cell population in suspension (Cai et al. 2013) especially for sensitve bioassays. Also, because of the multiple cell types, there is no particular section of the sponge tissue where aseptic inoculum can be obtained, thus extra steps are required to eliminate as much as possible associated symbionts and non-symbionts in sponge cell culture. (Cai et al. 2013; Cai and Zhang 2014; Rinkevich 2005). Sponge cell enrichment, and careful choice of antibiotics, are also required steps in addition to basic cell culture requirement for successful sponge cell culture (Pomponi 2006a; Pomponi and Willoughby 1994). Because of this, current trend in sponge cell culture studies are now geared towards improving the primorph (functional sponge cell aggregates) sytem, and sponge media optimization. Presently, there are no commercial sponge cell line and this is a major draw back in sponge molecular research. However, the discovery of the ability of single sponge cells squizzed through cheese cloth or nylon mesh to re-aggregate back into tiny microspic unit that grows and remains vaible in culture has led to an increased interest in sponge cell culture primorph formation, also to minimise inter-species variability and time spent on field trips for sponge collection, cryopreservation of both single sponge cell susupension and aggregates is also widely adopted (Custodio et al. 1998b; de Caralt et al. 2007). Primorphs are functional sponge aggrgates with similar features as obtainable in adult sponge. Thus the primorph system (details in chapter 1), represnts a promising future for susutainable production of sponge biactive compounds, and environmental monitoring of pollutant exposures and effects (Custodio et al. 1998a; Pomponi

and Willoughby 1994). This system also serves to further purify the sponge single cell suspension from other epi and endobionts (Rinkevich 2005).

3.2 Aims and objectives

The aim of this study therefore, is to investigate best media and culture condition for sponge cell culture and to develop an '*in vivo*' exposure model (primorph: primary sponge cell aggregates) using cryopreserved cultured sponge cell aggregates to detect DNA damage in sponge cultures using the comet assay techniques.

3.3 Materials and Methods

3.3.1 Sample Collection and Processing

For method development, samples of *H. perlevis* (Figure 3.1) were collected at low tide from exposed rock pools and under rock boulders from two Pembrokshire beaches in Wales; Manorbier bay and Tenby Bay castle beach. Sponge samples are in abundance and nearer shore in Tenby Bay beach compared to Manobier Bay, where they are more sparsely distributed. Large rock boulders characterize Tenby bay Castle beach, which provides shades from direct sunlight at low tide for in situ species. Generally, sponges prefer the shades away from direct sunlight, hence their abundance on Tenby castle beach. Manorbier bay beach on the other hand is an open beach characterized by wide spread rock pool with almost no shade at low tide. Sponges on this beach tend towards the deeper waters making their distribution near shore sparse and sample collection more difficult. Hence, apart from the first sample collection, all other sampling was from Tenby Bay castle beach.

Single sponge cells were isolated and processed following established protocols (Mussino et al. 2013) as described in section 2.3 and figure 2.3, in Chapter 2. The resulting pellets formed were first assessed for viability using Trypan Blue Exclusion assay (Section 2.6) and thereafter cryopreserved in vapour phase liquid Nitrogen until required.

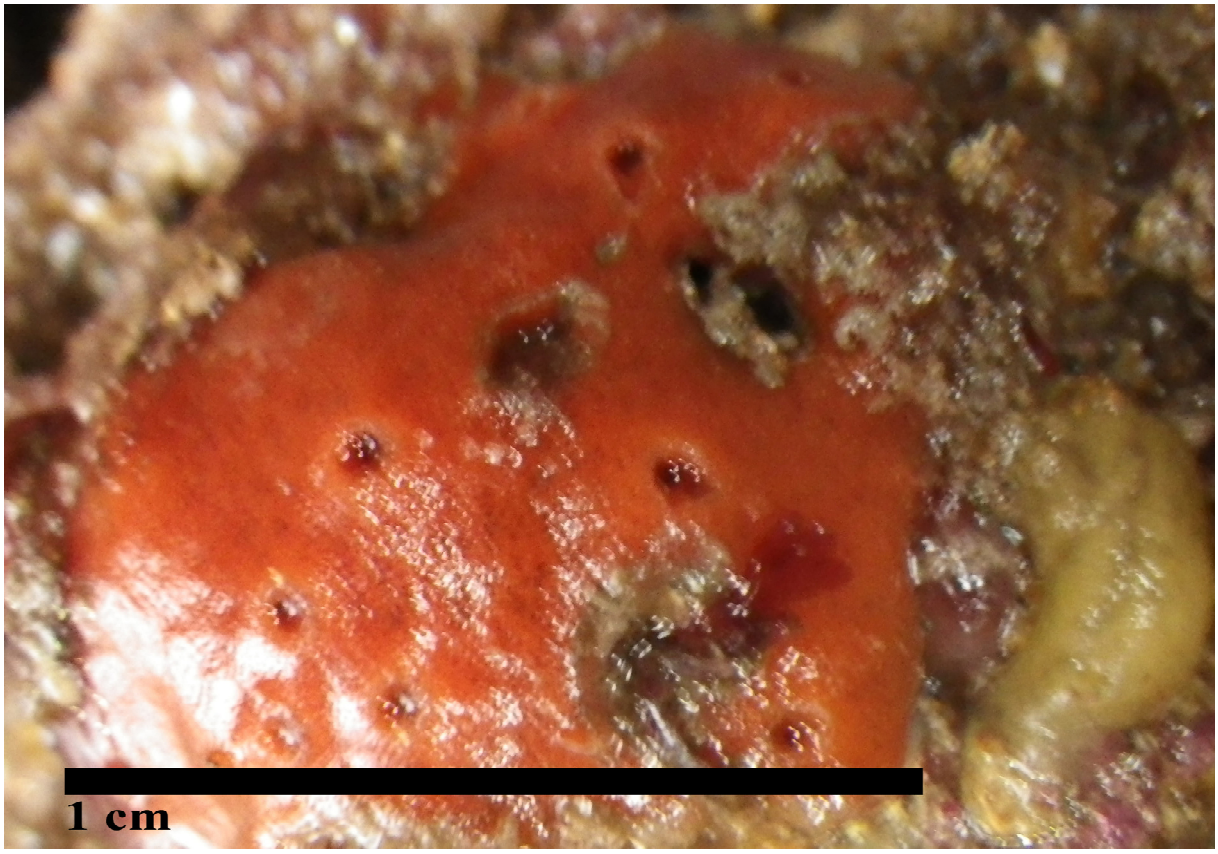


Figure 3.1 Sponge collection (H. perlevis) on exposed rock pool in Tenby Bay at low tide

3.3.2 Sponge cell culture and media optimization

Following investigation of different media constituents (Table 3.1), media 3 (3.3% Instant ocean in ultra-high quality water, 1mg/mL PSG cocktail, 0.2% RPMI and 0.1% v/v pluronic F-68) was adopted for future studies. In this media, cells remained viable as assessed by MTT assay (Figure 3.4) for more than 14 days in culture and there were no visible microbial growth within this period, which was a common observation in other media types investigated. Media 2 (3.3% Instant ocean in ultra-high quality water, 2% Amphotericin and 0.2% RPMI) however, was not suitable as amphotericin appeared to have a toxic effect on the cells including change in colour of culture media and aggregates from orange to green (data not shown). This is in agreement with the report of (Sipkema et al. 2003a), as they were unable to generate primorphs in culture media containing amphotericin. Media 1 (3.3% Instant ocean in ultra-high quality water, 1mg/mL PSG cocktail and 0.2% RPMI) was highly susceptible to microbial contaminations and fungal growth.

Table 3.1: Sponge culture media development and optimization

Media 1	Media 2	Media 3
➢ **3.3% IO (Instant Ocean salt)** (Hovanec and Coshland 2004) ➢ **1mg/mL PSG** ➢ **0.2% RPMI**	➢ **3.3% IO** ➢ **10mg/mL Amphotericin** ➢ **0.2% RPMI**	➢ **3.3% IO in ultra-high quality water.** ➢ **1mg/L PSG cocktail** ➢ **0.2% RPMI and** ➢ **0.1% v/v pluronic F-68 detergent** (To minimise shear effects from continuous shaking (Camacho et al. 2005)
PSG: Penicillin-Streptomycin-Glutamine (100X) RPMI: Roswell park Memorial Institute Medium1640		

3.3.3 Selection single sponge cells for throughput assays

In order to establish best source to obtain single sponge cell for all experiments, single sponge cell populations from two sponge cell sources were investigated using Feulgan reagents (Section 2.5.1, chapter 2) according to the methods outlined in (Hardie et al. 2002; Jeffery et al. 2013). Cells were isolated from freshly collected sponge tissues and from cultured aggregates (Section 2.7, Chapter 2).

A method for disaggregating sponge aggregates into single cell suspensions using Calcium Magnesium free seawater containing EDTA (CMFSW-E) which is compatible with the comet assay was thereafter developed. This was used as an 'in vivo exposure model' (Section 2.7.4, and 2.8, Chapter 2), for all experiments including the comet assay for assessment of both biomarkers of effects and biomarkers of exposures to genotoxicants.

To validate the amenability comet assay with sponge system and to test their response to a known genotoxicant, sponge cell aggregates were exposed to three concentrations of cadmium chloride, previously used by (Schröder et al. 1999) and viability of aggregates assessed using the MTT viabilty assay.

3.3.4 Assessment of sponge aggregate viability by the MTT assay

The MTT assay was performed to investigate the cellular effects of cadmium treatment (0, 0.01, 0.1 and 1 mg/l) on sponge aggregates. Briefly, following 12 hours treatment of sponge aggregates with cadmium chloride, aggregates were removed, washed with 1 x 5 ml fresh media and excess liquid was carefully removed by gently dabbing on dry paper towel. Next sponge aggregates were incubated with 100 µl of 0.5 mg/ml 1x MTT in six well plates at 37^{o}C for 3 hours. MTT reduction was observed by visual observation of the purple colour and

quantified by measurement of the absorption of DMSO solubilised (by gentle up and down pipetting) sponge aggregates at 570 nm in a corning 48 flat bottom transparent well plate, using an infinite 200Pro spectrophotometer against a DMSO blank.

3.3.5 Preliminary Comet assay experiment with resuspended cryopreserved sponge cells

. Cryopreserved sponge cells were quickly thawed in a water bath (Grant OLS200) at 37ºC and re-suspended firstly in 5.5 mL of sponge media 3. Cell pellets were then collected by slow centrifuge at 300 x g for 5minutes with a Bench top Centrifuge (Heraeus Multifuge 3SR Plus 75004375) at room temperature, and then finally re-suspended in 6mL sponge media. Sponge cells were then processed for comet assay following the standard protocol as described in Section 2.10, Chapter 2. Cell density appears to play an important role in sponge cell culture. To assess effective sponge cell density for the comet assay, sponge cell were serially diluted to a final volume of 1mL (5 $x10^6$ cells/mL) single sponge cell aliquot from the 6mL cell suspension stock by 90% ($5x10^5$ cells/mL) , 75% ($12.5x10^5$ cells/mL), 50% ($2.5x10^6$ cells/mL), and 0% ($5x10^6$ cells/mL) with sponge media

3.3.6 Non cyto-toxic concentrations of cadmium cause DNA strand breaks

Following the establishment of sponge cells amenability with the comet assay (Figure 3.7), subsequent attempts to replicate the assay in treated sponge aggregates was initially unsuccessful (aggregates were treated with H_2O_2, H_2O_2 + Fpg - formamido pyrimidine glycosylase protein, $Na_2Cr_2O_7$, and $Na_2Cr_2O_7$ +Fpg). Considering how crucial the electrophoresis step and the electrophoresis buffer are to the workability of the comet assay (Fairbairn et al. 1995); we modified the pair to optimised the use of the standard comet assay

protocol of Singh et al. (1988) in sponge cells exposed to known genotoxicants. First, following the report of Schröder et al. (1999) who observed maximum DNA damage by the fluorometric microplate assay in 12 hours 1mg/L cadmium incubated sponge cubes, we increased the exposure time of our '*in vivo'* model to 12 hours' time point instead of 1hour (previously used in previous unsuccessful trials). Extra filteration step following disagregation of treated sponge cell aggregates was also employed (Details in sponge cell preparation for comet assay section in chapter 2). Next, both the DNA unwinding time and the run time (See comet assay principle, chapter 1) were increased to 45 minutes and 30 minutes respectively, instead of 20 minutes in the standard protocol. Finally, the electrophoresis buffer was chilled to almost freezing each time and this was useful for maintaining a cold temperature in the buffer for the duration of electrophoresis. Consideration the extended 'run' time and the heat generated, lowering the temperature of the buffer would help to inhibit DNA repairs and also prevent the agarose gel on the comet slides from defrosting in the process.

3.3.7 Assessment of cadmium bioaccumulation by ICP-MS

Cadmium uptake by sponge aggregates was measured in triplicates by inductively coupled plasma mass spectrometry (ICP-MS) as previously quantified by (Cebrian and Uriz 2007a). Briefly, after treatment with cadmium as described above, sponge aggregates were removed from culture, washed in fresh media (5 mL) for 5 minutes and stored at -80^{o}C until required. Next, sponge samples (approximately 40–50 mg) were added to 2mL of 65% supra-pure nitric acid (HNO_3) and 660 µL of 37% v/v hydrogen peroxide in a Pyrex tube and samples digested at 95^{o}C for 20 h in a fume cupboard. Next 4 ml of ultrahigh quality (UHQ) water was added and the samples transferred to vials of known weight and weighed to determine the total

volume of the sample and correct for evaporative losses. The contents of the vial were diluted 1:20 dilution with 1% HNO_3, a blank solution was also prepared and all samples analysed for cadmium using an Agilent 7500ce ICP-MS (inductively coupled plasma optical emission-mass spectrometer) operated in standard mode. The isotope measured was ^{114}Cd, using ^{115}In as internal standard. Calibration standards (0–100 µg/L) were prepared from a VWR 1000 mg/l stock solution.

3.3.8 Systematic and Molecular speciation of *H. perlevis*

Following identification of the study sponge species as *H. perlevis* by systematic taxonomy (By Bernard Picton, Department of Natural Sciences National Museums Northern Ireland), Genomic DNA was isolated from ethanol preserved sponge sample. PCR amplification of the product was with a mixture of forward and reverse Cytochrome Oxidase C 1 and 28S primers. The presence of PCR amplicon in the extract was verified by gel electrophoresis (Figure 3.11B & Figure 3.12C) using 2% agarose gel. Sequenced data (Figures 3.11C & 3.12A) from purified DNA band were read from the NCBI BLAST (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and a phylogenic tree plotted as a cladogram using codon code 1.1 software (Figures 3.11A & Figures 3.12 B).

3.4 Results

3.4.1 Viability assessment of sponge cell isolate.

Cryopreserved single sponge cells were assessed for viability using the trypan blue exclusion assay. Result showed that greater than 99% sponge cells remain viable following thawing and re-suspension of cryopreserved single cells isolates (Figure 3.2a). An important feature of sponge cells is their ability to form functional aggregates from viable individual cells; this indicates an active communication signal between all cell types in suspension and the skeletons (spicules). To further confirm the viability of cryopreserved single sponge cell aggregate formation was assessed. Cryopreserved cells suspended in sponge media formed bright orange aggregates, within one hour (Figure3.2b). These progressively developed into a more organized and compact structure (Figure 3.2c). Over longer periods of up to 14-21 days structures that appeared morphologically to be like previously reported "primmorphs" (Pomponi and Willoughby 1994; Pomponi 2006b) were observed (Figure 3.3). These were stable for more than one week in culture after formation and remained viable by MTT viability assay (Figure 3.4). In all subsequent experiments, we used sponge cell aggregates no older than 2 days to assess the effect of cadmium chloride on cell viability and DNA strand breaks. This is to ensure that variability resulting from utility of cells at different developmental stage, is substantially reduced.

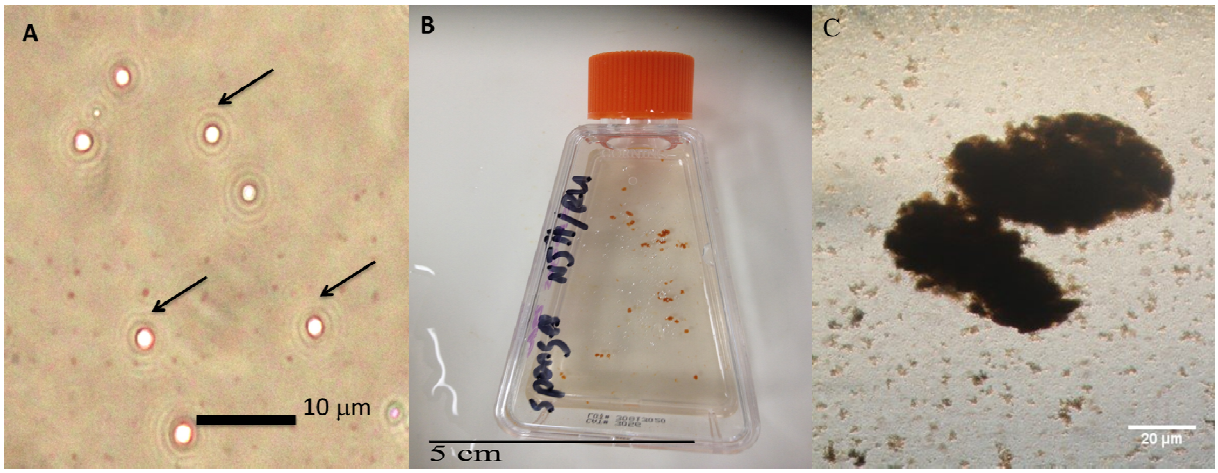


Figure 3.2 Viability assessment of sponge cell isolates A) Trypan blue viability assay of cryopreserved sponge cells. B&C) Rapid Sponge aggregate formation within one hour of suspension in sponge media. An important feature of sponges is their ability to re-aggregate back into tiny aggregates with the potential for developing into new sponge. This also indicates viability of the individual cells from dissociated sponge tissues.

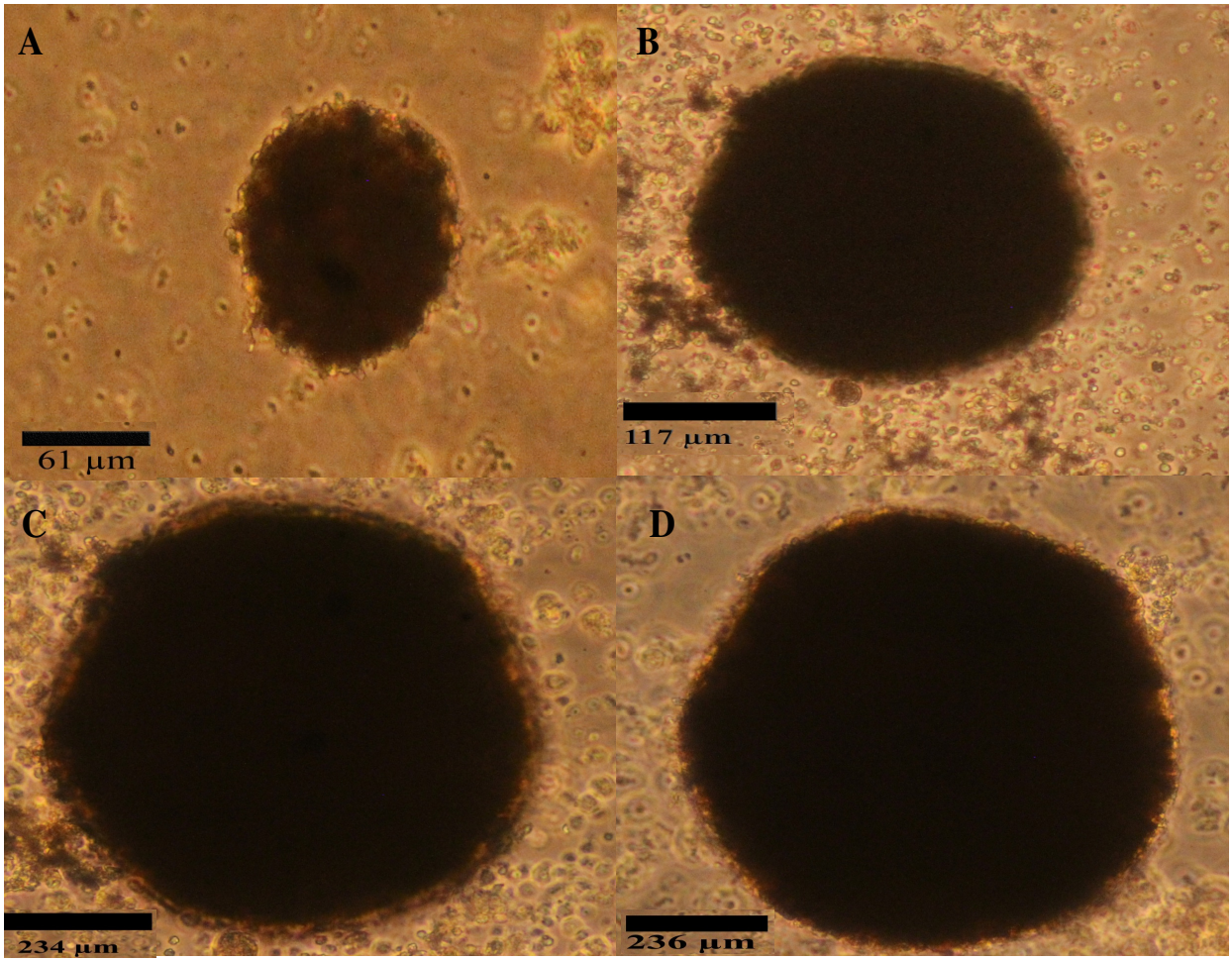


Figure 3.3: Microscopic appearance of sponge cell cultures; A) 48hours B) 72hours C) 7 days D) 14 days in culture. Successful primorphs formation achieved in up to two weeks culture. Aggregates progressively developed from loose cells to primary aggregates to structures with well-defined features. The Primorph is thought to be the potential future for sponge research, it serves both as a purified system as well as the source for sustainable production of bioactive compound and as a model for bomonitoring of environmental xenobiotics.

3.4.2 Viability assessment of sponge aggregates

Given the sensitivity of the comet assay and other assays in subsequent experiments in this thesis, the primorph (cultured sponge aggregate), which is a near axenic system with homogeneous cell population, was adopted for all experiments. MTT assay (Figure 3.4) showed viability of cultured sponge aggregates and DMSO solubilized MTT reduction was visualized as purple coloured formazan following a 3hours aggregate incubation with MTT dye (Figure 3.4A).

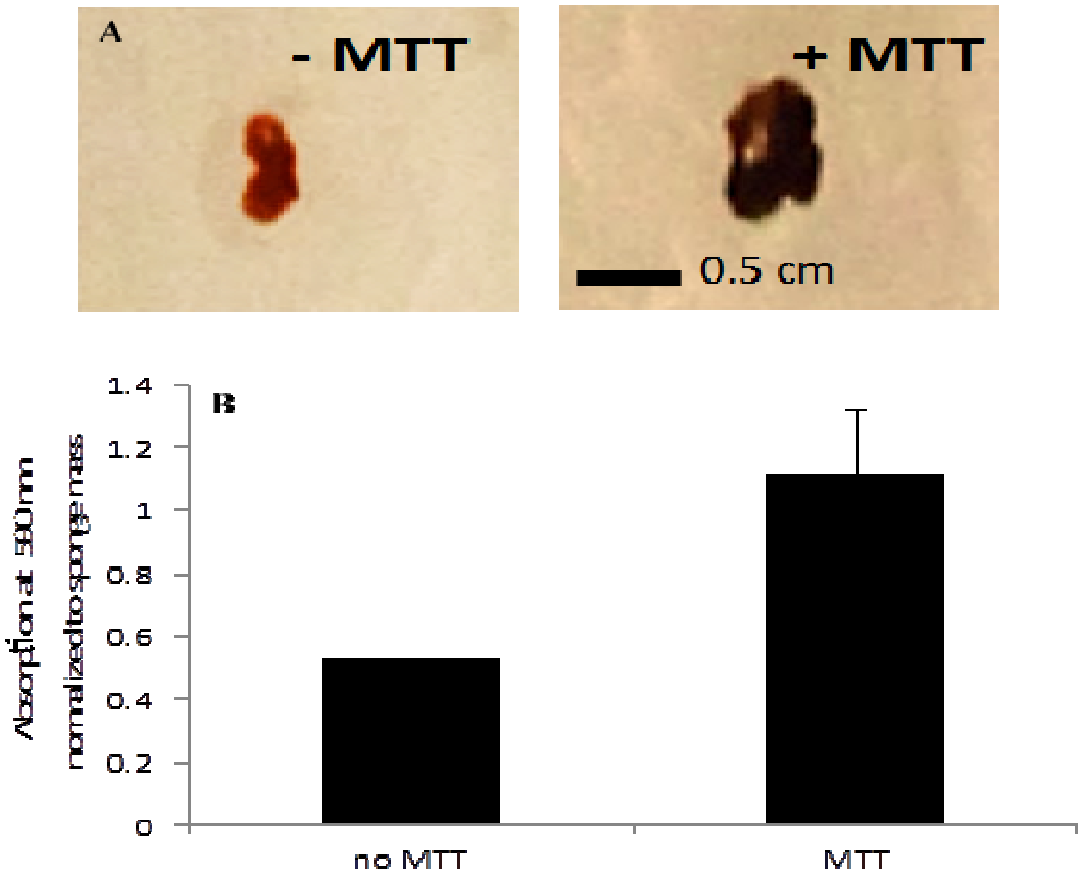


Figure 3.4 **Assessment of sponge aggregate viability** A) Sponge cell aggregates after 3hrs incubation with and without MTT **B)** DMSO absorption at 595nm of reduced MTT

3.4.3 Preliminary cell culture results and the effect of cell density on aggregate formation

Results obtained from preliminary sponge cell culture also showed that aggregate formation is dependent on cell density. Cell density in the original cell suspension (6mL) was approximately 7.6 x 10^6cells/mL. The effect of cell density was determined by plating approximately 10, 15, and 20 million cells (Figure 3.5) and aggregates formation monitored for up to two weeks (Figure 3.3) with an inverted Nikon microscope equipped with a camera. Mostly, compact aggregates were observed in cultures of 20 million cells and some in those of 15 million-cell culture after 72 hours. For subsequent experiments, approximately 20 million cells (3mL) from 6mL stock suspension were cultured for aggregate formation and for utility in all experiments.

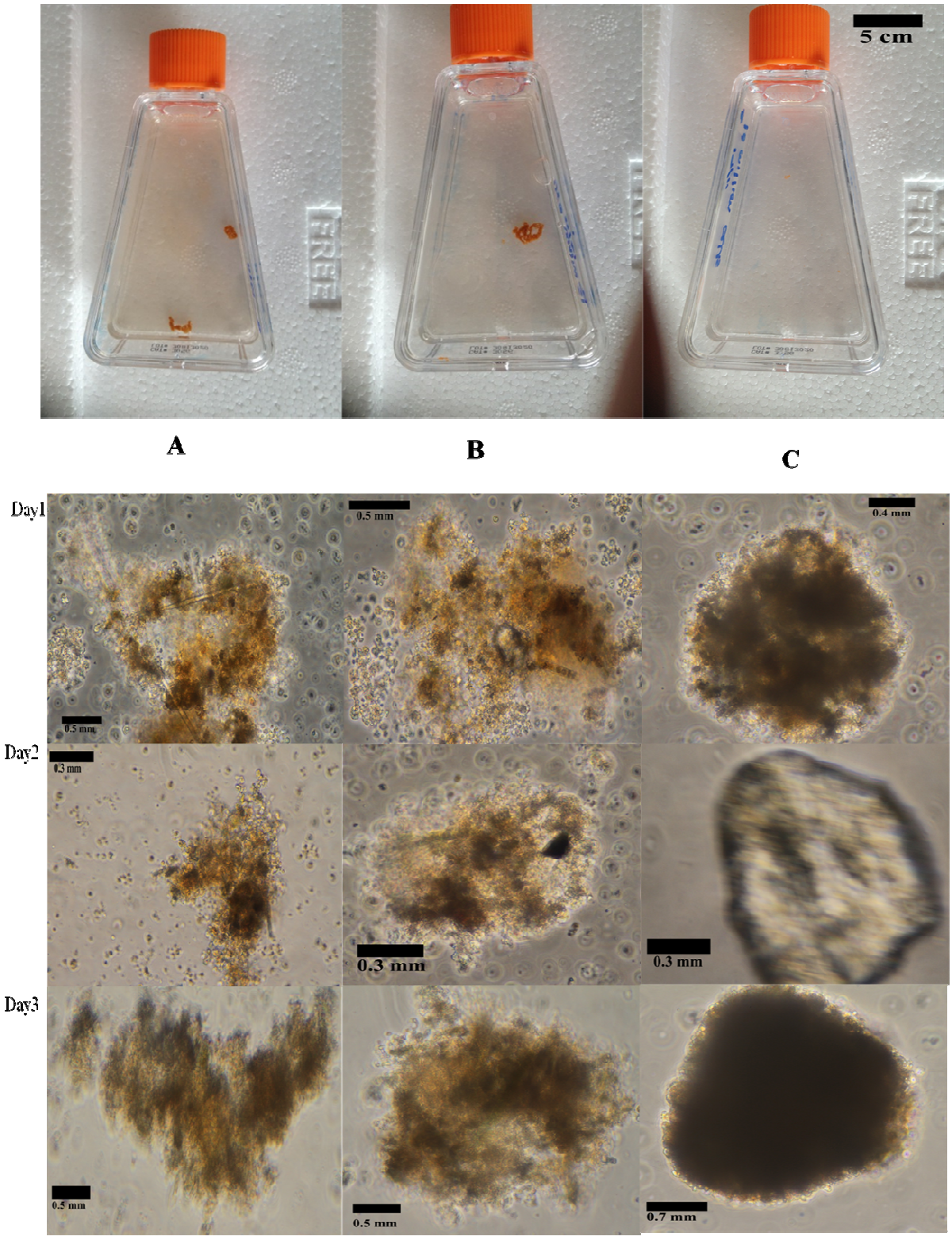


Figure 3.5: Gross microscopic appearance of sponge cell culture showing effect of cell density on sponge cell aggregation A) 20 B) 15 and C) 10 million sponge cells in 6ml of SSW per flask monitored over a three-day period. Primorphs (functional aggregates) were observed in 10 million cell culture which were viable for up to two weeks in culture, least number of cell needed for primorph formation.

3.4.4 Analysis and Differentiation of endosymbionts and epibionts from sponge cells

Investigation of sponge cells from single sponge cell suspensions (non-cultured sponge cell isolate extracted directly from sponge tissues) and dissociated aggregates (single cell isolates from cultured aggregates using CMFSW-E (Section 2.3, chapter 2) showed fully stained sponge nucleus of sponge cells (approx. 3µm) while those of epibionts were much smaller with partially stained cytoplasm (Figures 3.6 A & B). In single cell suspensions, however there was high background mainly from non-sponge materials making this cell source less suitable for nucleoid scoring in the comet assay and other assays in this thesis. Cells obtained from cultured primorphs (structural sponge cell aggregates) had very little non-sponge materials in the background compared with cells from single cell suspension (Figure 3.6B). In all subsequent experiments cryopreserved mixed sponge cell population dissociated from cultured aggregates were used.

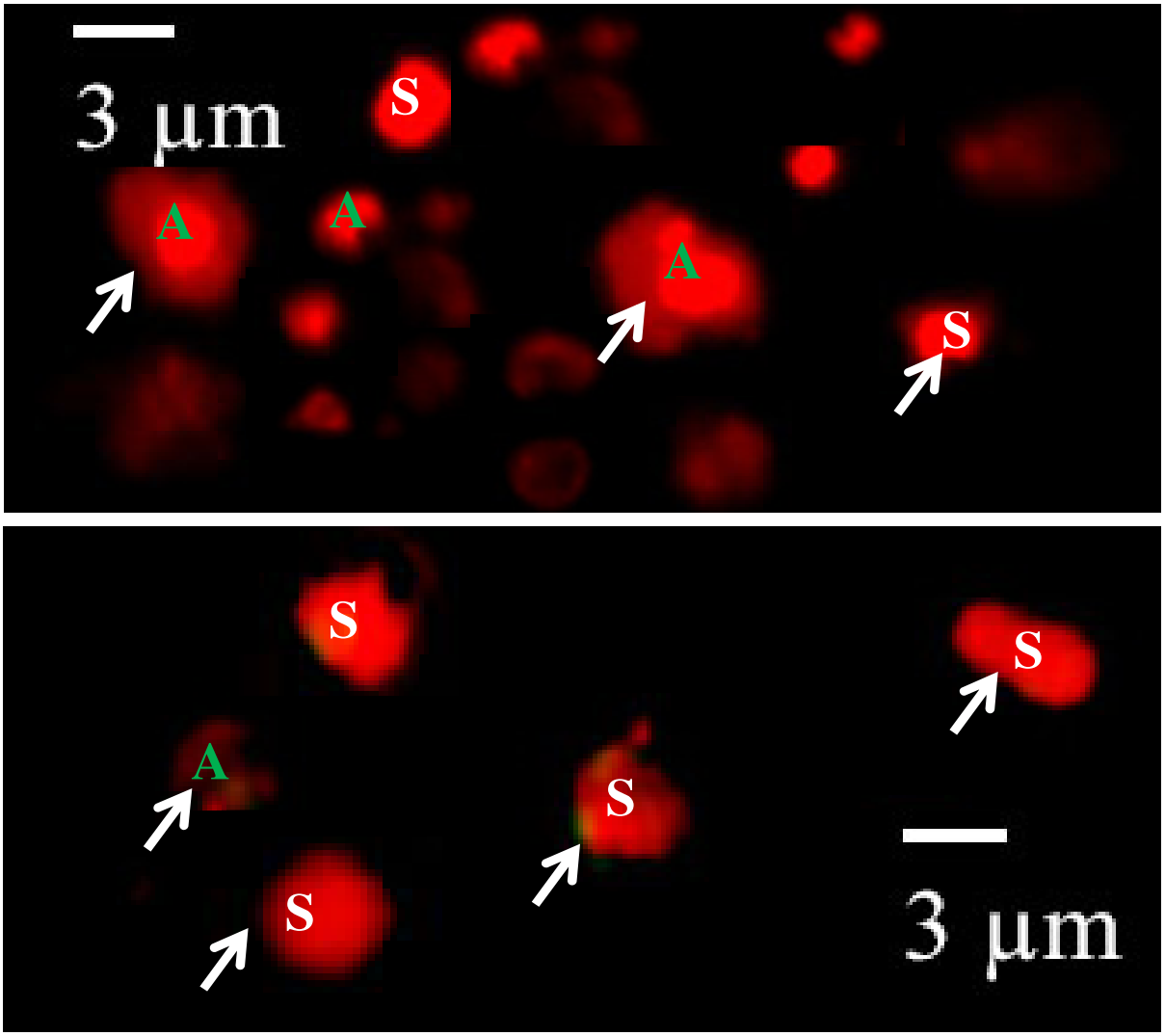


Figure 3.6 Differential Analysis of sponge cell nuclei from Algal symbionts by Feulgan staining. (A) Dissociated sponge cell suspension. S (arrowed)-sponge cell nuclei fully stained with the dye, A (arrowed)-Algae with partially stained cell wall and nuclei, other non-sponge materials in the background much smaller than sponge nuclei and easy to differentiate visually. B) Dissociated sponge cell aggregate (primorph). Compact and completely stained S-sponge cell, this is a much more axenic suspension with little or no non-sponge materials in the background.

3.4.5 Preliminary comet assay experiments and effect of cell density

Preliminary experiments demonstrate that sponge cells are amenable to analysis by comet assay. Both damaged and undamaged nuclei were observed in preliminary comet assay experiments with untreated control sponge cells (Figure 3.7). However, cell density appears to

be an important factor for sponge cells comet assay. Using the 40x oil immersion objective, cells with some comets were only detected on slides having high cell density. Very low (less than 50 nucleoids) and in most cases no cells were seen in slides with low cell density with the same magnification. Visible nucleoids were mainly observed on slides with undiluted 1mL cell suspension (5million cells per millilitre). Comet slides with 75% and 50% diluted cells had very few cells less than 100 nucleoids while no cells were detected on slides with 90% diluted cell suspension.

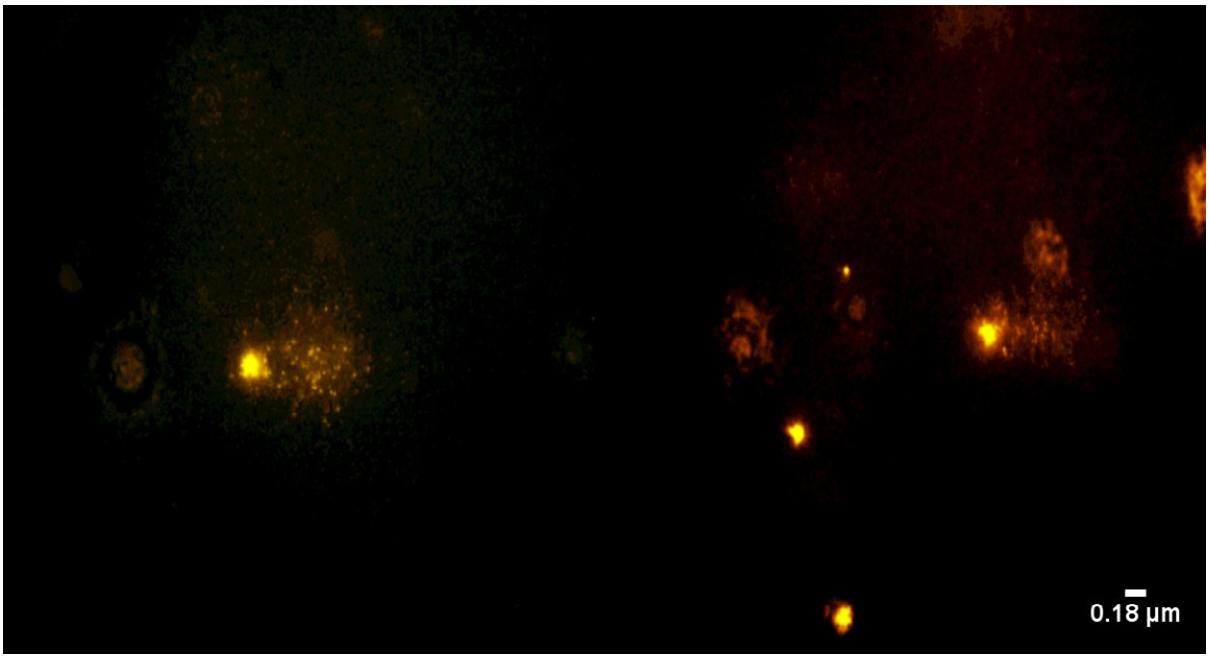


Figure 3.7 Preliminary comet assay experiment with single sponge cells. Initial results using sponge cells re-suspended from previously cryopreserved cells, showed amenability of sponge cells with the comet assay. There were evidences of both normal undamaged cells and obvious comets. Results demonstrates for the first time, successful assessment of DNA damage in sponge cells using the alkaline comet assay technique with some modifications.

3.4.6 Exposure of sponge cell aggregates to cadmium chloride and MTT viability assessment

Exposure of sponge cell to concentration of cadmium chloride (0, 0.01,0.1 and 1mg/L) showed no statistically significant effect on cell viability, assessed with the MTT viability assay and analysed using a 1 way ANOVA (Figure 3.8).

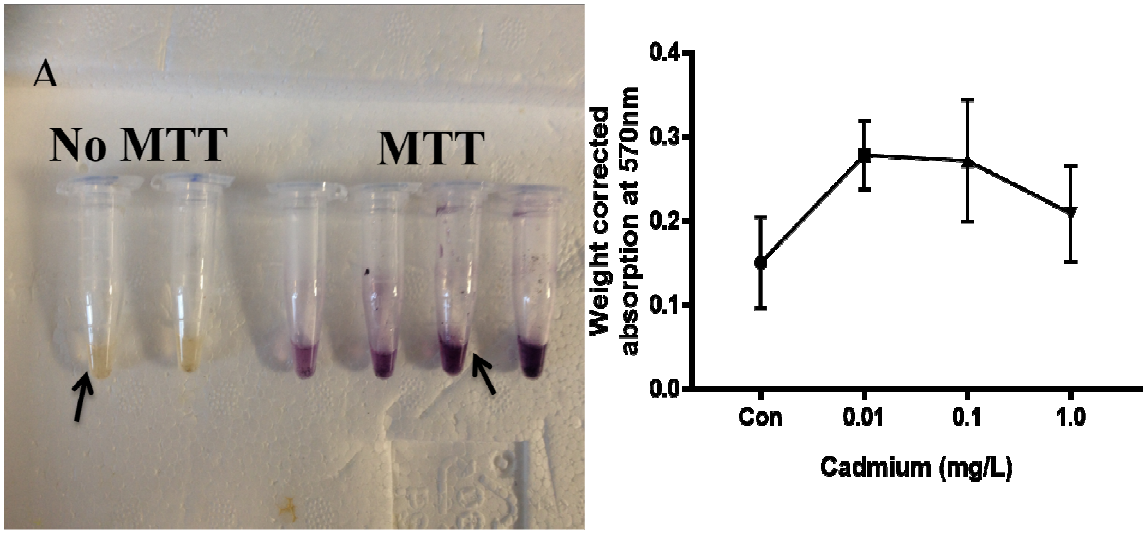


Figure 3.8: MTT Viability assay of Cadmium exposed sponge cell aggregates A) MTT assay B) Absorption of reduced MTT at 570 nm. Sponge cell aggregates exposed to cadmium chloride for 12 hours were assessed for viability using DMSO dissolved MTT -ve aggregates as blank. Results were both blank and aggregate weight in mg corrected. Mean values ± SEM; Cadmium exposure had no significant cytotoxic effect on sponge cell aggregates $P > 0.05$.

With the modifications outlined above in section 3.3.4 in place, DNA damage using the comet assay technique was assessed in sponge cells incubated with cadmium chloride for 12 hours. Preliminary exposure results shows that non-cytotoxic concentration of cadmium chloride results in concentration-dependent increase in the level of DNA strand breaks (Figure 3.9 C). In untreated control cells, the median tail DNA % ± SEM was 0.68±0.28, in contrast, this increased to 0.97±0.40, 1.24% ±0.51and 1.80±0.73 following treatment with 0.01, 0.1

and 1mg/mL cadmium chloride exposure. Representative images of 0.1mg/L cadmium treated sponge cells and control are shown are shown in Figures 3.9A and B respectively.

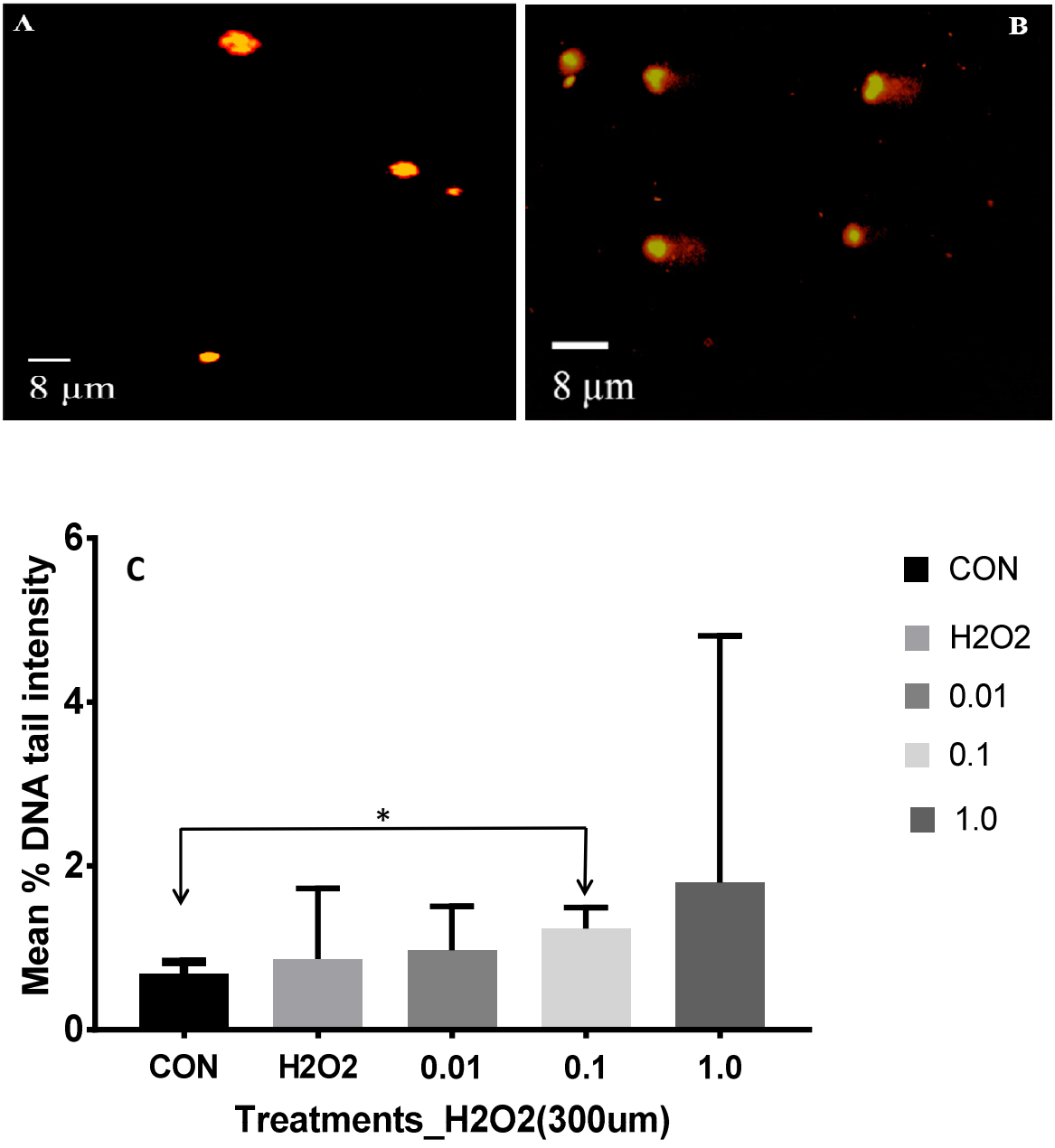


Figure 3.9 $CdCl_2$ Induced DNA damage in sponge cell aggregates. A) Undamaged control sponge cells B) $CdCl_2$ exposed sponge cell nucleoids with comets and C) % mean tail intensity assessed by 100 comet score in the Comet assay. Sponge cell aggregates exposed to cadmium for 12 hours showed a dose dependent response. Kruskall-Wallis multiple rank comparison post-hoc test showed statistically significant difference between 0.1mg/l cadmium treatment and the untreated control exposures with Mean ± SEM; P* < 0.05. Concentration of hydrogen peroxide used seems not to have had any statistically significant effect on the cells.

3.4.7 Cadmium chloride uptake and bioaccumulation assessment in *H. perlevis*

Measurement of cadmium uptake by ICP-MS (Inductively Coupled Plasma Mass Spectrometry) in sponge cell aggregates, to investigate bioaccumulation their potential, showed a statistically significant (R^2 >0.99, P<0.05) linear correlation between cadmium levels in the exposure media and levels in sponge aggregates. The ratio of concentration in sponge tissue compared to the exposure media was approximately 3:1. This is consisitent with the ability of sponge cells to accumulate toxic metals as previously reported (Cebrian et al. 2007) (Figure 3.10).

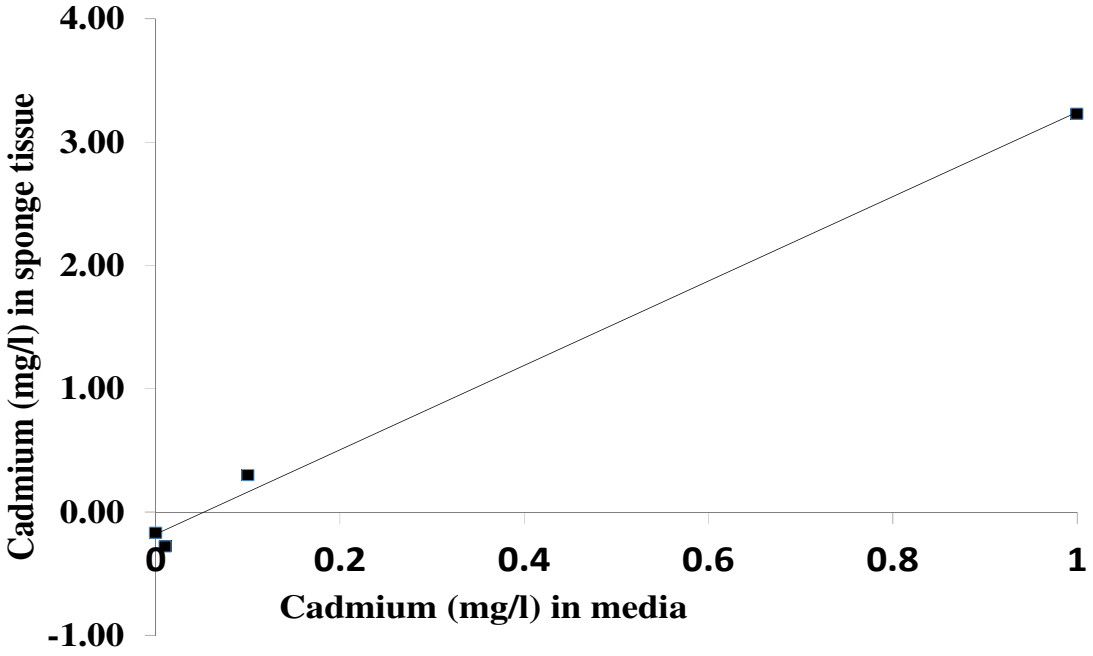


Figure 3.10 Bioaccumulation potential of Sponge cell aggregates demonstrated by: Cadmium uptake in sponge cell aggregates after 12 hours incubation. Positive correlation (R^2 = 0.9955)between cadmium in culture media and in sponge tissue

3.4.8 Molecular Characterization of the Type Specimen: *Hymeniacidon perlevis*

Genomic DNA PCR amplicons (Section 2.4.2, chapter 2), showed clear bands on a 1% agarose gel compared with a 1kb ladder (Figure 3.11B & Figure 3.12C) . Both cytochrome C oxidase 1 sequence of a 317 bp fragment (Figure 3.12) and 28S gene sequence of a 431 bp fragment MF685334 (Figure 3.11) clearly aligns the species with the same clades as those identified by systematic taxonomy and confirmed the identity of the type species as *Hymeniacidon Perlevis*.

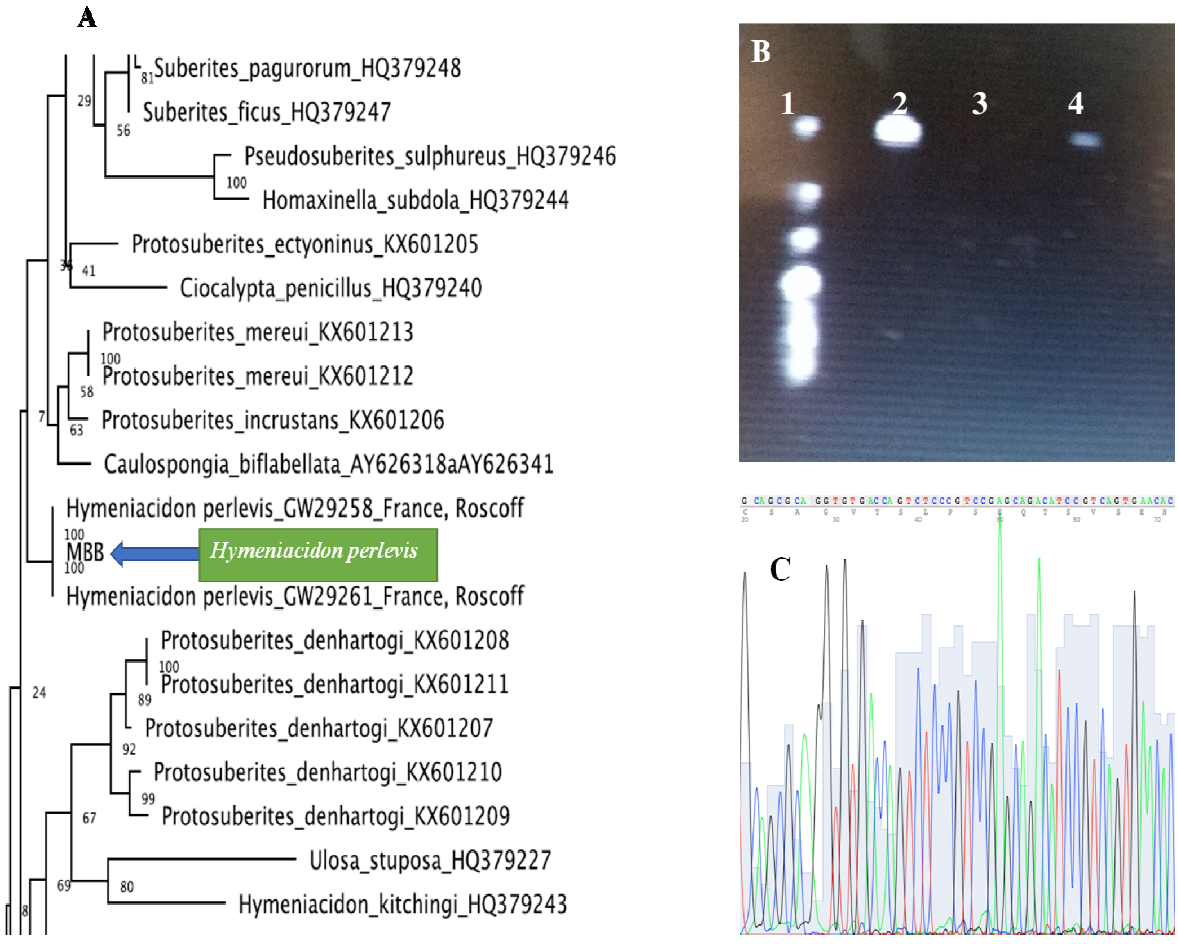


Figure 3.11 28S Gene Sequence of *H. perleve.* A) 28S Sequence analysis and molecular phylogeny confirmed samples as *H. perleve.*B) PCR amplification of cytochrome of RNA gene fragments C) PCR products Sequence reads. Data available on the NCBI gene bank (MF685334) confirming systematic taxonomy result

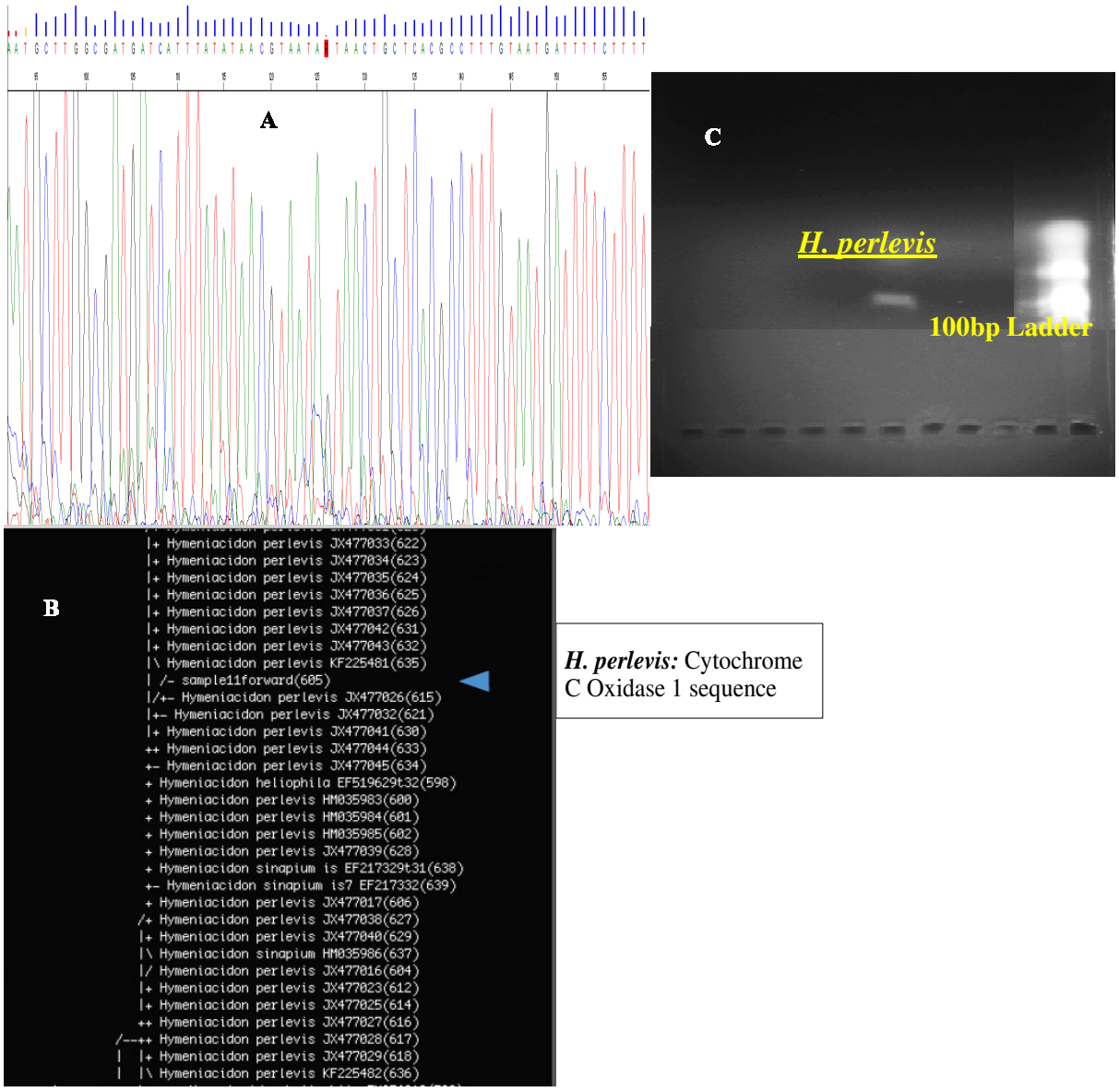


Figure 3.12: Cytochrome C Oxidase sequence analysis identifies Manobier bay sponge sample as *H. perleve* A) CO1 sequence read of *H perlevis* B) 2% agarose gel electrophoresis of *H perlevis DNA amplicon* C) Phylogenetic cladogram showing sibling species of *H. perlevis*

3.5 Discussion

The Phylum porifera is one of the most diverse metazoan groups, hence the need to correctly identify individual species to ensure appropriate applications in scientific research. Until recently, sponge identification was mainly by systematic taxonomy, however given the complex and diverse nature of sponges, morphological identification has proven insufficient in identifying closely related epithet, mainly due to overlapping morphological features of these sibling species (Wörheide and Erpenbeck 2007). The introduction of molecular taxonomy (Vargas et al. 2012) which brought about the use of molecular signatures in characterising individual sponge species, has greatly improved the taxonomy of taxa and helped to clear the ambiguities that have 'bedevilled' morphological speciation of sponges (Van Soest et al. 2012). In this study, the identity of *H. perlevis,* a near shore species cosmopolitan in the British and Irish marine systems was verified by both morphological and molecular taxonomy. *H. perlevis* is available all year round and it is an extremely easy to sample species as it is abundantly found on exposed rock pools at low tide near shore (Figure 3.1). A number of studies have identified interesting features and applications of *H perlevis* in both in molecular and environmental investigations. For example, *H perlevis* has also been reported as a suitable bioindicator with bioremediation potential (Gentric et al. 2016) and also as indicator of water quality (Mahaut et al. 2013). This thesis presents for the first time the suitability of the comet assay technique in the sea sponge *H. perlevis* using cells obtained from 1) cryopreserved single sponge cells and 2) single cells from dissociated sponge cell aggregates, previously cultured from cryopreserved single cells. For all experiments, sponge cell aggregates (primorph system) was utilised as a novel *in vivo* exposure model. Previous studies have established the use of mixed cell population sponge cell culture as also having

the potential for proliferation, DNA synthesis and primorph formation (Zhang, Cao et al. 2003).

Sea sponges belonging to the phylum porifera are an emerging sentinel group in environmental toxicology and have been applied in biomonitoring and risk assessment studies in the aquatic ecosystem (Pan et al. 2011; Patel et al. 1985). Recent ecotoxicological reports suggests that they are comparable to well established invertebrate models (such as mussels and other bivalves) used in aquatic biomonitoring (Perez et al. 2003). Gentric et al. (2016) reports that sea sponges, particularly *H. perlevis* accumulated more trace metals and PaHs than oyster (*Crassostrea rhizophorae*), an established sentinel species. In the current study *H. perlevis;* a near shore sponge species was utilised to investigate the ability of a known environmental pollutant (Cadmium) to cause DNA damage in cultured sponge cells. DNA damage in sentinel aquatic invertebrates, is a reliable biomarker of exposure and effects from environmental genotoxicants and it is widely employed in environmental risks assessment (ERA) and biomonitoring (Martins and Costa 2014). Because sea sponges occur in association with other aquatic organism, it is important to ensure as much as possible a homogeneous sponge cell population that is near aseptic (Pomponi 2006b). In this study, single sponge cells were isolated from freshly collected field samples (Figures 3.1, 2.2. and 2.3) via slow speed centrifugation. Usually, this is the first step towards purifying sponge cell suspension of epibionts such as cyanobacteria and protozoan cells, because they are less dense than sponge cells and are likely to be left behind in the supernatant (Cai and Zhang 2014; Mussino et al. 2013). Viability results of re-suspended cryopreserved single cells showed more than 99% cell viability using the Trypan Blue Exclusion assay (Figure 3.2A). A unique feature of sea sponges which also determines viability is the ability of dissociated single sponge cells to re-aggregate back into functional units (primorphs) (Custodio et al. 1998a; de

Caralt et al. 2007). Aggregate formation also helps to eliminate non-sponge materials, which are discarded with the culture media. To confirm the viability of sponge cells used in this experiement- *H. perlevis*, and as a second purification step, we cultured in artificial sea water (Media 3 in Table 3.1), freshly defrosted cryopreserved sponge cells to see if they would form viable aggregates. In less than an hour bright orange aggregates were observed in culture media (Figure 3.2B). Sponge aggregates progressively developed from simple primary aggregates to secondary structures with well-defined features; 'little sponge' (primorph-like) (Figure 3.2C Figure 3.3) as previously reported (Goh 2008a; Muller et al. 1978) and viability was confirmed by the MTT assay (Figure 3.4).

Because sponge cell suspension is highly heterogeneous, being able to make preparations of sponge cells without contaminating endosymbionts is extremely challenging (Jeffery et al. 2013). Commonly reported endosymbionts often found in association with sponges are bacteria (Cynanobacteria) and algae, of these two bacteria cells (being less dense and much smaller with less genomic material in comparison to the sponge) are easier to identify and remove e.g. by slow speed spinning. Algal cells are however, almost the same size as sponge cells and therefore more difficult to identify from sponge cells. Two methods are suggested in (Jeffery et al. 2013) for identifying and distinguishing non sponge cells; flow cytometry (FCM) and Feulgan image analysis desitometry (FIAD) using Schiff's reagent. They however, reported that non-sponge nuclear material appeared as noise in flow cytometry data and therefore difficult to compare size differences, we also confirm this as our attempt to distinguish cell types by flow cytometry proved challenging due to background noise and no clear size difference in the scatter plot (data not shown) . FIAD on the other hand provided a useful method to distinguish the two groups by identifying algae cells with partially stained cell wall and sponge material which takes up the stain completely. This, we were also able to

confirmed; algal cells in both direct cell suspension (defrosted cryopreserved cells) and cell suspension from cultured aggregates were partially stained by the Schiff's reagent (Figure 3.6 A & B).

The comet assay is only applicable in single cells, and care is needed in the process of single cell isolation(Fairbairn et al. 1995) and culture manipulation. To determine which sponge single cell source was best for the comet assay we conducted Feulgan staining test. In this analysis, sponge cell nuclei from aggregates were clearly identified as compact and completely stained structures (Figure 3.6B) while non sponge cells were identified as algal epibionts and endosymbionts with partially stained cell walls (Figure 3.6A). This is consistent with the report of (Jeffery et al. 2013). Comparing single cell population from two sponge cell sources (Figure 3.6), cell suspensions obtained from dissociated cultured sponge cell aggregates were found to have very little background from non-sponge material compared to cells from 'uncultured' cell suspension; this is mostly due to the extra purification step of culturing. Thus, single cells obtained from dissociated primary aggregate cultures were adopted as the most suitable source of sponge single cells for the comet assay and used for all future experiments. As part of the final modification steps, homogeneous cell suspensions from completely dissociated cells were carefully transferred into fresh Eppendorf tube while non-dissociated residues were discarded; this step was particularly useful for reducing microscopic background noise from debris and epibionts, which was been an issue in previous trial experiments with the undiluted cell suspension.

To test, whether our model would respond to a known genotoxicant, we exposed cultured sponge aggregates to selected cadmium concentration based on studies (Schröder et al. 1999). Cadmium is a prevalent heavy metal pollutant in coastal environment and an established genotoxicants with no known benefits in living systems (Nriagu 1980; Stohs and Bagchi

1995). It mainly exerts its toxicity via depletion of cellular glutathione, production of reactive oxygen radicals (regulated in oxidative stress) and DNA strand DNA damages (Giaginis et al. 2006; Hassoun and Stohs 1996). In this preliminary study, the genotoxic and cytotoxic effects of three concentrations of cadmium chloride (0.01mg/L, 0.1mg/L and 1mg/L) were investigated with the alkaline comet assay and MTT assay, respectively. Result obtained showed that treatments with cadmium had no statistically significant effects on the viability of sponge cells (Figure 3.8). However, assessment of DNA damage using the comet assay showed that non-cytotoxic cadmium concentrations (0.01-1mg/L) caused a statistically significant dose dependent increase in the level of DNA damage in single sponge cells, after 12 hours in culture (Figure 3.9). To also ascertain the potential of sea sponges to bioacummulate pollutants at low concentration from their surrounding environment, cadmium uptake in sponge cell aggregates incubated for 12 hours was assessed.

Measurement of uptake cadmium concentrations in cultured sponge aggregates using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) showed a linearly correlated cadmium concentration in both cultured sponge aggregates and in culture media. Up to 3 fold increase in the level of accumulated cadmium in the aggregate tissues compared to cadmium in culture media was observed (Figure 3.10). This confirms previous bioaccumulation potential report of *H. perlevis* (De Mestre et al. 2012; Gentric et al. 2016). This is also very important in showing that, deleterious effects resulting from exposure to xenobiotics is more related to the biologically available level rather than the levels in environmental matrices, such as sediment and water column.

Our culture model may serve as a tool for understanding the effects and the mechanism of action of cadmium and other environmental xenobiotic exposure on sponges in situ. Current ecotoxicology research trend is in understanding the direct effects on aquatic biota following

exposure to environmental xenobiotic; this information are required to reliably assess the risks and hazards posed to aquatic organisms. Information on the direct effect of environmental xenobiotic on local organisms are needed as informed tools for the enactment of sustainable and realistic environmental policies and regulations. and for pollution monitoring in the aquatic ecosystem (Martins and Costa 2014). The primorph system being a controlled system with near aseptic and axenic cell population is potentially a useful model for in environmental monitoring of pollutants. Potentially, other useful applications are in studying the biochemical processes of sponge bioactive metabolites and for understanding the process cell proliferation and apoptosis in long term sponge cell culture (Custodio et al. 1998b; Müller et al. 1999). Thus Previous attempts by (Mussino et al. 2013) to obtain aggregates from cryopreserved sponge cell was however not successful. Hence, apart from the novelty of the comet assay amenability with sponge cells in this study; the achievement of the primorphs system from cryopreserved single sponge cell and the development of the 'in vivo' exposure model are particularly important achievements and major strides in this research.

In conclusion, although the alkaline comet assay protocol is most widely used in mammalian biomonitoring studies, it has however in recent times seen a significant application in sentinel non-model invertebrate species e.g mussels (Martins and Costa 2014). The use of non-model sentinel organisms is not without limitations; especially given their poor genomic annotations and the potential for inter species differences. However, application of sentinel invertebrates in environmental assessment studies is increasing, this is mainly because they provide direct information on both the level of and bioavailable contents of environmental pollutants and also serve as surrogate for understanding biochemical and molecular processes in higher organisms. Sea sponges are not exempt from these limitations, especially because different sponge species are known to bio accumulate pollutants differently, and till date there is only

one sponge species with complete genome annotation (Srivastava et al. 2010). Attraction to sea sponge in environmental assessments has been linked to a number of unique features such as; the expression of biomarkers of exposures to environmental xenobiotic such as heat shock proteins, Metallothioneins, DNA damage using alkaline elution assay and other stress response genes. Given the general challenge associated with the application of the comet assay in invertebrate models, here we present sponge primorph system as a suitable model for investigating biomarkers of effects and exposures to trace metals and other environmental pollutants using the comet assay technique. This system has the potential to serve as model for assessing the risks and hazards associated with exposure to known genotoxicants and it is further explored in the following chapters.

Chapter 4 Xenobiotic Induced DNA Damage and Oxidative Stress in Cultures of the Marine Sponge *Hymeniacidon perlevis*: Potential tool for Environmental biomonitoring and risk assessment

4.1 Introduction

Sponges are simple invertebrate animals with ecological importance in the aquatic ecosystem; their existence in the fossil record dates back to the Cambrian period, although some studies have reported the existence of some Poriferan members during the Cryogenic and Ediacaran Precambrian periods (Li et al. 1998; Xiao et al. 2000). A prominent feature of sponges that have advanced their ecological usefulness is their ability to filter and pump large volume of water through their body tissues. (Stevely and Sweat 2015) suggested that a gallon size sponge could filter and fill up a residential swimming pool in a day. This feature apart from its relevance to environmental studies is particularly important to the sponge itself as most of its physiology and biology are dependent on the inflow and out flow of water within the animal. Sponges feed by filtering in large amount of particles both in their dissolved and suspended phase and sexual reproduction is by the flow of gametes in course of water filtration. Sea sponges, through their water filtering potential accumulate over 90% of bacterial serving as host to most endo and epi-symbionts in the coral reef community and in the process also serve to purify the coral reef community (Rützler 1978). Sponges are also unique in their ability to accumulate and retain xenobiotics, inadvertently filtered in in the process of scavenging for food materials. Some of which are eliminated via metabolic process while others remain trapped within the organism and are either detoxified (stored up as metal binding proteins or solid particulate matter in lysosomes) or biomagnified into tolerable compounds (excreted via metabolic pathways) (Berthet et al. 2005b). Among pollutants accumulated in sponges, ionic and organic pollutants have been extensively studied; for example, heavy metals bioaccumulation in sponges are reported even when concentrations are very low and undetectable in other environmental samples such as surrounding water and

sediments (Hansen et al. 1995; Hill et al. 2002; Olesen and Weeks 1994). Marine sponge exposure to environmental pollutants have been on increase since the advent of industrialization and main sources implicated with this are wastes discharge and spillage from petroleum products (Ferrante et al. 2018).

Because of the links between exposures to environmental xenobiotic, DNA damage, reactive oxygen formation and neoplastic diseases , environmental monitoring in ecotoxicology has advanced from monitoring the environment just for the levels of these pollutants to regular assessment of genome integrity and for biomarkers of exposure and effects in both vertebrates and invertebrates groups (Fairbairn et al. 1995; Martins and Costa 2014). Previously reported genotoxicity studies in sponges involved the measurements of strand scission factor (SSF) in the sponge *Suberites domuncular*, exposed to 5mg/L cadmium (Müller et al. 1998). Fold increase 1.7 to 8.6 were reported following 5 days exposure. Other investigations have also reported the presence of metal binding proteins in sponge, examples are heat shock protein 70 (Hsp70), and metallothioneins as biomarkers of exposure (Berthet et al. 2005a; Schröder et al. 2006) following exposure to cadmium, copper and zinc. Measurement of DNA strand breaks in sponge using the Fast microplate assay has also been reported (Schröder et al. 2006). Further details on the impact of genotoxic xenobiotic on sponges and their subsequent application in biomonitoring research are explored in the discussion session. Environmental pollutants with DNA damaging potentials are grouped into endogenous and exogenous sources, (Griffin 1996). Both endogenous and exogenous sources mainly induce damages to the DNA through two main pathways, production of reactive oxygen species (ROS) radicals and introduction of error in DNA polymerase activities in normal metabolic process. Heavy metals such as chromium, vanadium, copper and iron are redox active metals which act as catalysts for the oxidative damage of macromolecules, main

mechanism being through a Fenton-like reactions within the cell membrane while a few other ionic pollutants (cadmium, nickel, mercury, lead) and organic xenobiotic (e.g. Benzo[a] Pyrene) induce oxidative and damage to cellular molecules via redox cycling and depletion of cellular sulfhydryl antioxidants (Stohs and Bagchi 1995). Both mechanistic processes produce large amount of ROS which undermine intrinsic cellular defences, resulting in different molecular aberrations such as single and double strand DNA damages, lipid peroxidation, cytotoxicity, genotoxicity, mutagenicity, neurotoxicity and alteration of calcium homeostatic pathways (Ercal et al. 2001; Stohs and Bagchi 1995). These are the main precursors to various disease conditions (Stohs and Bagchi 1995).

DNA damage however, can be prevented and corrected by cellular defence mechanisms (repair enzymes such as glycosylase, peroxidases, glutathione molecules, dismutase) and repair processes (proof reading of copied bases and DNA repair processes) (Bernstein and Bernstein 1991; Griffin 1996). However, some endogenous and exogenous sources are produced in quantities and rates that overwhelm and confound normal cellular correction system, thereby by passing the defence systems. These processes are the main precursor for DNA damages in eukaryotic cells and if not corrected results in neoplastic diseases (Jackson and Loeb 2001). Hence in this chapter, we investigated the DNA damaging effects of cadmium, chromium, nickel, aluminium and benzo [a] pyrene and their ability to generate ROS in aggregate cultures in our previously developed 'in vivo' exposure model (*H. perlevis).*

Genotoxicity in ERA is a recurrent biomarker of pollution and it is widely employed in environmental monitoring and risk assessment especially in coastal systems. Application of DNA strand breaks as genotoxicity endpoint and as biomarkers of exposure to pollutants is also well established. Reactive Oxygen species formation is linked with the mechanism of

toxicity of most xenobiotic; hence it is also reliable biomarker of effect extensively studied in effect focused research. A number of studies have employed the comet assay in the investigation of both metals and organic compounds in both vertebrates and invertebrates; however to the best of our knowledge this study is the first to develop the comet assay for use in single sponge cells. Increasingly, there is the popular demand for the use of sessile biogenic epifauna in assessing the levels and effects of environmental pollutants in the aquatic ecosystem, sea sponges in this regards are also gaining significant attention as they compare favourably with well-established invertebrates models. Thus, given the increasing application of the comet assay in studies using indigent sessile biota, DNA strand breaks in sea sponges assessed by the comet assay techniques could serve as a reliable tool for aquatic pollutant monitoring and risks assessment.

4.1.1 Cytochrome (CYP) P450 Metabolism

CYP-P450s are multivarient iso-enzymes, constituting the terminal end of the mixed function oxygenase (MFO) system located in the endoplasmic reticulum. P450 enzymes are involved in catalysing the monoxygenation reaction of most xenobiotic and endogenous substrates. They are extensively utilised as biomarkers of organic pollutants, mostly in vertebrates and a few invertebrates. The catalytic reaction of CYP is requires reducing co-factors and molecule of oxygen; in animals, electrons are transferred from NADPH to CYP from CYP-reductase and from NADH to cytochrome-b_5 to cytochrome b_5-reductase. In ecotoxicology, P450 (CYP1A) is popularly studied due to its recurrent induction in vertebrates following exposure to organic pollutants. Thus, CYP1A serves as a reliable biomarker of organic pollution in the aquatic environment.

Not very much is known of CYP activities in lower invertebrate phyla, like sea sponges. Preliminary experiment to investigate CYP-P450 in the sea sponge *H. perlevis* was conducted using P540-Glo Assay kit.

4.2 Aims and objectives

The aim of this study was to investigate the genotoxic effects of selected environmentally relevant xenobiotics previously employed in ERA; cadmium, chromium, nickel, aluminium and benzo[a] Pyrene in sponge cell aggregates..

4.3 Materials and Methods

4.3.1 MTT Viability assessment of sponge cell aggregates

Sponge aggregate cultures from cryopreserved sponge cells were incubated with 0 mg/L, 0.1mg/L, 0.3mg/L and 0.4mg/L $CdCl_2$, $NiCl_2$, and $Na_2Cr_2O_7$ solution for 12 hours. For $AlCl_3$ and B[a]P exposures, the concentrations used were 0, 0.1,0.2,0.3 & 0.4mg/L and 0,1% DMSO(diluent), 0.1µM, 0.5µM, 2.5µM, 5µM, and 10µM, respectively. Untreated aggregates cultured simultaneously with the exposed aggregates were used as control. As positive control, sponge aggregates were exposed to 100µM of the test metals. Duplicate treatments in three repeat experiments were investigated for all exposures. The viability of sponge cells treated with genotoxicants was assessed; using MTT reduction assay as described in Sections 2.8.1 -. 2.9.1, Chapter 2.

4.3.2 Alkaline Comet assay assessment of DNA damage induced by genotoxic metals and B[a]P

Sponge cell aggregates previously treated with Al, B[a[P, Cd, Cr, and Ni were dissociated into single cell suspension as described in Section 2.8.1; Chapter 2 and processed for comet assay following the modifications developed for sponge cells (Sections 2.10.1 and 2.10.2).

4.3.3 Assessment of Oxidative stress in treated sponge cells

DCFH-DA assay was utilised to assess the formation of ROS (H2O2) in treated sponge cells, as a biomarker of oxidative stress. As illustrated in Figure 2.4, Chapter 2, treated sponge cell aggregates were dissociated into single cells using EDTA containing CMFSW and thereafter,

pelleted at 1800 x g for 5 minutes. Cell pellets were then re-suspended in 1mL mixture of 3 µL of 100 µM DCFH-DA and DMEM (Dulbecco's Modified Eagle Medium) in Corning® Costar® 6-wells, clear, polystyrene, flat bottom Plates (Sigma Aldrich: Merc). Well plates were incubated at 37oC for 1 hour. After incubation, pellets were obtained again by centrifugation at 1800 x g for 5 minutes, washed with PBS and digested in 100µL of 10mM NaOH for 15minutes at room temperature. Finally, the digests were pelleted again to remove cellular debris at 4000 x g for 5minutes, and the supernatants carefully transferred into a Corning® Costar® 96-wells, clear, polystyrene, flat bottom Plates (Sigma Aldrich: Merc). Fluorescence intensity was then measured with 200 pro infinite spectrometer at 485nm excitation, 535nm emission and 10nm band width.

4.3.4 Cytochrome (CYP) P450 metabolic activity in sponge cell S9 extract

Preliminarily, CYP1A1-P450 activity was investigated in B[a]P exposed sponge cells following the method described in (Solé and Livingstone 2005). Approximately 1.3g sponge cell aggregates were homogenised in buffer 1 (ratio 1: 4, sponge to buffer) using a Potter-Elvehjem pestle tissue grinder. Next sponge cell pellets were obtained by differential centrifugation (1st 500x g for 15 minutes and supernatant was collected, 2nd 10,000x g for 30 minutes again supernatant was collected and finally 100,000x g for 90 minutes and this time pellets were collected , re-suspended in buffer 3 and stored in -80^{o}C until required).

P450 Assay buffers and procedures

Buffer 1-Sample preparation buffer

- 10mM Tris-HCL(pH7.6)
- 1M DTT (Dithiothreitol)-made fresh before use
- 150mM KCL
- 0.5M Sucrose

Buffer 2

- 2x NADPH regenerating solution (22.0µL H_2O, 2.5 µL solution A, 0.5 µL solution B)
- 4x CYP reaction mixture (5 µL 1M KPO_4, **200 µM Luciferin CEE (to be added last just before use)**, 7 µL H_2O)
- 50 µL Luciferin detection reagent
- 11.5 µL H_2O
- 20 µg S9 protein (approx.1 µL) Buffer 3- microsomal buffer
- 10mM Tris-HCL
- 20% (w/v)Glycerol

Assay Procedure

A. *Three experimental set-up in triplicates*

1. Control (Blank-Water)
2. Rat Liver S9 (Positive control)
3. Sponge Cell extract.

B. *12 Reaction set up*

Buffers

Buffer 1: 264 µL H_2O, 30 µL solution A, 6 µL solution B

Buffer 2: 60 µL 1M phosphate buffer, 6 µL Luciferin CEE, 84µL H_2O.

25 µL of Buffer 1, 1 µL S9 + 11.5 µL H_2O (in triplicates), 10 µL sponge extract + 2.5 µL H_2O (in triplicate), 12.5 µL H_2O, 12.5 µL Buffer 2 were added to a 96 well plate (Corning 96 well Flat Bottom plate, clear bottom Polystyrol). Plate contents were given a gentle mix and incubated at 37ºC for 30 minutes. After incubation, 50 µL Luciferin detection reagent was

added and plate incubated for another 20minutes after a gentle mix. Luminescence was estimated using infinite 200Pro plate reader.

4.3.5 Statistical analysis

Shapiro-Wilk's test and Lavene's tests were utilised to determine normality and homogeneity of variance, respectively. Mean difference between treated and control samples were analysed using one way ANOVA in combination with Bonferroni multiple comparison in IBM SPSS version 22.0 and graph pad version 7.0.1. For B[a]P, Kruskal-Wallis H Test was utilised to test for mean difference between test concentrations and control treatments.

4.4 Results

4.4.1 Sponge Cell culture and viability assessment

Cultures of sponge cell aggregates (previously described in chapter 3, section 3.4.3) treated with 0.1mg/L, 0.2mg/L(only Al), 0.3mg/L and 0.4mg/L Cd, Cr, Ni and Al for 12 hours were assessed for viability using the MTT assay. Viability of B[a] P exposed aggregates was also measured and concentrations used were 0-10µM with 1% DMSO as a negative control. Treated aggregates reduced MTT to insoluble purple formazan solution and viability was quantified by measuring absorption at 570nm (Figure 4.1). Results obtained using one way analysis of variance at $P < 0.05$, showed that concentrations of the test compounds used in this study do not have any significant cytotoxic effect on *H. perlevis* aggregates (Table 4.1). As a positive control, aggregates were also treated with known cyto-toxic concentration of cadmium (100µM) as previously reported in other invertebrate systems (Mitchelmore and Hyatt 2004) (Table 4.2). And same concentration of 100µm was also adopted for other test compounds, The MTT assay showed significant cyto-toxicity of this higher concentration of all test in sponge cells except for Aluminium exposure (Table 4.2).

4.5 Xenobiotics Induced DNA strand break and reactive oxygen formation in sponge cell cultures

Using our novel 'in vivo' exposure model described in chapter 3, aggregates of *H. perlevis* were exposed to non-cytotoxic concentrations of cadmium, chromium, nickel, aluminium and benzo[a] Pyrene. Investigation of genotoxicity (using the comet assay) and oxidative stress induction (using 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) assay) following exposure to individual contaminants showed a concentration dependent increase in both the level of DNA strand breaks and in the amount of reactive oxygen species (ROS) generated.

Table 4.1: Non-cytotoxic concentration of test metals as assessed by MTT viability assay: No statistically significant cytotoxic effect on sponge cell aggregates following 12-hours exposure to Al, B[a]P, Cd, Cr, and Ni,

Xenobiotic	Concentrations	P*≤ 0.05	Mean±SEM
	Cadmium Exposure		
Cd	Control	1.00	1.52±0.18
	0.1mg/L	No statistical difference	1.29±0.13
	0.3mg/L		1.19±0.11
	0.4mg/L		1.21±0.18
	Chromium Exposure		
Cr	Control	P =1.00	1.59±0.01
	0.1mg/L	No statistical difference	1.52±0.03
	0.3mg/L		1.45±0.09
	0.4mg/L		1.32±0.09
	Nickel Exposure		
Ni	Control		1.65±0.09
	0.1mg/L	P =1.00	1.69±0.08
	0.3mg/L	No statistical difference	1.64±0.11
	0.4mg/L		1.67±0.07
	Aluminium Exposure		
Al	Control		0.74±0.06
	0.1mg/L	P =1.0	0.788v0.15
	0.2mg/L	No statistical difference	0.67±0.10
	0.3mg/L		1.05±0.20
	0.4mg/L		1.10±0.05
	Benzo[a]Pyrene		
B[a]P	Control	P= 0.058	0.96±0.18
	1% DMSO	No statistical difference	1.39±0.24
	0.1µM		1.70±0.19
	0.5 µM		1.71±0.20
	2.5 µM		1.24±0.25
	5.0 µM		1.29±0.24
	10 µM		1.11±0.20

Table 4.2: Comparison of the Cytotoxic Effects of test Ionic compounds

Xenobiotic	**Concentrations**	**P* Value**	**Mean ±SEM**
	Cadmium Exposure		
Cd_Positive control	100µM	0.012 * Only statistically different from control untreated measurement.	0.71±0.01
	Chromium Exposure		
Cr_Positive control	100µM	0.00* Control 0.00* 0.1mg/L 0.00* 0.3mg/L 0.00* 0.4mg/L Significantly different from all test concentrations at P<0.05	0.88±0.07
	Nickel Exposure		
Ni_Positive control	100µM	0.00* Control 0.00* 0.1mg/L 0.00* 0.3mg/L 0.00* 0.4mg/L Significantly different from all test concentrations at P<0.05	0.65±0.05
	Aluminium Exposure		
Al_positive control	100µM	1.0 No statistical difference at P≤0.05	1.04±0.26

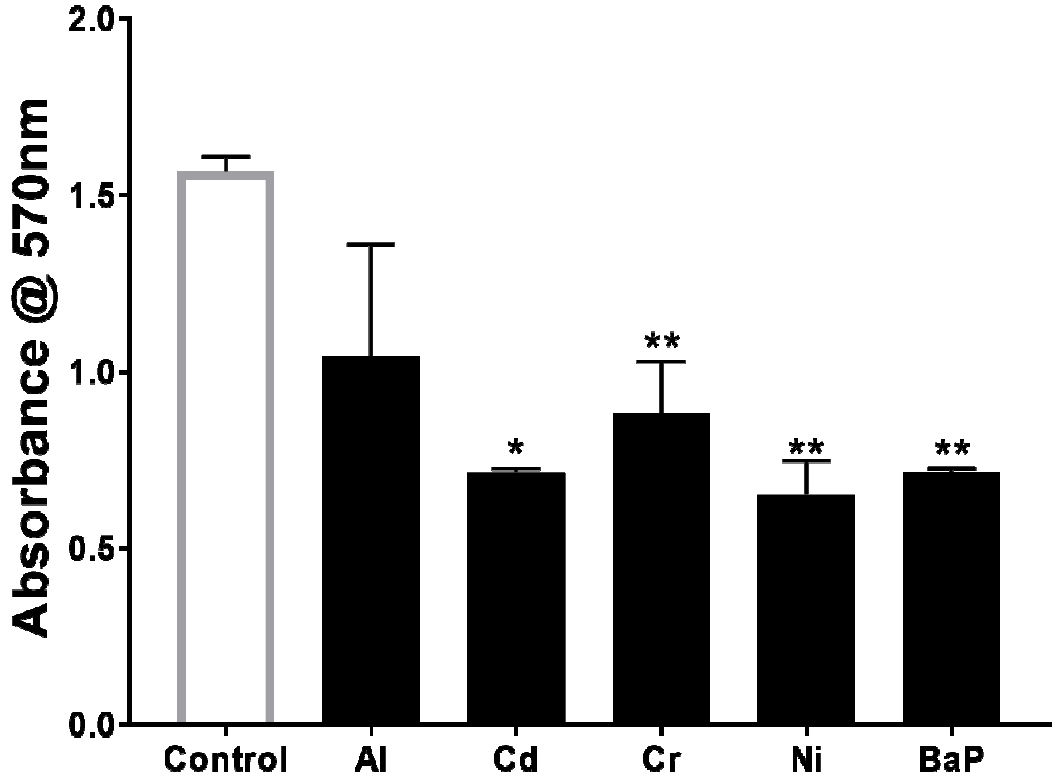


Figure 4.1: MTT viability assessment of 100µM (positive control) treatment**.** Comparison of Mean differences between positive control treatments of all test compounds. Results of 1 way ANOVA with Dunett's multiple comparison test, analysed with Graph pad prison 7.03 shows a statistically significant difference between the absorbance of sponge cell aggregates treated with 100µM of Cd, Cr,Ni and 10µM B[a]P and untreated control aggregates, no statistical difference was observed with Aluminium exposures. Data represent Mean absorbance ±SEM, n=3; $P < 0.05$. Results shows a decreasing order of xenobiotic induced cytotoxicity in sponge cells as Ni < Cd < B[a]P < Cr< Al.

4.6 Cadmium Exposure

4.6.1 Viability assessment of test cadmium chloride concentrations in sponge cells

Absorption result at 570nm shows that cadmium concentrations used had no statistically significant ($P > 0.05$) cytotoxic effect on sponge cell aggregates in comparison to the control (Figure 4.2 and Table 4.1). As positive control, sponge aggregates were treated with 100µM $CdCl_2$ and the results obtained showed that 100 µM is significantly cytotoxic on sponge cells with up to 2-fold increase compared to the control in MTT reduction (Figure 4.2 and Table 4.2) in sponge cells.

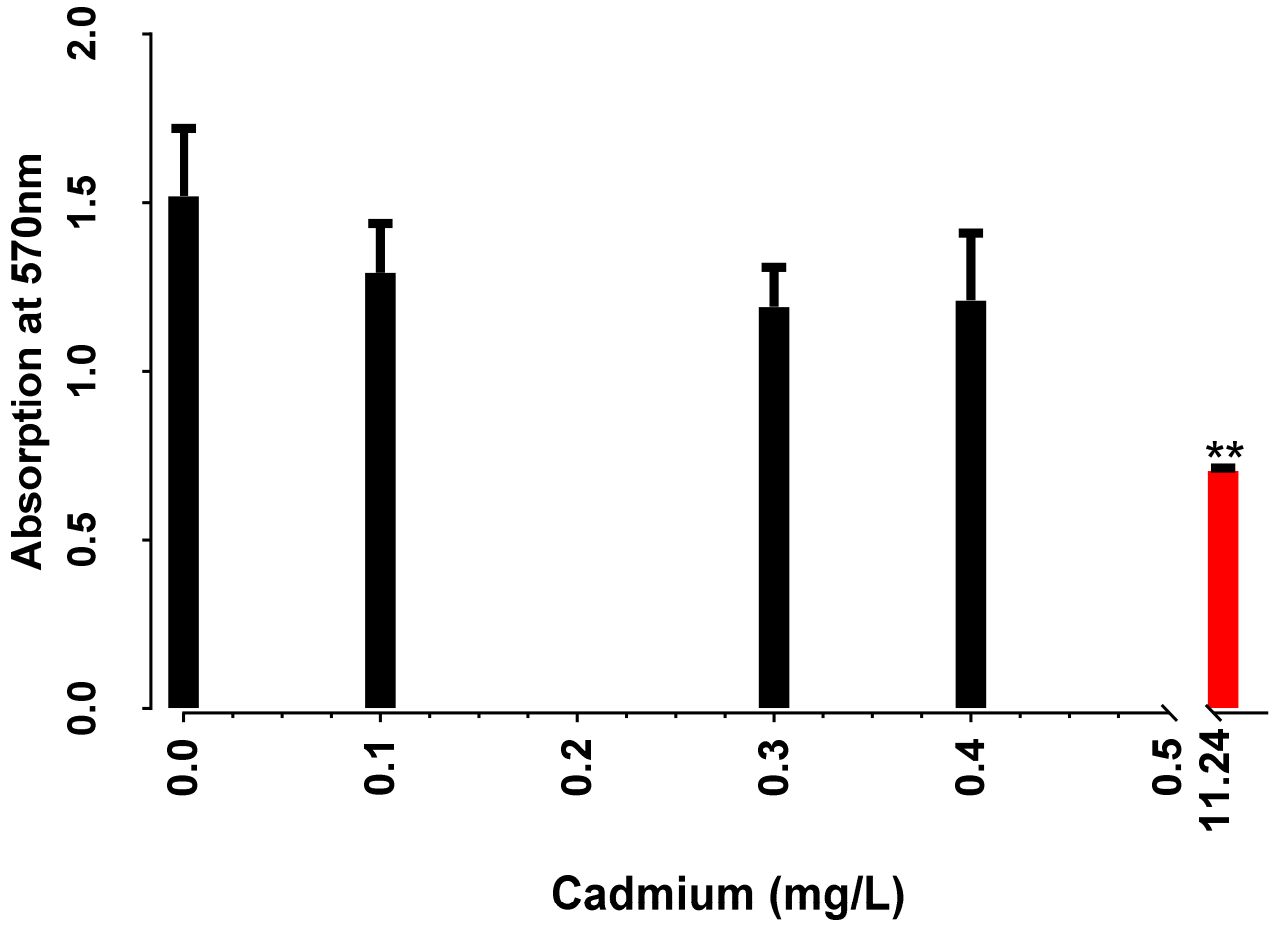


Figure 4.2. Viability assessment of cadmium treated sponge cell aggregates with 0-0.4 mg/L showed no statistically significant cytotoxicity by 1 way Analysis of variance. Mean ± SEM (Values in table 2), Cadmium cytotoxicity at higher concentration was confirmed by 100 µM positive control exposure under similar conditions, P = 0.012 at 95% CI , 0.71 ± 0.001.

4.6.2 Assessment of Cadmium Induced DNA Strand Breaks in Sponge cell Cultures

Using percentage tail intensity as the calculated endpoint, differences in DNA strand breaks between control (untreated-0mg/L) samples and treated samples (0.1-0.4mg/L) were compared. Results showed extensive DNA strand breaks that increased proportionally with increasing cadmium concentrations (Figure 4.3). Data analysis using 1-Way analysis of variance with Dunnett's multiple comparison test was statistically significant at 95% confidence interval, $P \leq 0.05$. In these exposures, % median tail intensities were measured over three experimental repeats in duplicates and concentration dependent data (Mean ± SEM) were obtained as, 1.45 ± 0.18 (untreated control); 4.04% ± 0.97 (0.1mg/L exposures), 6.98% ± 1.77 (0.3mg/L exposures) and 10.30% ± 2.61(0.4mg/L exposures). Effects of cadmium exposures were also visible as comets with migrated loop as the 'tail' (Figure 4.3a).

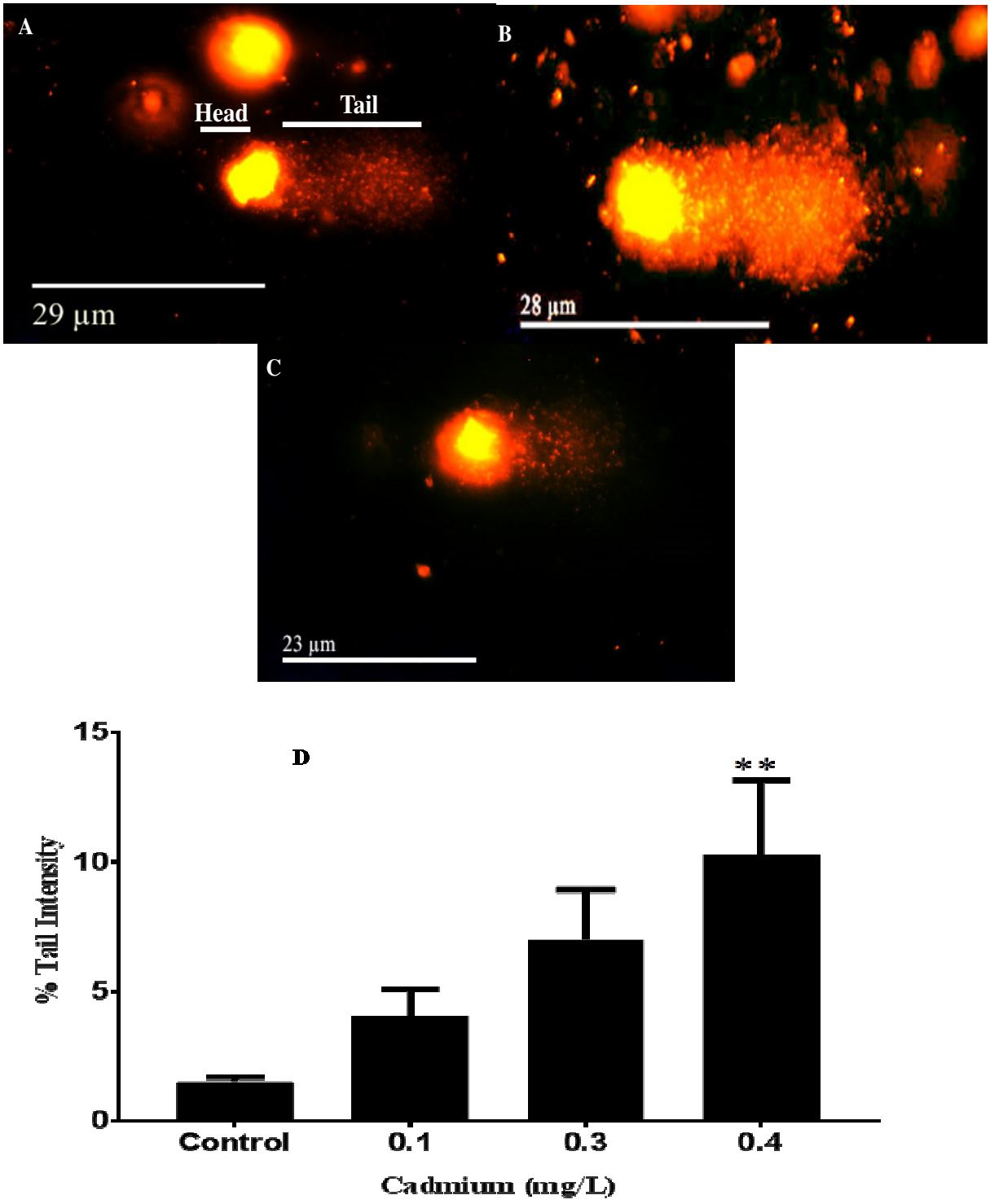


Figure 4.3 A) Representative comet images of sponge cells following 12-hours cadmium chloride exposure; a) 0.1mg/L exposure; b) 0.3mg/L exposure c) 0.4mg/L exposure . B) Comet assay assessment of concentration dependent increase in DNA strand breaks of sponge cell aggregates following 12-hours treatment with 0-0.4 mg/L cadmium. Displayed data are mean values of the percentage median tail intensity ± SEM; P*<0.05 and P**<0.01 (Statistically different from the untreated control) obtained using one way ANOVA and Bonferroni post-hoc multiple comparison test.

4.6.3 Reactive Oxygen Species (ROS) Formation in Cadmium Exposed Sponge cell Aggregates

ROS formation was quantified by measurement of oxidation of H2DCF-DA to the fluorescent product – DCF. Fluorescence intensities measured in triplicate exposures over three experimental repeats showed a concentration dependent increase in the level ROS formation. Mean DCF-fluorescent intensity ± standard error of mean (SEM) increased with increasing cadmium concentration as 12578 ± 1731 (Control or unexposed sponge cells), 16890 ± 4065 (0.1mg/L exposure); 22847 ± 3564 (0.3 mg/L) and 30764 ± 462 (0.4mg/L exposure). Using a one way analysis of variance and Bonferroni correction multiple comparison post-hoc test, there was a statistically significant increase in the amount ROS generated in cadmium treated sponge cell aggregates compared with untreated control cells at $P < 0.05$ (Figure 4.4). There was a statistically significant positive correlation ($R^2 = 0.9774$) between the mean DCF-fluorescent and percentage DNA tail intensity (Figure 4.5).

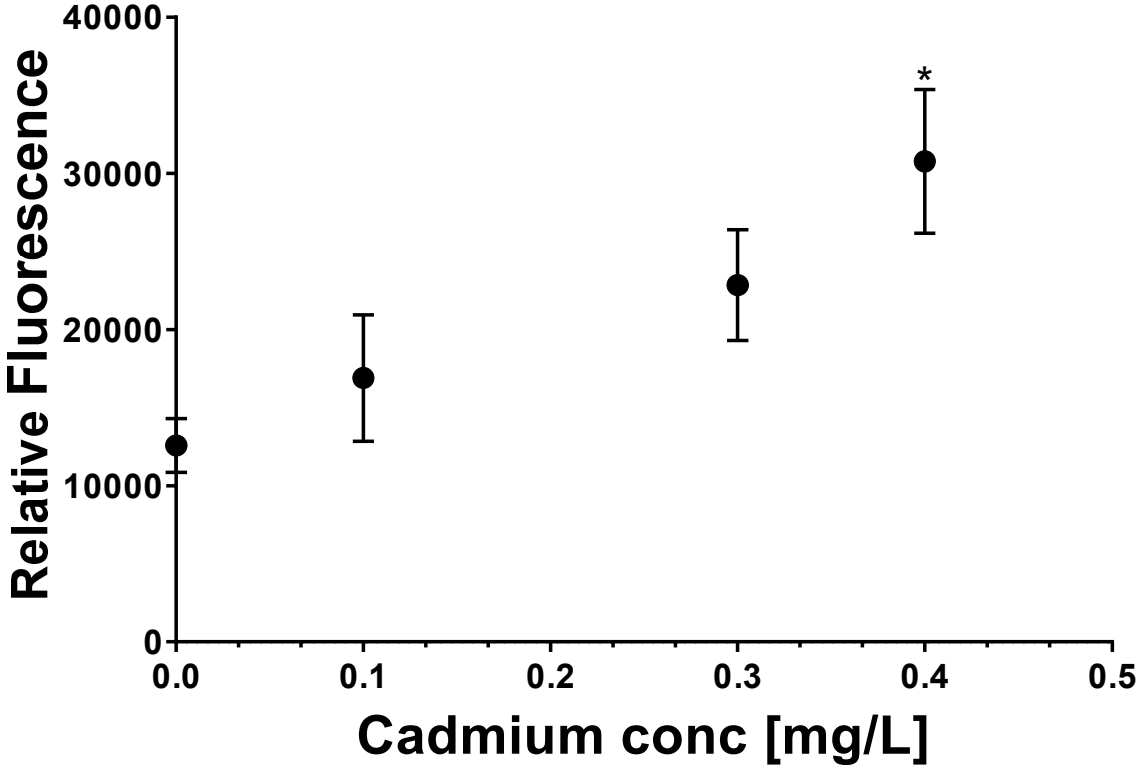


Figure 4.4 Reactive oxygen species formation in cadmium treated sponge cell aggregates. Result shows a concentration dependent increase in the amount of ROS produced with increasing cadmium concentration. Data displayed are Mean values ± SEM, n=9; statistically significant increase in DCF-fluorescence; P*<0.05; 1-way ANOVA with Bonferroni correction multiple comparison post-hoc test; triplicate exposures in three repeat experiments.

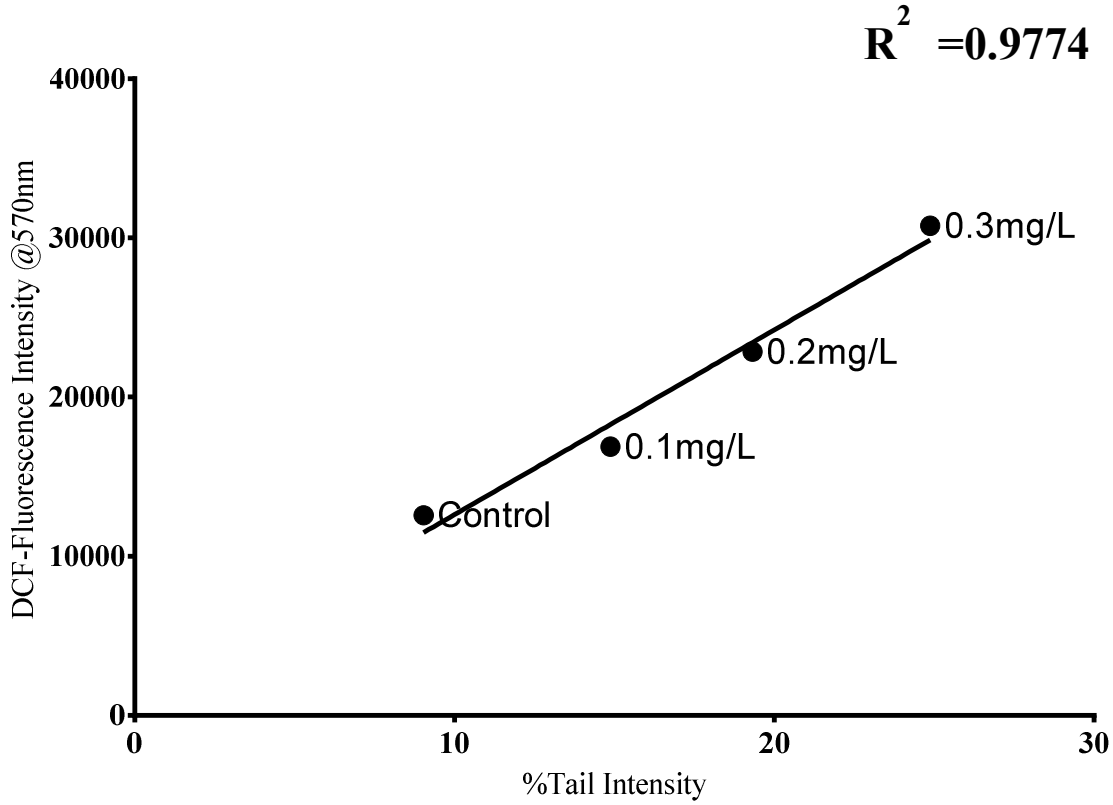


Figure 4.5 Correlation coefficient of reactive oxygen species formation versus level of DNA strand breaks in 0, 0.1, 0.3 and 0.4mg/L, respectively cadmium exposures measured over 3 repeats, showed a very strong and positive correlation (R^2=0.9774) between the two biomarkers of oxidative stress.

4.7 Chromium Exposures

4.7.1 Viability assessment of Chromium treated sponge aggregates

MTT viability assessment of chromium exposed sponge cell aggregates showed that the test concentrations (0-0.4mg/L) had no cytotoxic effect on sponge cells. One way analysis of variance of the DMSO solubilised MTT absorbance showed no statistical difference between chromium treated aggregates and control data at P>0.05 (Figure 4.6). Sponge cell aggregates were also treated with 100µM chromium as positive control to verify the workability of the assay, result showed a statistically significant difference in comparison with the control (Figure 4.6 and Table 4.2).

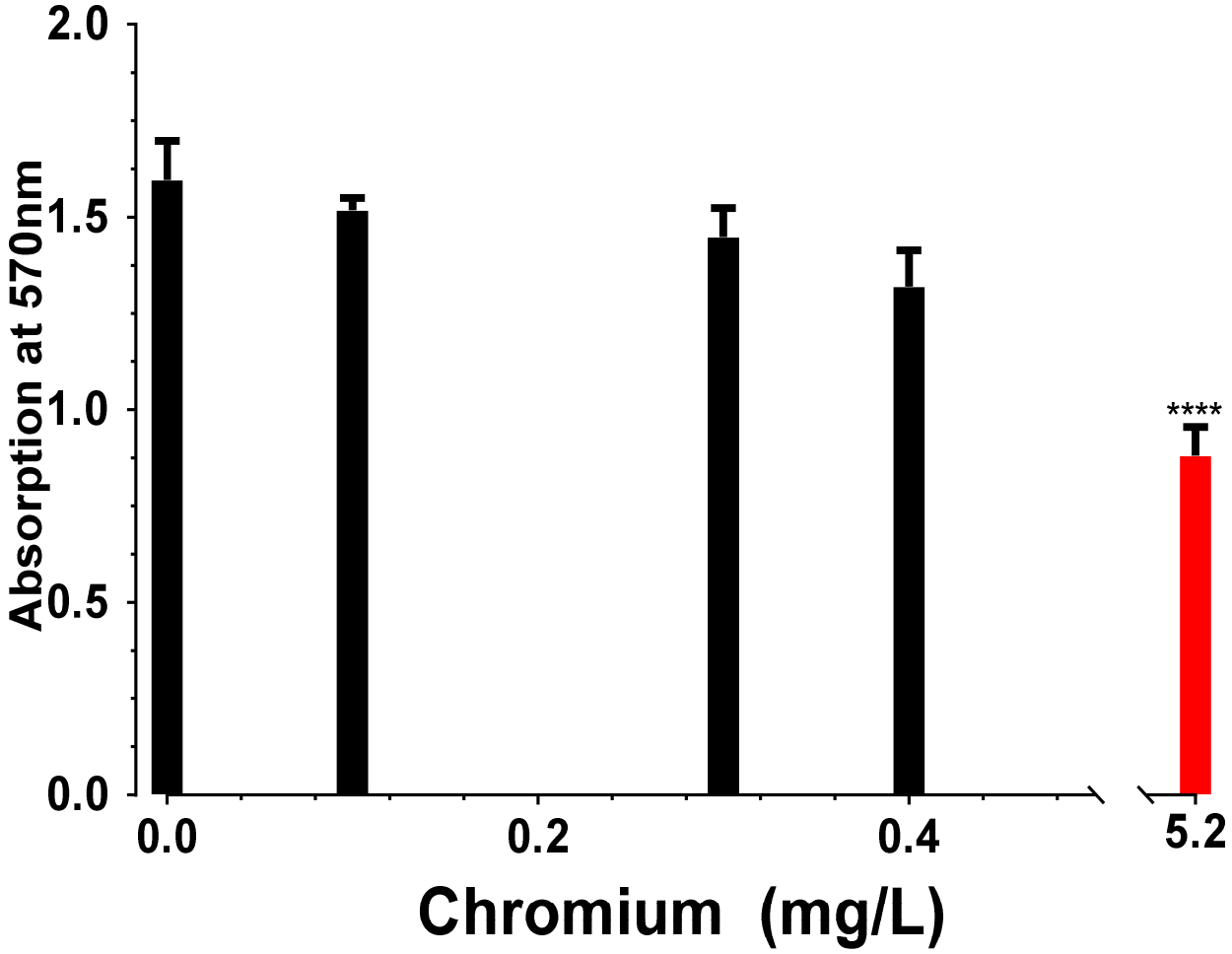


Figure 4.6 Viability assessment of $Na_2Cr_2O_7$(Sodium dichromate treated sponge cell aggregates with 0-0.4 mg/L showed no statistically significant cytotoxicity by 1 way Analysis of variance. Mean± SEM (Values in table 2), 100 µM Chromium was used as positive control under similar laboratory conditions, $P < 0.05$, n =9 Mean absorbance ± SEM; 0.88 ± 0.07.

4.7.2 Measurement of Chromium Induced DNA strand breaks in Sponge cell Aggregate cultures

Exposure of sponge cell aggregates to 0-0.4mg/L chromium solution for 12 hours showed an increasing level of DNA damage following increase in chromium concentration (Figure 4.7). In all exposure, sponge DNA with obvious comet tails was observed in all chromium exposures (Figure 4.7a). Analysed values are those of median percentage tail intensity measured over three experimental repeats in duplicate using the comet assay technique. There was a statistical difference between 0.4mg/L treatment and the control at 95 % confidence interval. Mean difference ± SEM of percentage DNA tail intensity was assessed by one way ANOVA with Dunnett's multiple correction, P = 0.0021 (as the calculated endpoint for DNA strand breaks). Result showed a statistically significant, concentration dependent increase in the level of DNA strand break. (Control – aggregates without chromium exposure) 1.93 ± 0.83, (0.1mg/L chromium treatment) 6.72 ± 3.07, 7.67 ± 2.68 (0.3mg/L chromium), and 18.09 ± 4.89 (0.4mg/L chromium treatment).

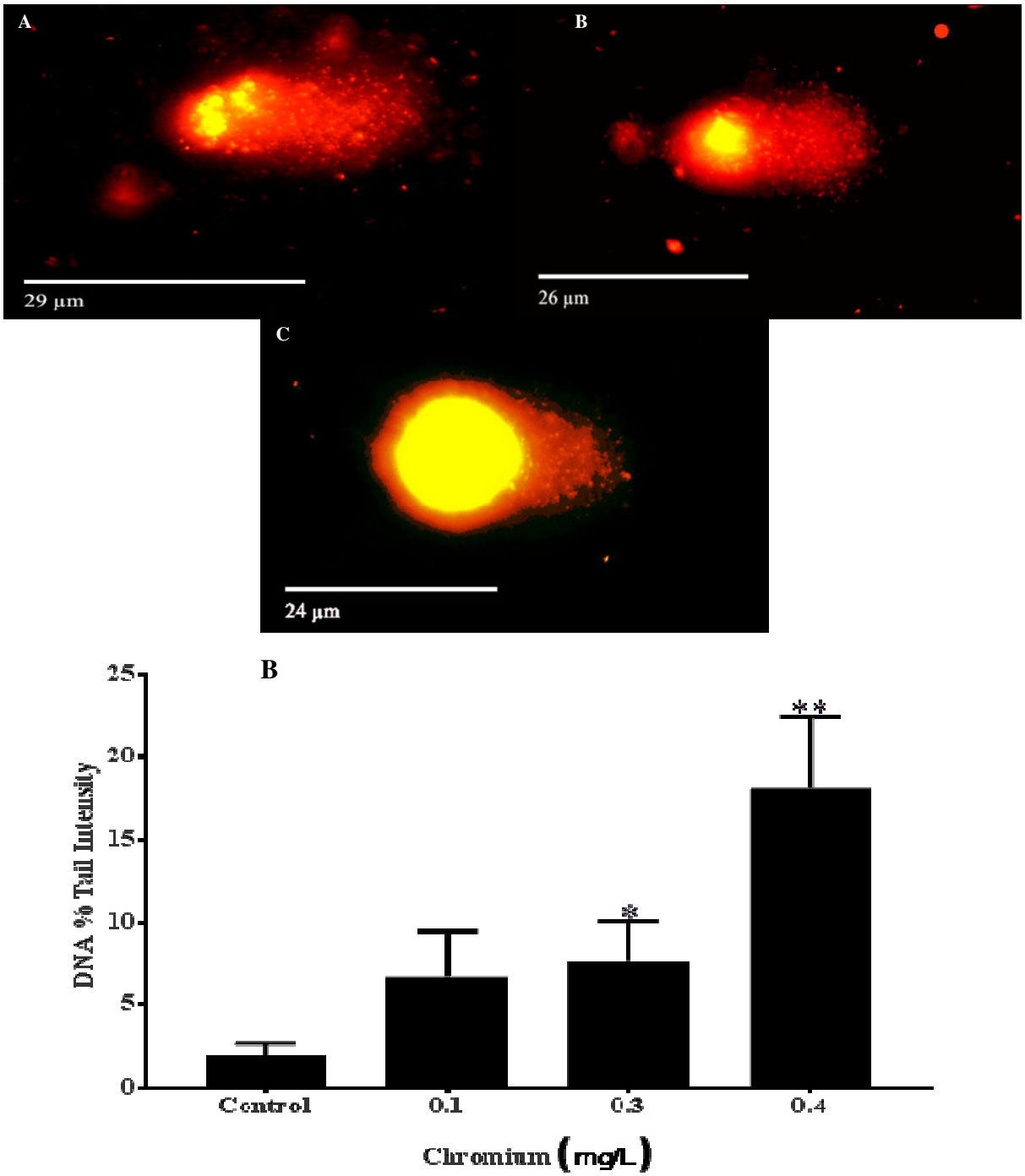


Figure 4.7 (A) Representative comet images following sponge cell exposure to chromium dichromate A) 0.1mg/L treatment; b) 0.3mg/L; C) 0.4mg/L. B) Treatment of sponge cell aggregates with 0-0.4mg/L of $Na_2Cr_2O_7$(Sodium dichromate) showed statistically significant increase in the level of DNA strand damage in treated aggregates compared to untreated control exposures. Displayed data is the Mean of duplicate exposures in three repeat experiments ± SEM (n =3); P* < 0.05; P** < 0.01 by 1-way Analysis of variance followed by Dunnett's multiple comparison test.

4.7.3 Reactive Oxygen species formation in chromium treated sponge cell aggregates

Measurement of DCF-fluorescence at 570 nm as biomarker for reactive oxygen species formation also showed a concentration dependent increase (Figure 4.8). Mean ± SEM, n=6 , were 19888 ± 1688 control; 24508 ± 1696 0.1mg/L chromium exposure; 30784 ± 1947 0.3mg/L chromium; and 31987 ± 1564 0.4mg/L chromium treatment (Figure 4.7) and correlated positively (R^2 = 0.821) with DNA strand breaks following treatments with the same concentration DNA strand damage data and reactive oxygen species formation (Figure 4.9). Assessment of Reactive Oxygen Species (ROS) with the H_2DCF-DA (2′, 7′-Dichlorodihydrofluorescein diacetate) assay statistically significant at $P^{**}< 0.01$; $P^{***}<0.001$ using one way analysis of variance with Dunett's multiple comparison test. ROS formation measured as DCF-Fluorescence was in measured in three experimental repeats in triplicate using the H_2DCF-DA assay. Analysis using 1-Way ANOVA with Bonferroni multiple comparison test showed that 0.3mg/L (P= 0.0006) and 0.4mg/L (P = 0.0002) chromium exposures were statistically significant from the control at $P^{***}<0.001$.

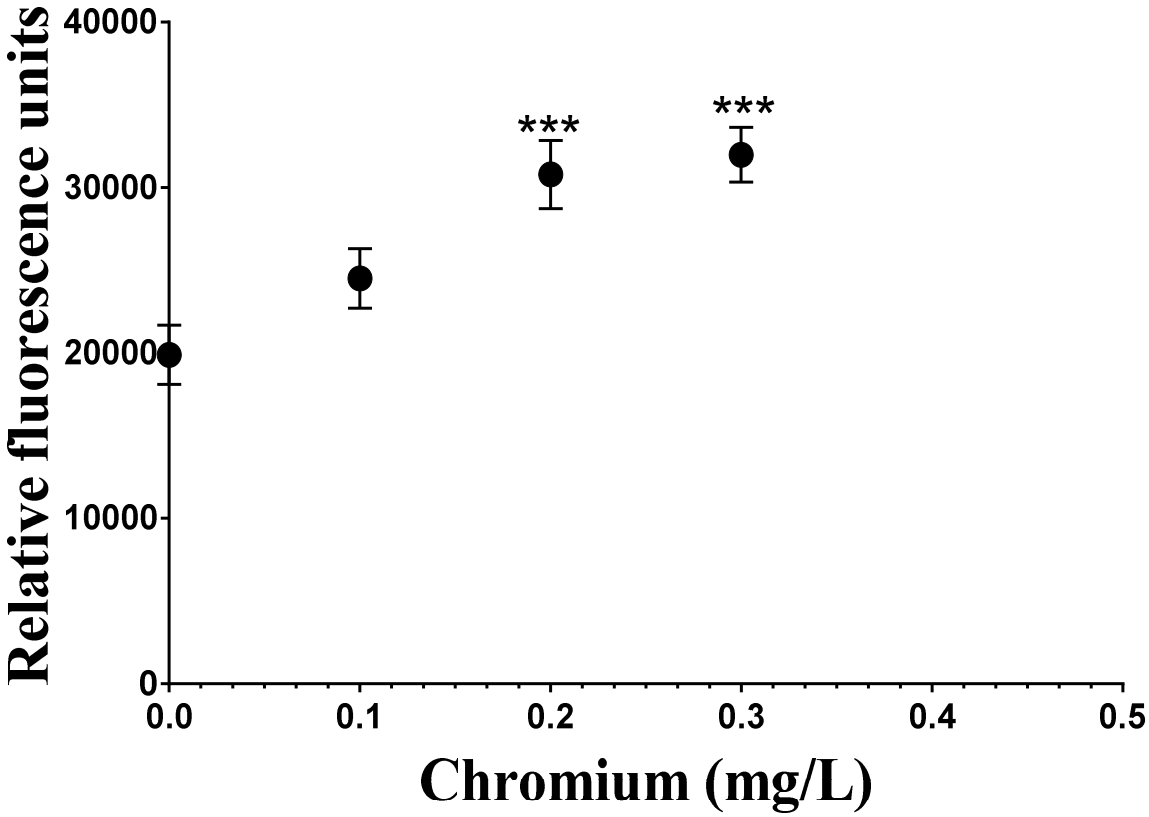


Figure 4.8 Non-cytotoxic concentrations (0-0.4 mg/L) of Chromium induces concentration dependent reactive oxygen species formation in sponge cell aggregate incubated for 12-hours. Results represent Mean DCF fluorescence of triplicate exposures measured over three technical experimental repeats (cultured sponge cell aggregates) ± SEM (n=3); statistically significant difference between chromium exposed aggregates and untreated control aggregates P =0.001 *** P < 0.001, 1-way ANOVA with Bonferroni multiple test correction.

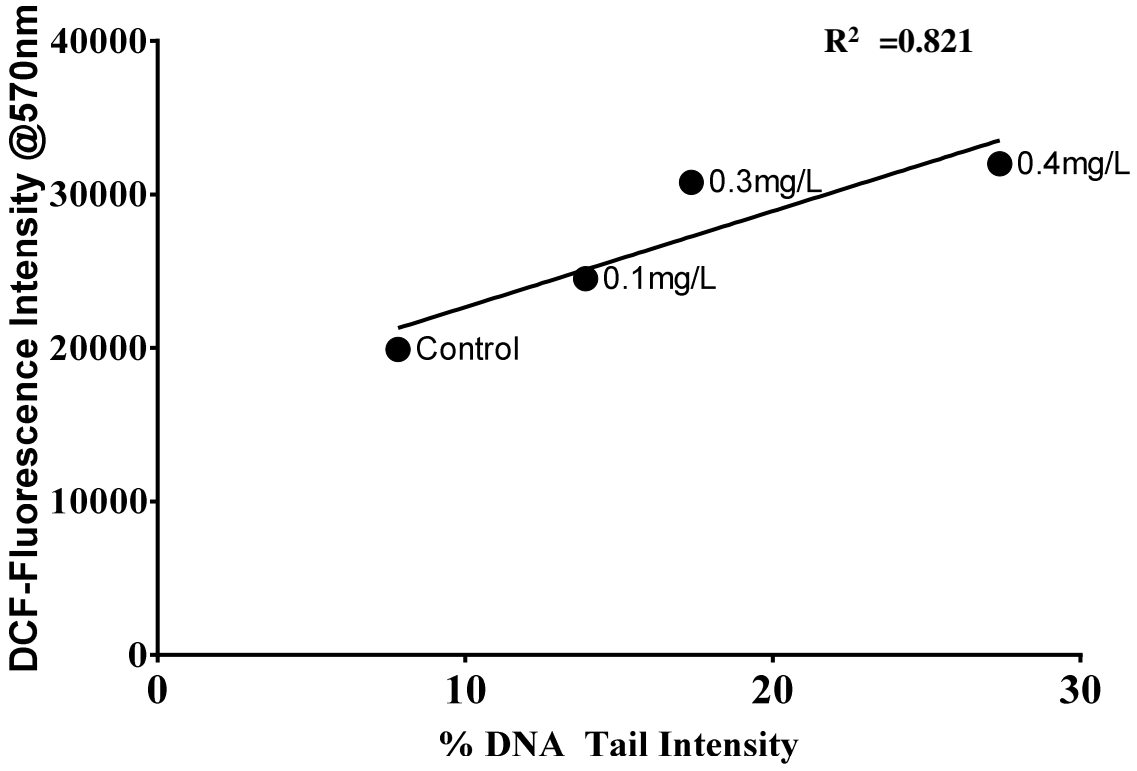


Figure 4.9 Pearson correlation coefficient of DCF- Fluorescent intensity (endpoint for Reactive Oxygen species formation), following treatment of sponge cell aggregates with 0-0.4mg/L $Na_2Cr_2O_7$, as Vs Median % DNA Tail Intensity. Results of both assays were significantly correlated (R^2 =0.821), however compared to the Cadmium data in Figure 4.5, less positive correlations were observed.

4.8 Nickel Exposure

4.8.1 MTT Viability measurement of Nickel exposed Sponge Aggregates

MTT viability assessment of sponge cell aggregates exposed to 0-0.4mg/L Nickel showed no cytotoxic effects from the exposures (Table 4.2, and Figure 4.10). Measurements were done in three repeats in triplicates, following a 12 hours' time point incubation. Data analysis using 1 way ANOVA showed no statistically significant cytotoxic effect on the sponge cell aggregates. Nickel toxicity in sponge cells was confirmed with a cytotoxic concentration of 100µM $NiCl_2$ incubation for 12 hours at room temperature. Result was statistically significant and different from all test concentration at $P < 0.05$.

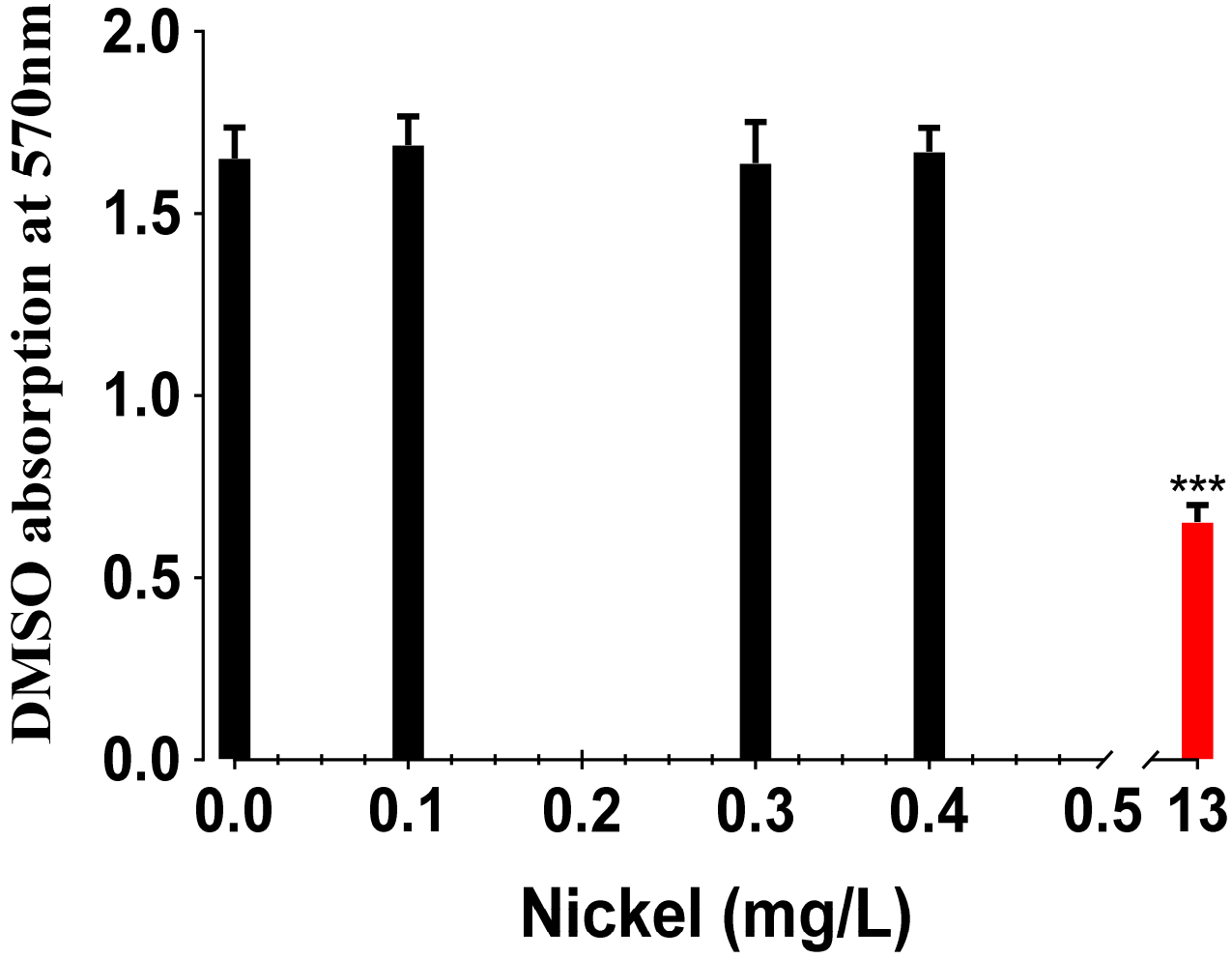


Figure 4.10 Viability of Cultured sponge cell aggregates exposed to 0-0.4mg/L Nickel (Ni) for 12 hours. Displayed data are mean absorbance at 570 ± SEM, n =9. Ni exposures had no cytotoxic effect on sponge cells by 1-Way ANOVA using Graph pad Prison version 7. 100µM positive Nickel control showed a statistically significant cytotoxic effect in sponge cell aggregates, $P < 0.05$.

4.8.2 Nickel Induced DNA strand breaks in sponge cell aggregate culture

In this study, the genotoxic effects of 0, 0.1, 0.3 and 0.4mg/L Nickel chloride in sponge cell aggregates exposed for 12-hours was investigated at room temperature, and was monitored under laboratory conditions. Observations were made over duplicate, triplicate repeat exposures, and DNA damage was quantified as Mean ± SEM of % median tail intensity using the comet assay techniques (Figure 4.11). Results analysed using Kruskall-Wallis non-parametric test and Kruskall-Wallis H test with post hoc, showed a statistically significant difference between control Nickel exposure and Nickel test exposures at $P^{*} < 0.05$. Data for DNA strand breaks at $n = 9$, $P < 0.05$, were: 1.34 ± 0.385; 2.90 ± 0.53; 3.54 ± 1.10 and 8.09 ± 2.62 for 0, 0.1mg/L, 0.3mg/L, and 0.4mg/L, respectively.

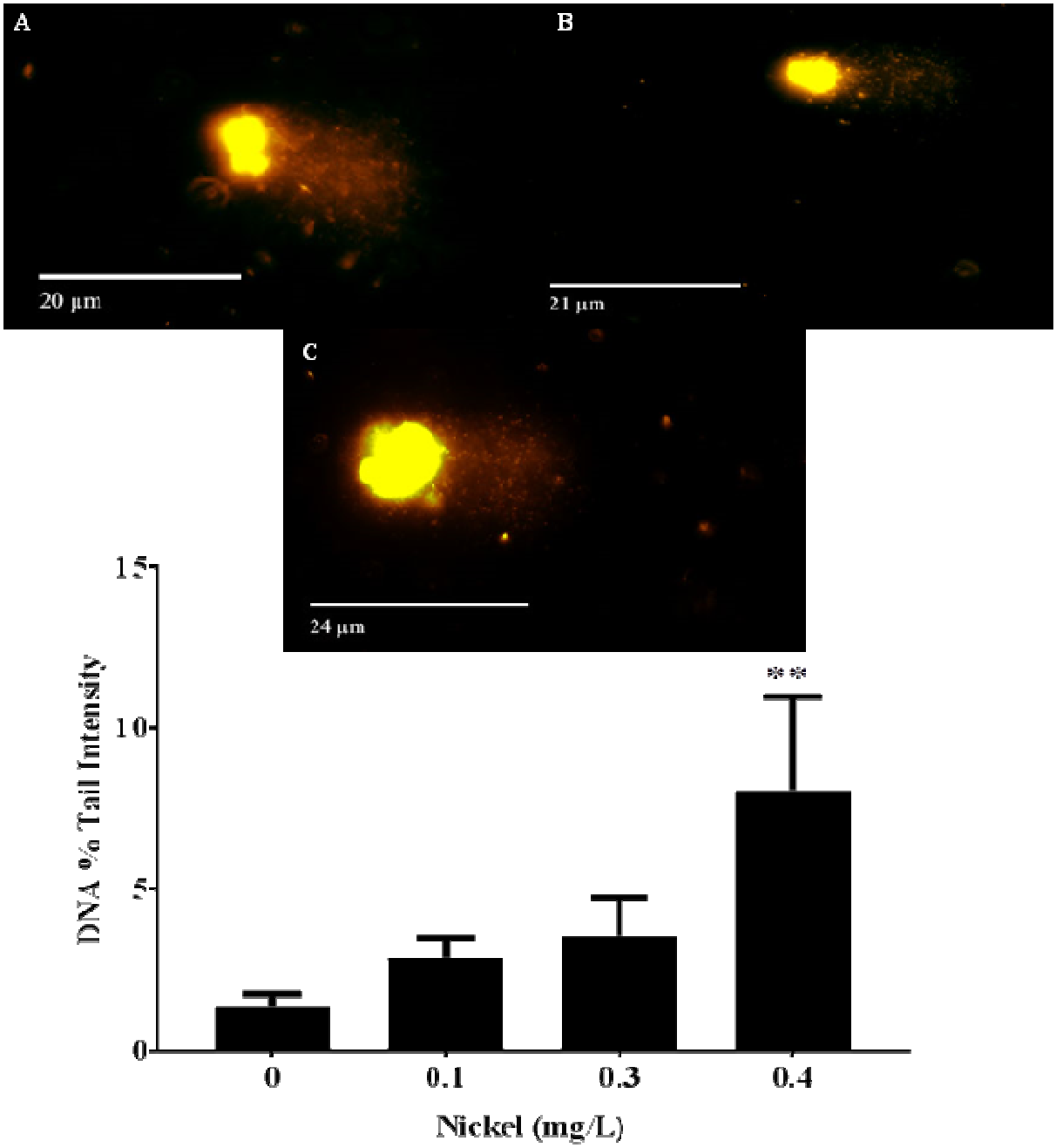


Figure 4.11.A) Representative comet images of sponge cells exposed to 0-0.4mg/L Nickel (Ni) for 12 hours. a) 0.1 mg/L b) 0.3 mg/L c) 0.4 mg/L B)DNA strand breaks in sponge cell aggregates treated with 0 -0.4mg/L of $NiCl_2$ (Nickel Chloride). Kruskall- Wallis test using SPSS 22.0showed a statistically significant increase in the level of DNA strand damage in treated aggregates compared with untreated control exposures. Displayed data are the Mean percentage tail intensity ± SEM (n= 3); P* <0.05; Kruskall-Wallis H test with Post Hoc.

4.8.3 Reactive Oxygen formation in Nickel treated sponge cell aggregates

DCF-fluorescent intensities were used as the measure for reactive oxygen species formation following exposure to non-cytotoxic Nickel concentration of 0-0.4mg/L. Mean difference ± Standard Error of Mean (SEM) were processed using I-Way ANOVA with Dunnett's multiple correction on Graph pad prism version7.03 and values obtained were ; 13796 ± 2507 for control samples. A fold increase in ROS formation of 1.16, 1.64 and 2.13 for 0.1mg/L, 0.3mg/L and 0.4mg/L, respectively were observed. Again, like previous reports of exposure to other test compounds, there was an increasing level of ROS formation with increasing Nickel concentration, (Figure 4.12) with a positive correlation ($R^2 = 0.9231$) between both data (Figure 4.13).

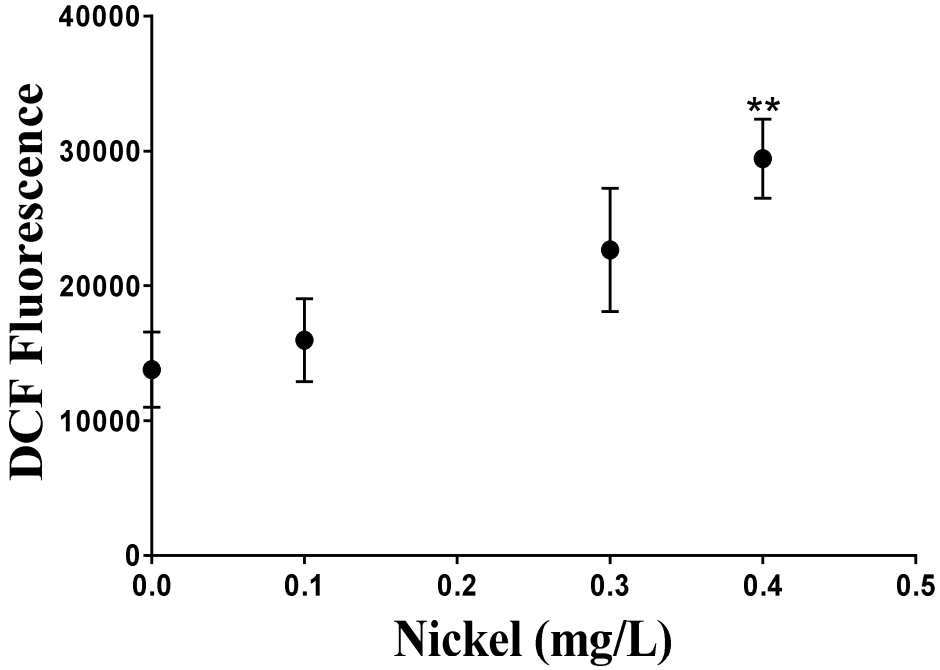


Figure 4.12 ROS levels in Nickel chloride (0-0.4mg/L) treated sponge cell aggregates over 12-hours' time points. Data represents mean ± SEM for triplicate exposures in 3 repeat experiments; n = 9, P** < 0.05 (Treatments were significantly different from untreated control samples), 1 Way ANOVA with Bonferroni correction.

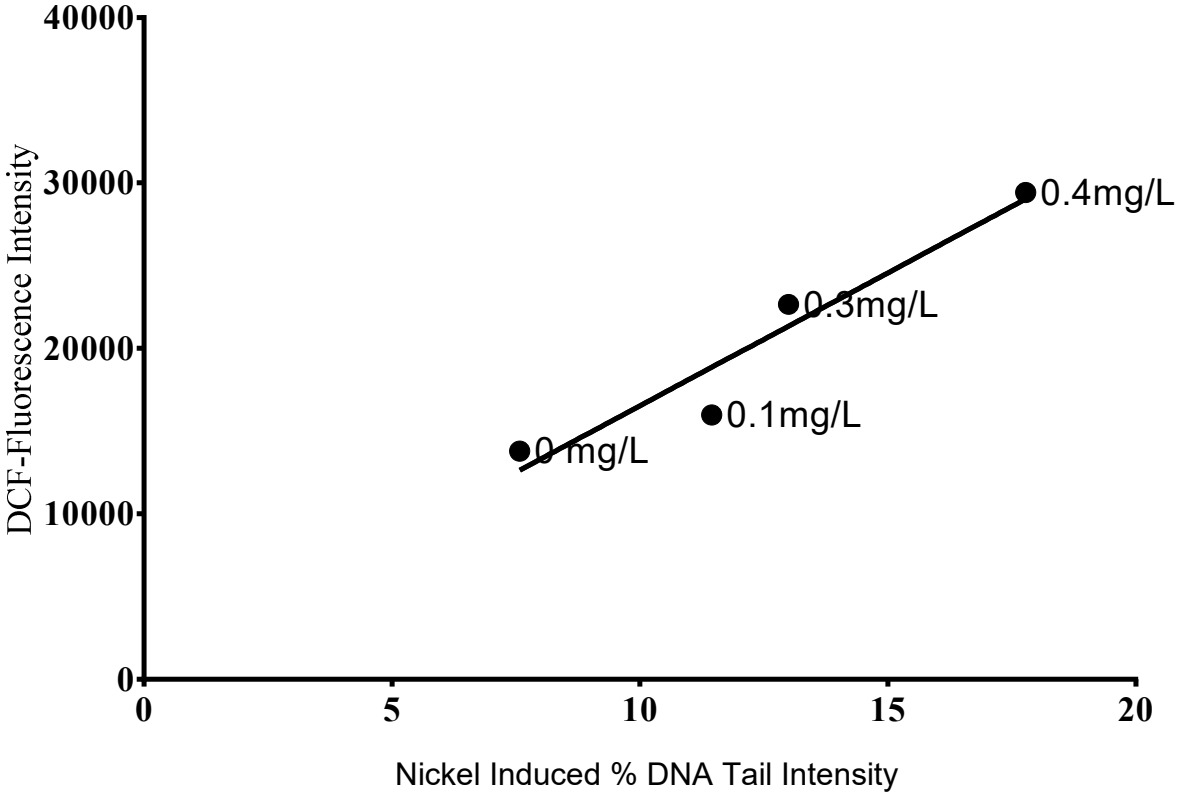


Figure 4.13 Pearson correlation coefficient of DCF- Fluorescent intensity versus Median % Tail Intensity in sponge cell aggregates exposed to 0-0.4mg/L Nickel chloride at a 12 hours' time point. Significant positive correlation ($R^2 = 0.9231$) suggest the involvement of ROS formation in DNA strand break mechanism of Nickel toxicity.

4.9 Aluminium Exposure

4.9.1 Viability assessment of Aluminium Incubated Sponge cell Aggregates

Sponge cell aggregates were exposed to 0, 0.1, 0.2, 0.3 and 0.4mg/L of aluminium chloride for 12 hours, MTT viability assessment shows that aluminium exposures (concentration and time investigated) used in these analysis had no significant cytotoxic impact on sponge cell viability (Figure 4.14). Treatment of sponge cell aggregates with 100µM of $AlCl_3$ for 12 hours also had no statistically significant cytotoxic effect on sponge cells (Table 4.2 and Figure 4.14).

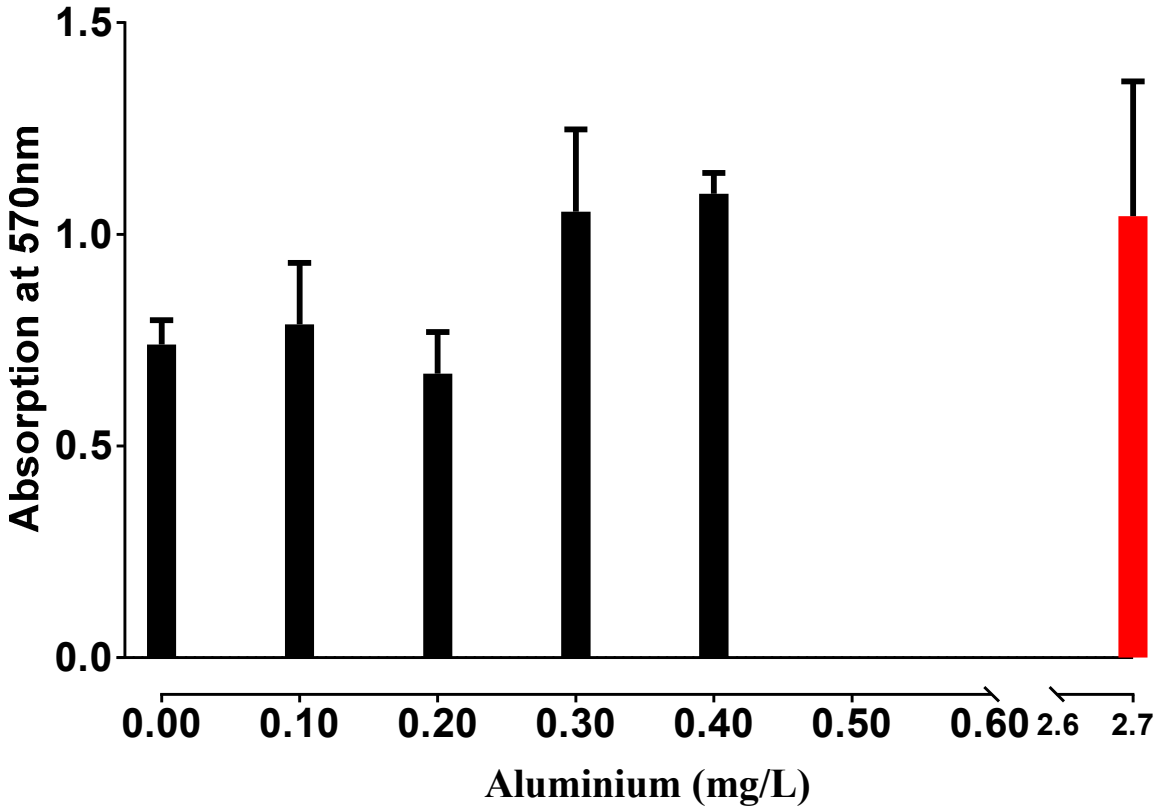


Figure 4.14: MTT viability assay of sponge cell aggregates exposed 12- hours in Aluminium III Chloride solution (0, 0.1, 0.2, 0.3 and 0.4mg/L). Data represent mean absorbance at 570 ± SEM, n =3. In all Aluminium exposures, including the 100µM had no cytotoxic effect on sponge cells by 1-Way ANOVA using Graph pad Prison version 7, $P < 0.05$.

4.9.2 DNA strand breaks and Aluminium toxicity in sponge cell aggregates

Aluminium toxicity was measured in our 'in vivo' exposure model following 12- hours' exposure of sponge cell aggregates to 0, 0.1, 0.2, 0.3, and 0.4mg/L $AlCl_3$. In all treatments (3 repeat experiments all in duplicates), sponge nucleoids with clear comets were visualised with the comet fluorescent microscope (Figure 4.15 A). 1-Way ANOVA with Dunnett's multiple comparison tests, $P < 0.05$ showed concentration dependent effects of Aluminium exposures on sponge DNA strand breaks (Figure 4.15 B) in all exposures. Aluminium toxicities in sponge cells following exposure to the concentrations above, were Mean ± SEM values of percentage median tail intensities, n=6 ; 1.79 ± 0.46 (control/untreated samples), 4.87 ± 0.93 (0.1mg/L treatment), 8.97 ± 1.59 (0.2mg/l), 14.88 ± 2.69 (0.3mg/L) and 23.09 ± 3.62 (0.4mg/L treatment).

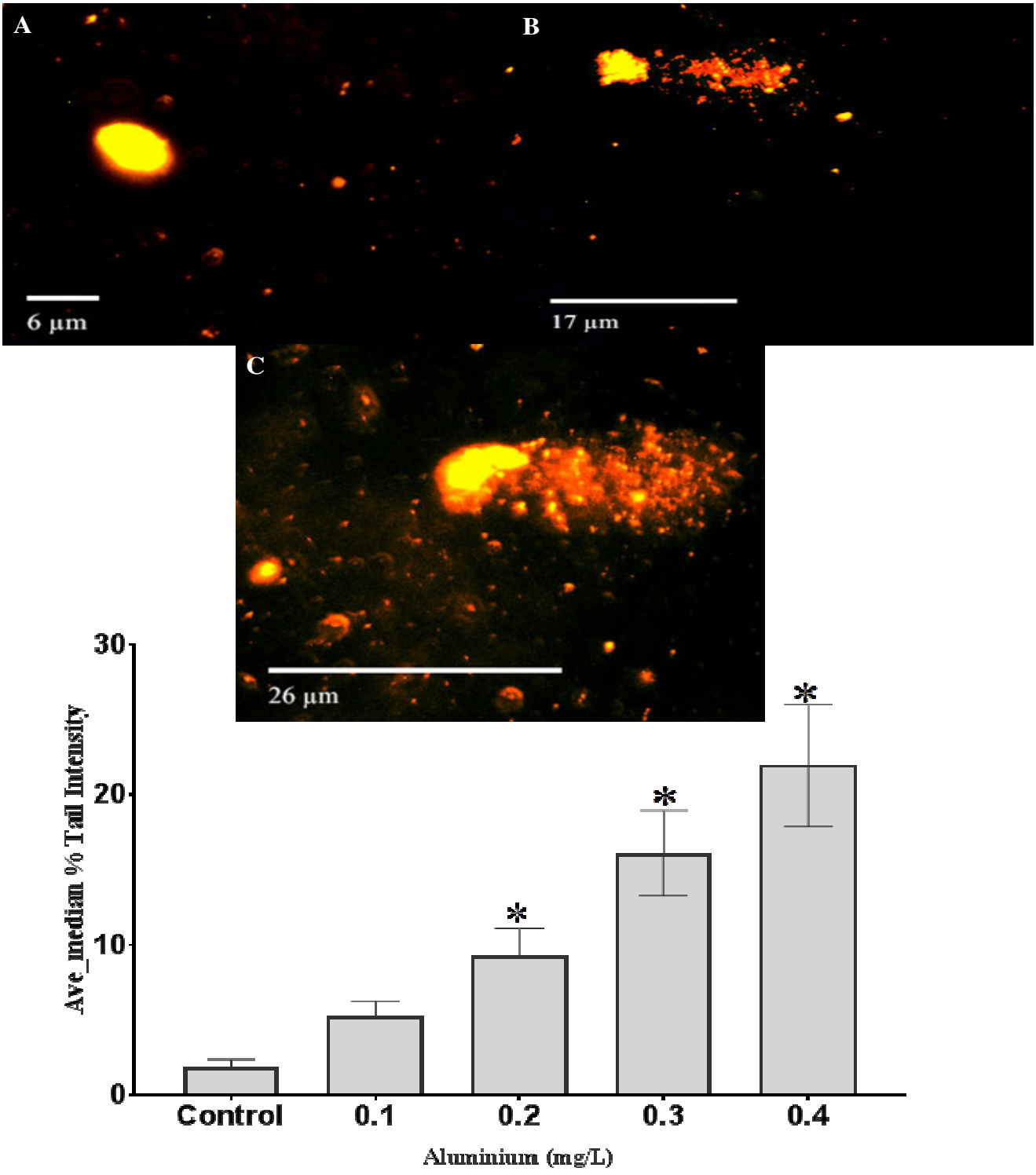


Figure 4.15 A) Representative comets images of Aluminium chloride treated sponge cells following 12 hours incubations at room temperature a) Control b) 0.1mg/L treatment; d) 0.3mg/L. B) DNA Strand breaks in sponge cell aggregate cultures following treatment with 0-0.4mg/L Aluminium Chloride for 12 hours. Displayed data shows Mean values ± SEM, n= 6; three experimental repeats each in duplicates; $P^{**} < 0.05$ 1 way ANOVA, Shapiro Wilk's test of normality $P^{*} < 0.05$. Result shows statistically significant concentration dependent increase in the level of DNA strand breaks from control samples.

4.9.3 Aluminium Induced Reactive Oxygen Species formation in sponge cell aggregates

Investigation of ROS formation consequent to Aluminium exposure was also statistically significant at $P^{*} < 0.05$ and $P^{****} < 0.0001$ (Figure 4.16) which also increased significantly with increasing Aluminium concentrations. Both DNA strand break and reactive oxygen species positively correlated (Figure 4.17) DCF-Fluorescence intensity measurements following the 12 hours Aluminium exposures were expressed as Mean fluorescence intensities ± Standard error of Mean; n =3, for control, 0.1, 0.2, 0.3, and 0.4 as 20410 ± 2956, 53776 ± 10960, 61308 ± 12071, 118495 ± 11088 and 136220 ± 4874. ROS formation was highest in Aluminium exposed sponge cell aggregates compared to other ionic test compounds, which suggest that aluminium toxicity could possibly be mainly due to reactive oxygen formation.

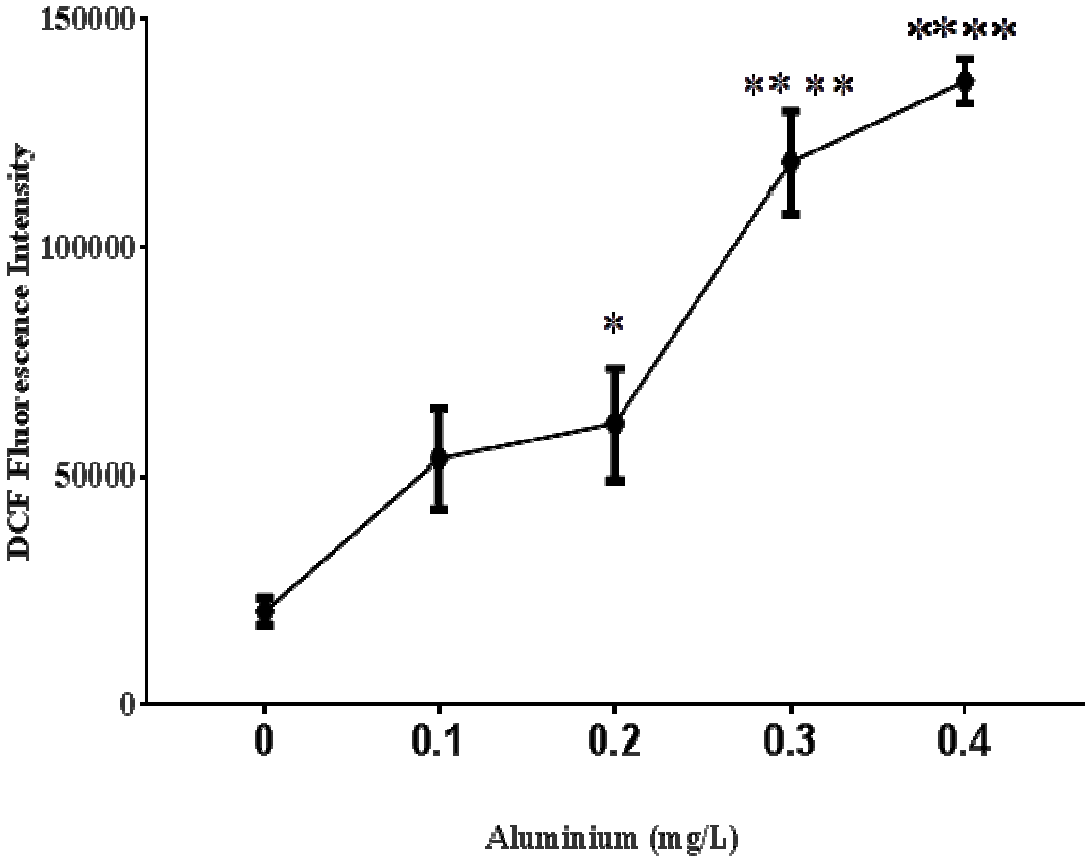


Figure 4.16 Aluminium Induced Oxidative stress in cultures of sponge cell aggregates. Amount of reactive Oxygen Species formed increased with Increasing Aluminium concentration. Data displayed are Mean values ± SEM, n = 3; statistically significant increase in DCF-fluorescence at P*<0.05 and P****<0.0001 was analysed using 1-way ANOVA with Bonferroni correction multiple comparison post-hoc test on Graph pad prison 7.0, Results represent triplicate exposures in three repeat experiments. Test of homogeneity of variance and normality performed with Levene's test and Shapiro Wilk's test using IBM SPSS.

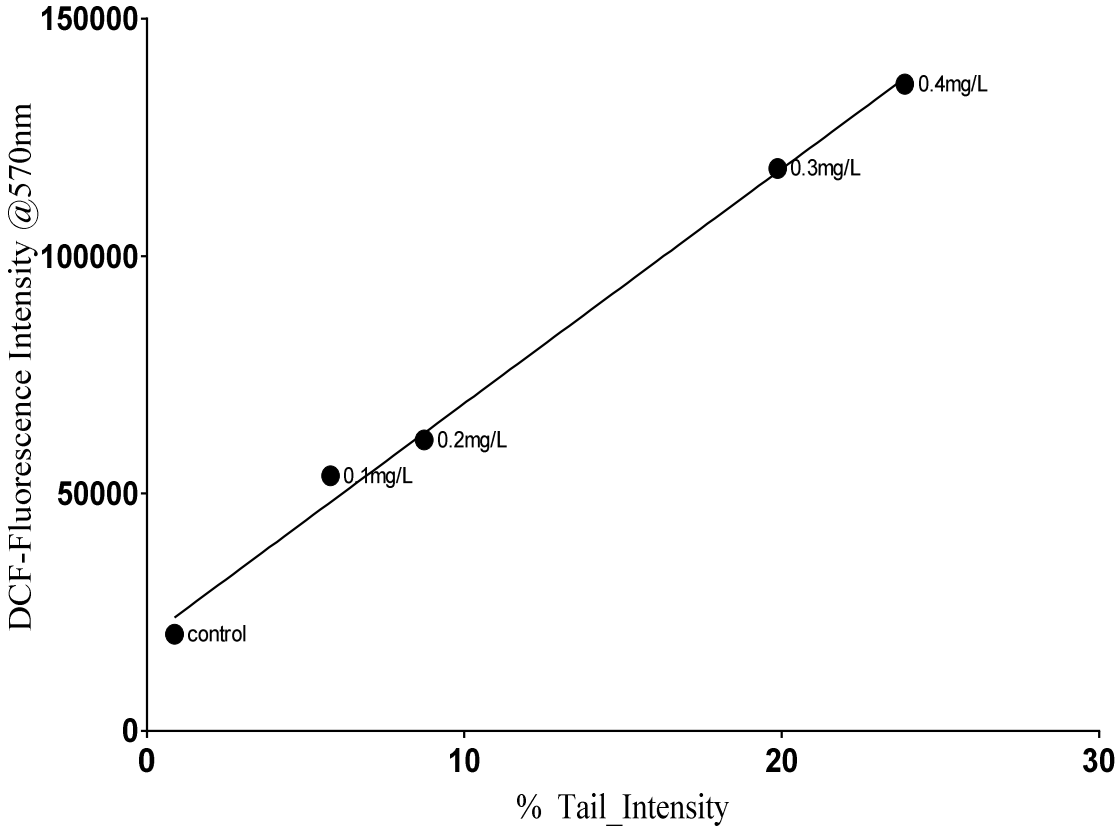


Figure 4.17 Correlation analysis of DCF- Fluorescent intensity versus Median % Tail Intensity in sponge cell aggregates exposed to 0, 0.2, 0.3 and 0.4mg/L Aluminium (III) chloride for 12 hours. Result showed very significant correlation between ROS formation and DNA strand breaks in Aluminium treated Sponge aggregates. $R^2 = 0.9974$, This is in confirmation that DNA strand induction following Aluminium exposure is primarily via reactive oxygen species formation (Henkler et al. 2010).

4.10 Benzo[a] Pyrene

4.10.1 P450 Metabolic activities in Sponges

Measurement of Benzo [a] pyrene induced DNA damage in sponge cells was compared with response following exposure to metal pollutants. Considering that B[a]P is a major inducer of the cytochrome P-450 monoxygenase family of metabolic enzymes, CYP1A1-P450 activity in sponge cell 'Microsomes' was investigated. As shown in figure 4.18, there was a clear evidence of catalytic activity towards a known substrate (Luciferin) for rodent CYP1A1.

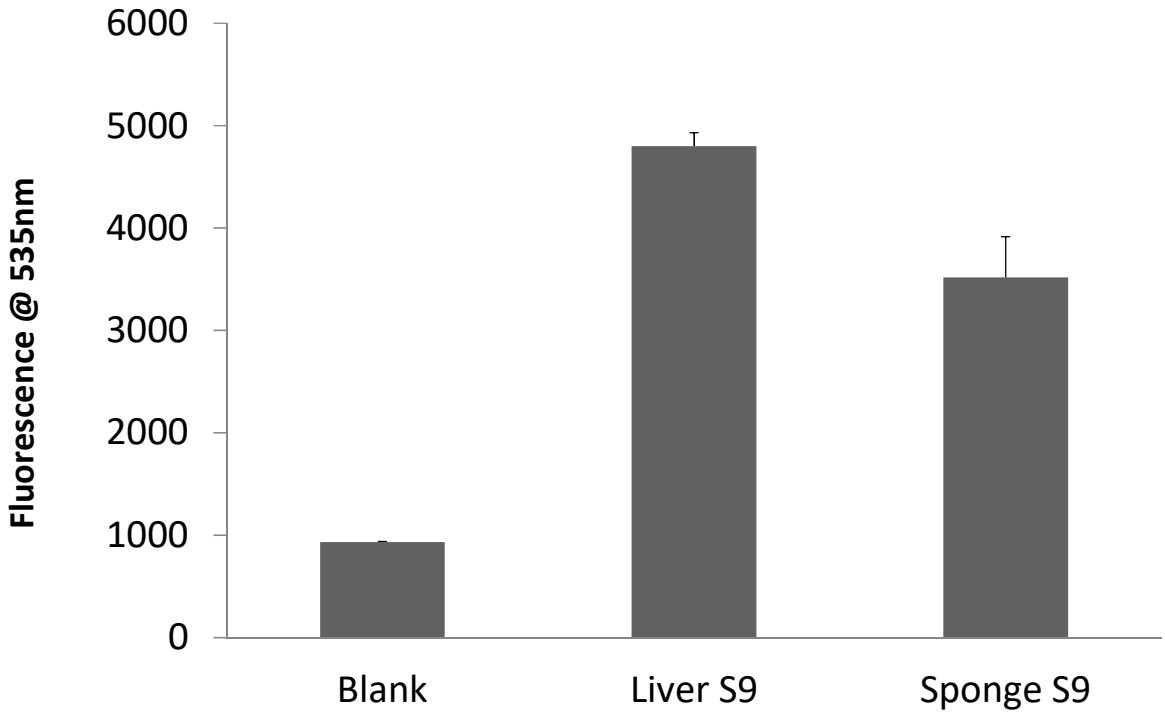


Figure 4.18 Assessment of sponge cell metabolic activity

4.11 B[a]P Viability assessment in sponge cell aggregates.

To assess B[a] P toxicity in sponge cells, cultured sponge aggregates were exposed to 2.5µM, 5.00 µM, and 10.00 µM B[a] P for 12 hours with 1% DMSO diluent exposed aggregates used as negative control. Kruskall-Wallis test of significance was employed to determine MTT viability of exposed aggregates, absorbance result at 570nm showed that test B[a]P concentrations compared to the controls had no statistically significant effects on sponge cell. However, compared to the metallic ions test compounds, B[a]P exposed aggregates were less viable, (Table 4.2, Figure 4.19).

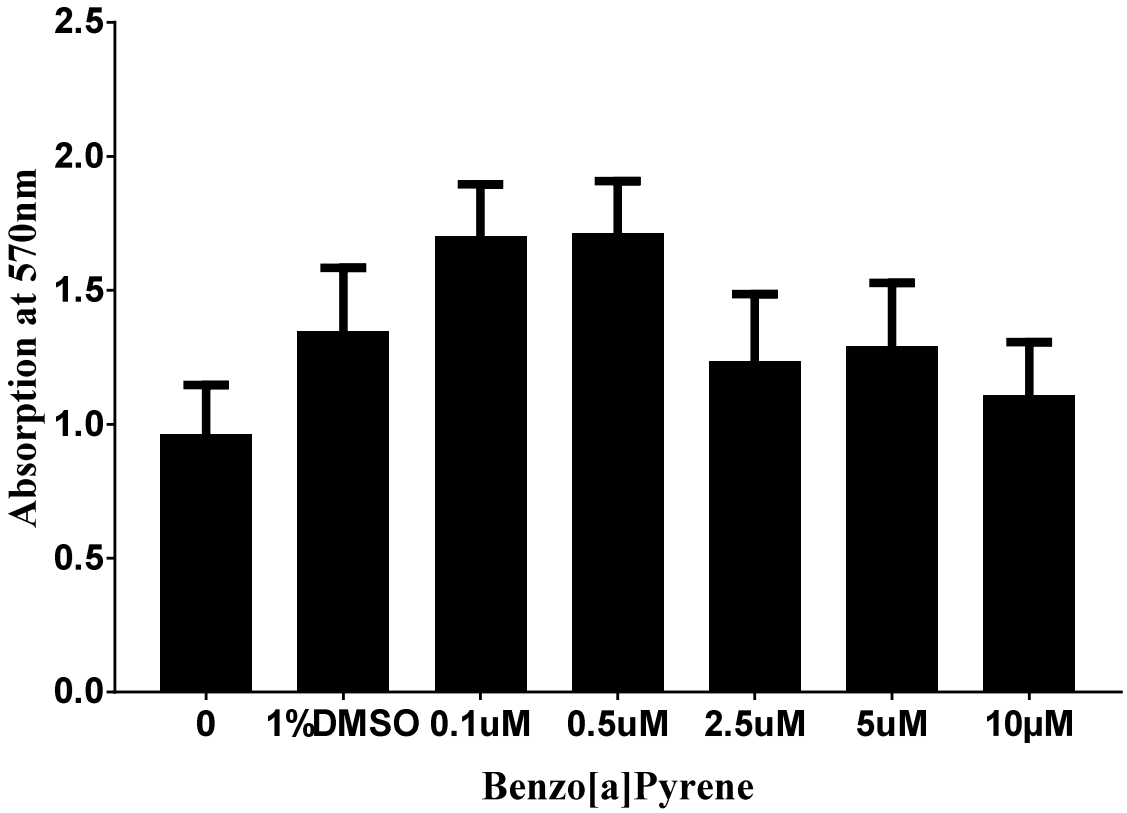


Figure 4.19 Treatment of sponge cell aggregates with 0-10µM B[a]P showed no statistically significant cytotoxic effect between exposures and control by 1 way Analysis of variance. Values are Mean ± SEM, n= 3.

4.11.1 B[a]P Induced Genotoxicity (DNA Damage)

DNA strand break investigation was performed over three experimental repeats; data analysis was with 1-way ANOVA with Tukey multiple comparisons post hoc test; Mean ±SEM measurements of percentage median tail intensity (Figure 4.20). Result showed a statistically significant difference between control treatments and 10μM B[a]P exposures over a 12-hours' time point.

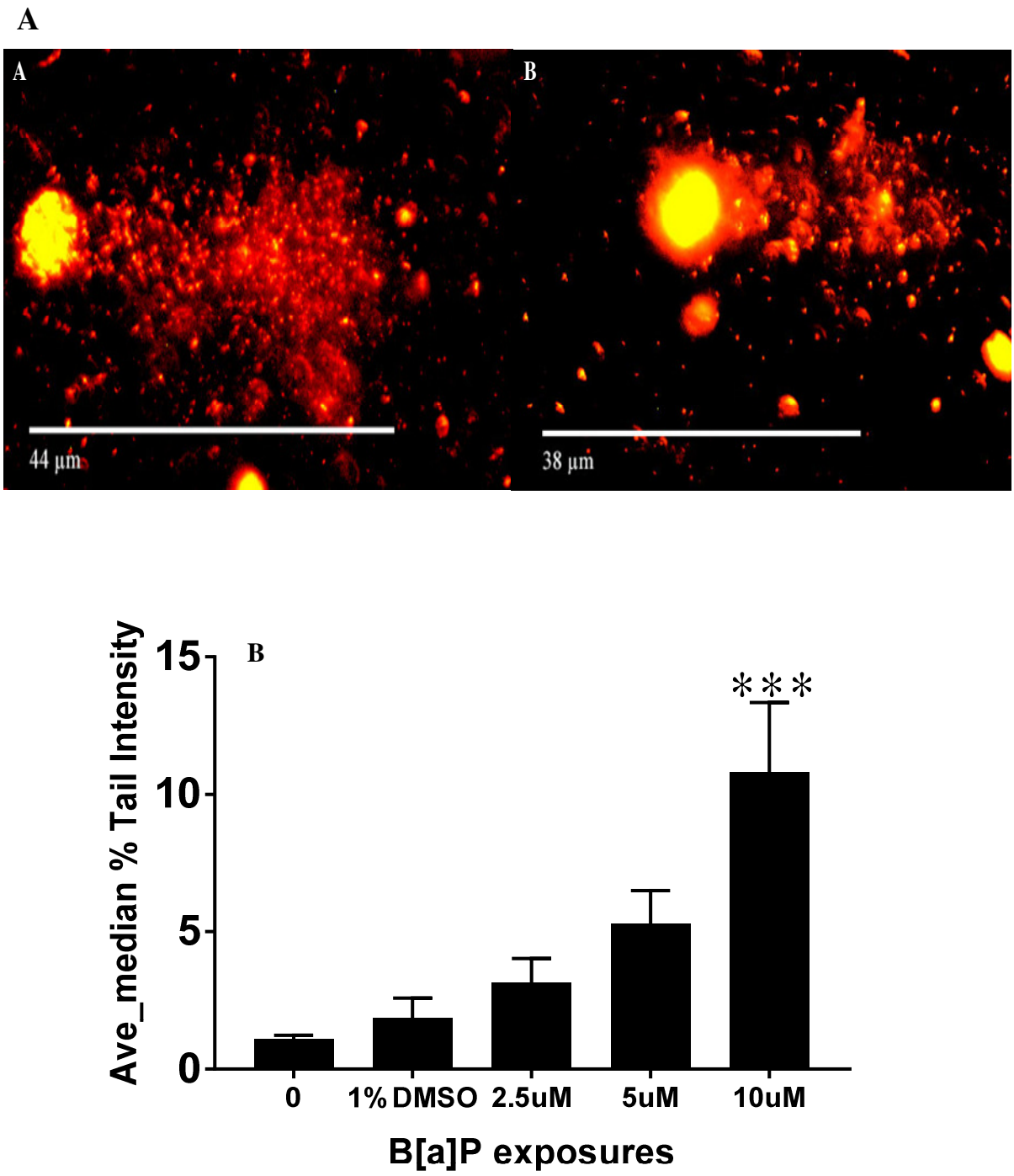


Figure 4.20 A) Benzo [a] Pyrene induced sponge cell comets. 1% DMSO: negative control b) 2.5 uM BaP. B) Comet assay assessment of DNA strand breaks in sponge cell aggregates following 12-hours treatment with 2.5-10µM B[a]P . Displayed data are mean values of the percentage median tail intensity ± SEM; P <0.05, n = 3. Kruskal-wallis analysis of data showed statistically significant difference between BaP exposures and untreated control) obtained using one way ANOVA and Bonferroni post-hoc multiple comparison test.

4.12 Variability Measure of sponge cells over two years sampling Period.

Sponge samples collected over a two year period were exposed to 0-0.4mg/L $CdCl_2$, Na_2CrO_7, NiCl2 and $AlCl_3$ and 0-10µM Benzo[a] Pyrene to assess the genotoxic effects of all test compounds and reactive oxygen formation following a 12 hours exposure time point to individual compounds. Experiments were performed using sponge samples collected over 8 months (February, March, April, May, July, August, September, and December) within the 2 year time from December 2014 to December 2016.

Inter-species variability is a major setback in the application of non-model systems in mechanistic investigations (Roberts et al. 2008). However, because of the rising demand for sessile invertebrate species in biomonitoring research and ERA in the aquatic environment, there is the need to manipulate the system to eliminate or reduce significantly interspecies variability. Currently, methods such as fragment transplantation, cryopreservation and in vitro laboratory culture with water samples from polluted environments are deployed to study biochemical processes using non-modular systems. In this project, although it was not possible to utilized just a single set of sponge cells, sponge samples were collected between the periods December, 2014 and December, 2016; isolated into single cells and cryopreserved in vapour phase liquid Nitrogen. Calculation of coefficient of variability (cv) in control data with background DNA damage showed no statistically significant percentage variation in cryopreserved single sponge cells collected and preserved for 2 years and more. This implies, that this model and this approach can be reliably reproduced in further studies and that cryopreserved sponge cells can remain viable for sensitive assays like the comet assay for up to 2 years.

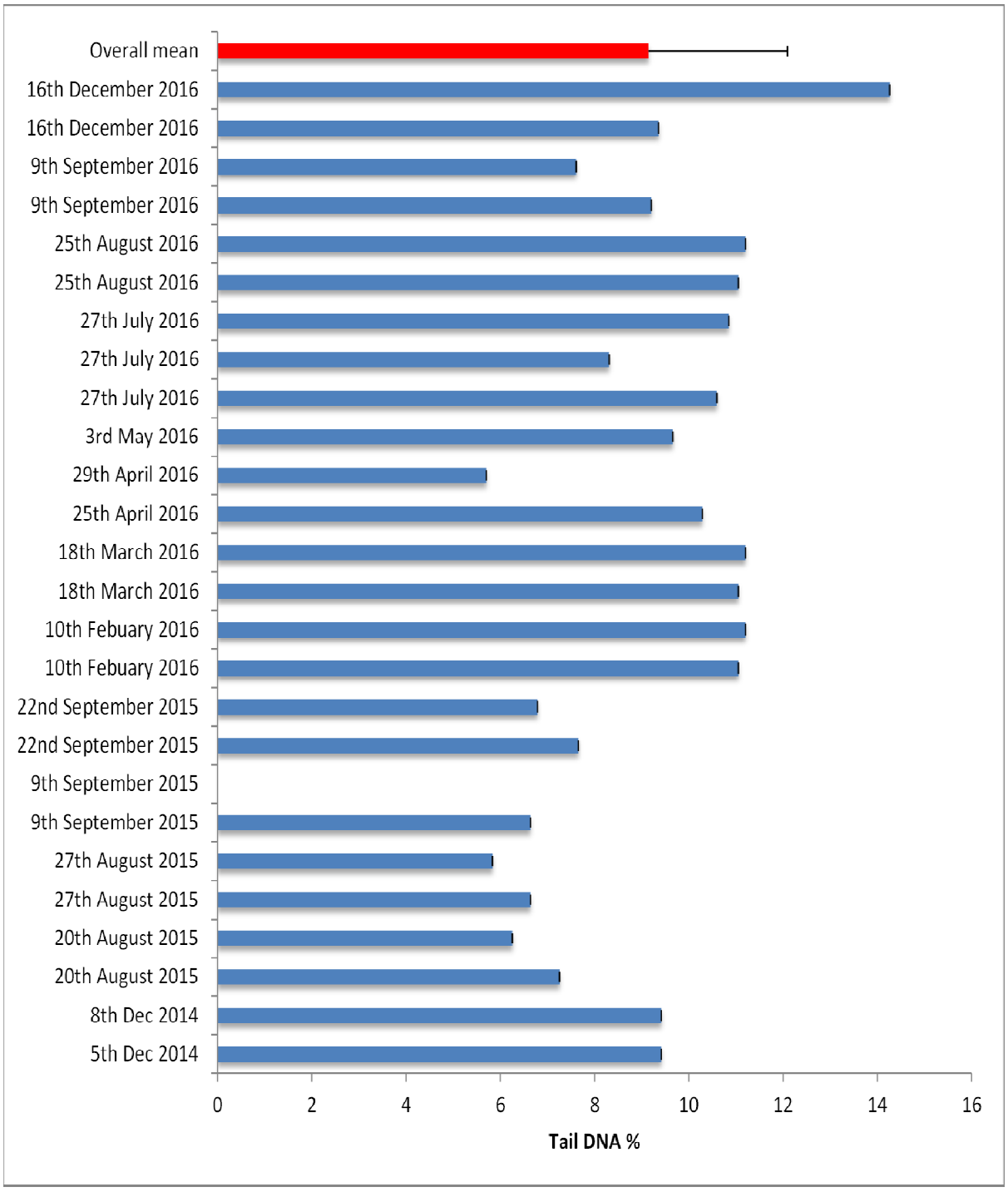


Figure 4.21: Chronological order of sponge cell control comet assay data, n = 32. cv = 29%.

4.13 Discussion

Common pollutants in the aquatic ecosystem and those with high propensity to accumulate in biogenic sentinel species include heavy metals, polycyclic aromatic hydrocarbons (PAHs), and organochlorines. There is increasing use of sessile invertebrate animals in pollution biomonitoring and Environmental risk assessment because they provide direct information of the health status of their immediate environment and information on the effects of pollutants exposures (Roberts et al. 2008). Heavy metals are of particular interest among aquatic xenobiotics for a number of reasons; first, unlike their organic counterparts, heavy metals are not readily metabolised nor are they subject to biochemical degradation, second they bio-accumulate in both sessile and mobile biogenic epifuana in levels higher than the ambient environment, Thirdly, heavy metal toxicity is influenced by a number of physico-chemical parameters in the aquatic system (Bryan and Darracott 1979; Phillips and Rainbow 1993; Rainbow 2017; Rainbow 2002); for example, increased water temperature, low pH , reduction in water hardness and oxygen depletion all aid in the enhancement of heavy metal toxicity (Shesterin 2010). In addition, heavy metals such as Cr, Cd, Ni, As, Pb, and Hg are also listed as class I (human carcinogens) IARC priority metals of interest, because of their toxic activities (IARC 1990). It is however important to note that different aquatic pollutants induce toxicities via different mechanistic pathways, for instance commonly reported metal toxicity mechanism are via the induction of metallothionein-like proteins and DNA strand breaks, depletion of cellular glutathione, lipid peroxidation, production of reactive oxygen species, induction of heat shock protein 70 (Hsp70) and glucose-regulated protein GRP78 (Wiens et al. 1998). Organic xenobiotic on the other hand are either metabolised or subsequently detoxified or they are biotransformed into non-toxic forms (Livingstone 1998).

Organic metabolism or biotransformation involves two stages; Phase I which is catalysed by Cytochrome P450 enzymes and Phase II involving conjugation enzymes. Organic xenobiotics are first oxidized in phase I and then the oxygenated xenobiotic gets conjugated by phase 2 enzymes, and eventually gets converted into hydrophillic or water soluble excreta (Goksøyr and Förlin 1992). The process of organic chemical biotransformation however, sometimes results in the activation of intermediate products of the biotransformed xenobiotic which then interacts with cellular macro-molecules (Livingstone et al. 2000). This interactions are the main precursors of organic pollutants toxicity, resulting in mutagenic, genotoxic and carcinogenic outcomes (Goksøyr and Förlin 1992).

Previously, environmental pollution reports were based just on the assessment of the presence and levels of pollutants in the environment, recent advancements in ecotoxicology however, are now geared towards understanding the hazards and risks associated with different levels of these pollutants and their deleterious effects on indigent species. This information as well as knowledge of the presence and levels of these contaminants is useful for application in ERA, Environmental biomonitoring and for enacting sustainable environmental policies (Martins and Costa 2014). Also, most of the data from which bulk of environmental policies are formed are mainly from model vertebrate species and from in vitro laboratory exposures, new guidelines and environmental standards now requires the deployment of 'batteries' of species across the entire aquatic strata, especially sessile aquatic invertebrates in order to fully observe and understand the distribution and the direct effect of different aquatic pollutants on the local biota (De Mestre et al. 2012). In this respect, commonly utilised sentinel invertebrates for environmental assessment of pollution and the associated risks is mussels and fish among sessile and pelagic vertebrates. This is because they share a number of biochemical and physiological features that can be easily extrapolated in higher phyla. Other

popular invertebrate groups with extensive application in biomonitoring research are gastropods especially bivalves (Phillips and Rainbow 1993; Phillips and Rainbow 2013). Sea sponges are another emerging group with increasing applications owing to their ability to accumulate wide range of aquatic pollutants in both suspended and dissolve phase. Sponges are one of the oldest metazoan groups still in existence today; they have survived different environmental changes that have seen the extinction of some individual species and even entire phyla (Van Soest et al. 2012). Given their sessile and extensive bioaccumulation and water filtration potentials, sponges have earned the right to be enlisted as bio indicators and as tools for environmental biomonitoring and tools in ERA. Because they are unable to escape pollution sources, sponges and other benthic aquatic invertebrates provide information on both bio accumulated (levels in surrounding water medium) and bioavailable (within the animal tissue and responsible deleterious effects) amount of aquatic pollutants. Information on bioavailable pollutants are required in effect based research and major tool ERA (Gentric et al. 2016; Van Soest et al. 2012). Also, in agreement with Marine Strategy Framework Directive on Environment, sea sponges are useful tools for bioremediating environmental pollutant owing to their ability to bioaccumulate environmental pollutants (Ferrante et al. 2017). And they have been recommended for applications in environmentally friendly biological techniques, especially heavy metals and organics pollutant monitoring (Berthet et al. 2005b).

Currently, assessing the genotoxic effects of environmental pollutants on indigenous biota has been the major target of ERA. Genotoxicity is a reoccurring biomarker of effects in ERA, it is a reliable endpoint for extrapolating the effect from exposure to environmental xenobiotic on living system and can be quantified using different biological assays such as the sister chromatid assay, Ames test, alkaline comet assay, alkaline elution assay etc. (Martins and

Costa 2014). Although each listed assay type has its advantage, the comet assay is advantageous for a number of reasons such as; increased sensitivity to stressors, ability to detect specific classes of DNA damage e.g. oxidative stress lesion and bulky DNA adducts, application in quantifying stress level in individual cells which allows for the study of the heterogeneity of the response, and assessment of genome integrity. In this study we deploy the comet assay technique to measure DNA strand breaks induced by environmentally relevant xenobiotics in cultured cryopreserved sponge cells.

In the coastal aquatic environment, pollution results from varying anthropogenic sources and although there may be some investigations with the potential for matching specific toxicants to their particular sources; the emphasis in ERA as it applies to the aquatic ecosystem is in whether or not an identified pollutant has an adverse effect on the biota and on humans by extension. Hence, although the comet assay has a few setbacks in terms of lack of specificity (Chapman et al. 2013), it is still a very potent tool extensively utilised in ecogenotoxicology; mainly because it is able measure heterogeneous stress response in individual cells exposed to environmental toxicants, in a clear, visible and easy to interpret manner (Martins and Costa 2014).

In this study, we have deployed the comet assay (previously developed in the preceding chapter) to investigate DNA strand breaks as a biomarker of genotoxicity following exposure of sponge cell aggregates to non-cytotoxic concentrations [0,0.1,0.3 and 0.4mg/l (Cd, Cr, and Ni)]; (Figures 4.3, 4.7, and 4.11 respectively) 0, 0.1,0.2,0.3 and 0.4mg/L (Al) and 0, 1%DMSO, 2.5µM, 5 µM and 10 µM (B[a]P)](Figure 4.19) of environmentally relevant genotoxicants (Cd, Cr, Ni, Al and B[a]P) . To the best of my knowledge, this study is first to investigate using the comet assay DNA damage induced by these environmental pollutants sponge cells. Also, although information exists in literature on the deployment of *H.perlevis*

for various mechanistic investigations (Kimball and Webster 2001; Nguyen and Tepe 2009; Zheng et al. 2006), this would be the first study to assess its response to the pollutants listed above using the comet assay technique and the H_2DCF-DA assay to quantify reactive oxygen species (ROS) (Akpiri et al. 2017) .

Comparative studies in literature on exposure of invertebrates to genotoxic pollutants and the use of bio assays to detect their oxidative stress impacts exist. In vivo exposure of the marine gastropod, *Neri chamaeleon* to different cadmium concentration showed significant increase in the level of DNA single strand break and DNA integrity by the comet assay and alkaline unwinding assay (Sarkar et al. 2015). Threefold increase were observed for exposure to 1, 10, 25, 50 and 75ug/L Cd for three days (Sarkar et al. 2015). Also, a dose dependent increase in DNA tail damage in the fresh water perch *Anabas testicudineus* exposed to increasing concentrations of cadmium has also been reported, with highest tail DNA damage recorded for 2.0mg/L exposure for up to 4 days (Ahmed et al. 2010). Coughlan et al. (2002a) also exposed single cells obtained from both gills and digestive glands of manila clam (*Venerupis philippinarum*) to sediment from both polluted and unpolluted sites for 3 weeks. Measures of DNA damage by comet assay showed a statically significant difference between cells exposed to sediments from polluted sites compared to those from clean sites. Ahmed et al. (2013) exposed the fresh water stinging fish to potassium dichromate for 48, 96 and 192 hr time points to assess both acute toxicity and genotoxicity of chromium 35.724mg/l LC_{50} value was recorded after 96hrs exposure. Concentration ranges of 8.931mg/L ($1/4^{th}$ of the LC_{50}), 3.5731mg/L ($1/10^{th}$ of the LC_{50}) and 1.6mg/L (chromium concentration in the study environment) were utilized to study chromium genotoxicity in the fish *Heteropneustes fossilis (Ahmed et al. 2013)*. Their results showed both dose and time dependent increase in percentage DNA tail damage.

In vitro exposure of earthworm (*Lumbricina*) coelomocytes to 2, 6, 12mg/mL $NiCl_2$ showed significant difference between Nickel treated cells and control untreated cells. However, no statistically significant difference was observed between the doses of 2,6 and 12mg/L (Reinecke and Reinecke 2004).

Main sources of cadmium in the environment are from electronic batteries, colour pigment from paints, and cigarette smoke (Nriagu 1980; Scarponi et al. 2000),. Considering that, cadmium has no known biological function and has a half-life of about 30 years; its rising environmental level gives serious cause for concern (Stohs and Bagchi 1995). Mechanism of cadmium toxicity ranges from inhibition of DNA strand damage repairs to depletion of intracellular glutathione and lipid peroxidation (Hassoun and Stohs 1996; Henkler et al. 2010). Although Schröder et al. (1999) reported the lack of clearly defined relationship between reactive oxygen production and DNA damage following cadmium exposure, and also suspected antagonistic relationship between cadmium activity and reactive oxygen scavengers; our result showed a strong positive correlation (Figure 4.4), which suggests that cadmium induced DNA damage observed in this study may have been triggered in part by reactive oxygen species. This is in agreement with other reports (Henkler et al. 2010). Both percentage DNA strand breaks and reactive oxygen species formation induced by cadmium were statistically significant in a concentration dependent manner. However, compared to other metal ions tested in this study, cadmium treated sponge aggregates had the least amount of reactive oxygen species production. Direct mechanism of cadmium induced oxidative stress have been linked with its potential to replace essential elements like iron and copper in biomolecules and enzymes, resulting in lipid peroxidation (Henkler et al. 2010; Manca et al. 1991). Thus, cadmium produces ROS via different mechanistic pathways, prominent path being lipid peroxidation via an indirect replacement of essential ions (Shimizu et al. 1997;

Stohs and Bagchi 1995). Cadmium induced ROS formation therefore, is not directly involved cadmium induced carcinogenicity. Inhibition of DNA damage repairs and DNA methylation alterations are considered more direct mechanism of cadmium induced toxicity (Henkler et al. 2010). We also report preliminary measurement of thiobarbituric acid reaction lipid peroxidation following exposure of sponge cells to 0-0.4mg/L cadmium chloride for 12 hours. Dose dependent increase in malondialdehyde (MDA) fluorescence was observed up to 3mg/L exposure which then decrease from 4mg/L exposure (Data not shown).

Chromium is useful in a wide range of industrial and domestic applications. It is a component of some industrial raw materials, such as steel, alloys, paints and several other materials; however some forms of chromium are established carcinogens and are toxic to both animal and human systems (IARC 1990; Stohs and Bagchi 1995; Wise et al. 2016). Chromium exist as valency of -2 to +6, with Cr (III), (V), and (VI) being the most stable forms and Chromate (VI) most dominant form in aqueous solution. The mechanism of chromium toxicity results from the formation of ROS through redox cycling (Stohs and Bagchi 1995). Chromate (VI) has been reported to cause varieties of cellular lesions, such as chromium-DNA adducts, Chromium- protein crosslinks, chromium-DNA-DNA crosslinks, DNA–Strands breaks and other forms of oxidative stress (Ahmed et al. 2013). In the presence of some reductase such as glutathione and NADPH, chromate (VI) is reduced to chromate (V) in cells; chromate (V) is also a known carcinogen with potential for causing various forms of oxidative stress (Stohs and Bagchi 1995). Several factors such as Concentration, duration of exposure, pH etc are known to play key role in Chromate (VI) genotoxicity and ability to cause DNA stand breaks (Ferrante et al. 2018; Kumari et al. 2014). Mainly chromate (VI) elicits cellular DNA strand breaks through the interaction of ROS specifically hydroxyl radicals with guanine residues which results in the formation of different DNA adducts. The most common and widely

studied adduct is 8-hydroxyl-deoxyguanosine (8-OH-d G) and has been implicated as the main cause of chromium genotoxicity and carcinogenicity. Thus it is a known biomarker of chromium induced oxidative stress (Valko et al. 2005). Other mechanism of chromium toxicity is the formation of chromium-DNA adducts and cross-links with proteins (Henkler et al. 2010). Here we report a concentration dependent increase in both DNA strand break (Figure 4.7) and DCF-fluorescence (Figure 4.8) following 12 hours treatment with 0-0.4mg/L sodium dichromate (VI). There was also strong positive correlation between DNA strand break and reactive oxygen species formation (Figure 4.9), however compared to cadmium less correlation coefficients between the two oxidative stress markers were observed. However, fold increase in ROS formation in chromium treated sponge aggregates compared to cadmium exposures was 1.45 fold, 1.35 fold and 1.04 fold for 0.1mg/L, 0.3mg/L and 0.4mg/L treatments.

Although Nickel's genotoxicity and carcinogenicity is already established, questions still exist regarding the mechanism of nickel toxicity in living system (Dallas et al. 2013). Nickel accumulates in living cells producing large amount of reactive Oxygen species (ROS) radicals via interaction with cellular intrinsic molecules. This interaction and the resultant production of ROS eventually overwhelm the cell's defence mechanism resulting in damage to intracellular macromolecules, such as genomic DNA.

Nickel is grouped as one of the EU WFD (Water Framework Directive) metals of interest owing to its carcinogenic effects on humans and animals (Dallas et al. 2013). Like most carcinogenic metals, a number of pathways are proposed as routes through which Nickel toxicity is established; examples are Intracellular redox homeostasis, Inhibition of DNA strand break repairs, Induction of hyper methylation of $P16^{InK4a}$ (Tumour suppressor protein) (Henkler et al. 2010). However, main mechanism Nickel toxicity is associated with reactive

oxygen species induction (Henkler et al. 2010). Nickel also acts in synergy with other ionizing agents like UV radiations and X-rays and inhibits the expression of the $P16^{InK4a}$ in oxidative stress condition (Henkler et al. 2010; Valko et al. 2005).

In this study, investigation of non-cytotoxic concentration of Nickel (0-0.4mg/L) using the comet assay technique and DCF-fluorescent assay produced both biomarkers of exposure (DNA strand breaks) and biomarker of effect (Reactive oxygen species) . Exposure of sponge cell aggregates to concentrations of Nickel chloride for 12 hours at room temperature resulted in a significant concentration dependent increase in the level DNA strand break (Figure 4.11). We also report a concentration dependent increase in DCF-fluorescence as endpoint for Reactive Oxygen Species formation (Figure 4.12). Although DNA strand breaks in sponge cell aggregates treated with Nickel is less compared to other test compounds in this study, the correlation coefficient (Figure 4.13) between the two biomarkers was relatively very high comparing favourably with that of Aluminium and Cadmium. This suggests that ROS induction contributes significantly to Nickel-induced DNA strand break and as such constitute the likely mechanistic nickel toxicity pathway. This is also in agreement which previous studies (Henkler et al. 2010; Stohs and Bagchi 1995). Sponge cell aggregates were also exposed to 0, 0.1, 0.2. 0.3, and 0.4mg/L $AlCl_3$ (Aluminium chloride) under similar laboratory conditions as those t previously discussed test metals. Viability results showed that aluminium concentrations used in this study do not have any significant cytotoxic or apoptotic effect on the cells. Interestingly, treatment with 100µM as positive control (concentration is within the range previously reported (Toimela and Tähti 2004) and those of other test compounds in this study), was also not cytotoxic as assessed the MTT mitochondrial assay (Figure 4.14). This result however, is in contrast with the report of (Lankoff et al. 2006), who reported increased cytotoxicity and apoptosis with 25µg/ml following treatment of human blood lymphocytes

with 1,2,5,10 and 25µg/ml of Aluminium chloride. (Lima et al. 2007) also reported cytotoxic effects of aluminium on human lymphocytes after treatment with 5-25µM $AlCl_3$. In both results however, the cells were incubated for 72 hours and 52 hours respectively. The implication of this is that, the threshold for aluminium cytotoxicity is probably more 'time' than concentration dependent. Assessment of percentage DNA strand breaks in sponge cell cultures exposed to aluminium chloride for 12 hours using the alkaline comet assay showed increased DNA strand breaks as aluminium concentrations increased (Figure 4.15). Results of percentage DNA strand break correlated positively with the amount of reactive oxygen formed (Figure 4.17). Compared to all test compounds in this study, sponge cell aggregates exposed to aluminium produced the highest amount of reactive oxygen species after 12 hours incubation (Figure 4.16). This might also mean that Aluminium induced DNA strand break and genotoxicity is through the production of reactive oxygen species. Benzo[a] Pyrene is a priority organic xenobiotic and it is also grouped into class I IARC pollutant (human carcinogen). Although multiple toxicity routes have been reported in literature, generation of BP -7,8-diol-9,10-epoxide (BPDE) is the predominant toxicity pathway. BPDE interacts with DNA guanine and adenine bases to form bulky DNA adducts which are measurable by the comet assay (Henkler et al. 2010). Also, although detoxification is the main biotransformation pathway for most invertebrates (De Flora et al. 1995), a P450 metabolite Erebusinone (EREB), biomarker of aromatic xenobiotic was recently identified in the Antartic sponge *Isodictya erinacea* (Moon et al. 2000). The mechanism of action being the inhibition of CYP3151A1 and binds preferentially to aromatic residues (Vankayala et al. 2017).

Here, Benzo[a] Pyrene induced DNA strand breaks were measured in cultured sponge cell aggregates. Percentage DNA strand break increased with increasing B[a]P concentration and there was a significant difference between B[a]P treated cultures and control/ untreated

samples (Figure 4.20). Preliminary assessment of metabolic activity in sponge cells using NADP fluorescence substrates showed the possibility of a P450 metabolic activity in sponge cells (Figure 4.18). Cytochrome P450 enzymes are particularly sensitive to petrolytic and petrogenic xenobiotic, especially those of the sub-family 1A1; thus, the measurement of CYP-P450 activity is still very valid in biomonitoring organic pollution (Livingstone 1991; Michel et al. 2001; Richards and Shieh 1986). Basically, P450 enzymes would biotransform organic pollutants into polar and water soluble compounds which are then eventually detoxified. However, in some instances the process of bio-transforming organic chemicals could result in the activation of organic chemical intermediates which are the mutagenic or carcinogenic components of the organic pollutants (Livingstone 1998). To best of my knowledge, this is first application of the comet assay to measure B[a]P induced DNA strand break in sponge cells.

In conclusion, I have established in this piece of work, the genotoxic effects on sponge cells of environmentally relevant xenobiotics (B[a]P, Cd, Cr, Ni and Al), and have also shown the positive correlation between reactive oxygen species formation and DNA strand breaks which defines their mechanism of toxicity. These are novel findings, as prior to this time, studies deploying sea sponges for biomonitoring purposes have only measured levels of pollutants in sponge tissues and a few which have utilised alkaline fluorometric methods to detect DNA damage. Here we have successfully established the viability (using MTT viability assay) of previously cryopreserved and cultured sponge cell aggregates and their amenability to the assessment of oxidative stress following exposures to environmentally relevant xenobiotic (using the alkaline comet assay to measures DNA strand breaks and H_2DCFDA assay to measures reactive oxygen species). Both biomarkers are reliable tools in ERA for biomonitoring and for environmental regulatory purposes. Current trend and focus of ERA in

the aquatic ecosystem is to investigate and determine deleterious effects of environmental xenobiotic exposure on indigent biota. The next chapter therefore, looks at levels of these pollutants and other established genotoxicants from potentially polluted environment in sea sponges compared to levels in other environmental matrices, such as sea water and sediments from same sites. The chapter would also correlate levels of pollutants with the level of DNA strand breaks in cryopreserved untreated sponge cells from these sites.

Chapter 5 Heavy metal Bioaccumulation in Aquatic matrices: Genotoxicity Assessment in the sea sponge *Amorphinopsis kalibiama* a potential biomarker for biomonitoring and environmental risk assessment.

5.1 Introduction

Since the advent of industrialization with the resultant increased urban migration, coastal regions have been immensely challenged with heavy metal pollution (Cebrian and Uriz 2007b; De Mestre et al. 2012; Iwegbue et al. 2018). Major contribution of heavy metals in the coastal waters is mainly from and effluents from both domestic and clinical sewers (Iwegbue et al. 2018; Wogu and Okaka 2011). Sessile organisms such as sea sponges owing to their affinity and propensity for metal bioaccumulation are mostly affected, with effects ranging from chronic (inhibition and deactivation of aggregation factor, increased cell division, interference with sponge water filtration mechanism and decrease in population growth) to acute (depletion of population and species diversity) (Kotelevtsev et al. 2009; Van der Oost et al. 2003). Other 'cause-effects' or biomarkers of environmental pollutants measured in sponge are listed in Table 1.5, Chapter 1.

Biomarkers are heavily relied upon in ERA; which involves the analysis and management of the potential risks resulting from the levels of different pollutant in the environment and their actual effects on living system. because they provide evidence based results on the adverse effect of exogenous pollutants and have over time become indispensable in ERA and in biomonitoring studies This is because they provide information on biologically available contents of pollutants, needed for an early warning signal (Livingstone et al. 2000; Martins and Costa 2014; Van der Oost et al. 2003). In coastal environment, there is high risk of exposure to carcinogenic pollutants and pollutants capable of inducing mutation to reproductive cells resulting in increased number of cancer outcome and reproductive complications (Kotelevtsev et al. 2009). Genotoxicity of pollutants therefore, is an important biomarker in ERA and a number of endpoints such as DNA strand breaks, sister chromatid

exchanges, micronuclei and chromosomal aberrations are utilised to assess the effects of environmental pollutants on biota. It is particularly important to monitor the genotoxic impacts of pollutants in the coastal environment, Among the genotoxicity endpoints deployed in ERA, DNA strand breaks because of its close connection with carcinogenic outcomes and the comet assay are mostly utilised (Martins and Costa 2014; Reinecke and Reinecke 2004). Thus both bioaccumulation and biomarkers are important tools for efficient and adequate assessment, of pollution (Van der Oost et al. 2003). It therefore important to efficiently monitor the genotoxic effects of pollutants in the aquatic environment using indigenous species and especially sessile benthos, as they have developed mechanisms for tolerating pollutant in the medium. Benthic aquatic organisms are the ultimate sink for aquatic pollutants and information on the health status of their environment can been extracted from them.

5.1.1 Overview of Niger Delta and pollution

The Niger Delta is Africa's largest delta, and third largest delta in the world next to American's Mississippi and Brazilian Pantanal (EKPO et al. 2012). It is a region characterized by a vast expanse of mangrove and coastal forests which serve as rich sources for different plant products, fish, shellfish and timber; it is made up of 9 political states of Abia, Akwa Ibom, Bayelsa, Cross River, Delta, Edo, Imo, Ondo and Rivers State.

Because there are no established public sewerages in the Niger Delta, wastes are disposed of into drainages and the river, which eventually make their way into the creeks and are trapped in environmental matrices (Sediment, water column and biota) (Ajao and Anurigwo 2002). Considering that sea foods make up a greater percentage of protein source for the resident of

the region (Udotong et al. 2017), there is the need to set up adequate and reliable biomarkers to monitor pollution in the Niger Delta region. Pollution sources in this region are mainly associated with oil and gas exploration, agriculture and metal smelting activities, with most contributions being attributed to the oil and gas industries (Ajao and Anurigwo 2002; Iwegbue et al. 2018).

In this study, metal levels in sponge, sediment and water samples obtained from 6 sites in the Niger Delta Nigeria (Site B: Kalio Ama in Okrika Local Government area; Site C: Ologbogbo All-marine creek ,Marine base Port Harcourt Local government area; Site D: Kalibiama creek in Bonny Island Bonny Local Government area; Site E: Egbomu River, in Andoni Local Government area; Site F: Isaka creek in Eagle Island Port Harcourt Local Government area and Pokokri Creek in Nembe/ Brass adjoining sea Bayelsa state) and a control site A; Topo creek in Badagry, Lagos state (Figures 2.5, Chapter 2) were independently analysed in three laboratories. DNA strand breaks as a biomarker of effects from metal exposure was measured in sponge cells from all sample locations using the comet assay technique and results correlated with metal levels in all matrices.

Although the comet assay has been used extensively in aquatic biomonitoring using both model and non-model organism (Table 1.3, Chapter 1), this study presents here for the first time, the application of the comet assay for the assessment of DNA strand breaks in the sea sponge *Amorphinopsis kalibiama* as a biomarker of metal toxicity. And also, this would be first assessment of the effects of pollutants in marine sponges in the Niger Delta system.

5.2 Aims and Objectives

This chapter looks at bioaccumulation assessment of toxic metals in sea sponges, sediments and surrounding sea water, and determination of their biological effect in the sea sponge *Amorphinopsis kalibiama* (DNA strand break) using the comet assay in cryopreserved single sponge cell. The aim of this study therefore is to establish DNA strand break using the comet assay in sea sponges as a reliable biomarker for environmental biomonitoring of aquatic pollution and as a potential tool in ERA.

Current trend and demand in environmental toxicology is for ERA studies especially in the aquatic ecosystem, to combine information on metal behaviour in living system such as exposure levels or bioaccumulated contents, biologically available concentrations, biomagnification or fate in organisms and the concomitant adverse effects at individual levels using biomarkers (Martins and Costa 2014; Rainbow 2002; Van der Oost et al. 2003).

5.3 Materials and Methods

5.3.1 Sample collection and processing

Sponge, sediment and water samples were collected from 6 Niger Delta sites and 1 control site outside the Niger Delta; in Badagry, Lagos state (Table 2.1 and Figure 2.5, Chapter 2) between the periods of October and January, 2016. Sponge samples were collected from exposed mangrove stomp at low tide and surrounding water column and sediment at a depth of approximately 0.3m. All samples were immediately processed and cryopreserved as described in Section 2.3, Chapter 2.

5.3.2 Systematic and Molecular characterization of Nigerian sponge samples

Type specimens were systematically identified by Professor Rob Van Soest, Naturalis Biodiversity Centre, The Netherlands as *Amorphinopsis* sp. Genomic DNA was extracted and confirmed by PCR amplification (Details in Sections 2.4.1 and 2.4.2, Chapter 2). Using existing primers from the sponge barcoding project https://www.spongebarcoding.org/primers.php, (which are moderately conserved homologous and suitable for speciation) cytochrome oxidase 1 gene and 28S gene were sequenced using Sanger Dideoxy sequencing method. Sequenced data was checked against NCBI GeneBank blast.

5.3.3 Sponge cell culture for aggregate formation, Viability assessment of aggregates and Comet assay assessment of DNA Strand breaks in untreated samples of Amorphinopsis

As previously described for *H.perlevis* (Section 2.7.2), cell suspension of *Amorphinopsis* were cultured for aggregate formation in synthetic sea water. Viability of aggregates was then assessed using MTT assay as in Section 2.9.1.

To assess DNA damage induced from environmental xenobiotics, untreated sponge cell (from the Niger Delta) aggregates that have been cultured from cryopreserved sponge cells, were dissociated into single cells following the method described in Section 2.8.1, Chapter 2. DNA strand breaks were then measured using the slightly modified alkaline comet assay for sponge cells (Section 2.10.1 and Section 2.10.2). Measurement of DNA strand breaks in *Amorphinopsis* was compared with strand breaks in *H. perlevis* cells which were processed under similar conditions.

5.3.4 DNA strand break repairs and Assay swap for water quality assessment

DNA strand break is induced among other factors by exogenous factors such as environmental and pharmaceutical xenobiotics (Tubbs and Nussenzweig 2017). Intracellularly, there are three main DNA repair pathways; i) DNA base excision repairs (for repairs of deleted, damaged or hydrolysed bases), ii) removal of mismatched DNA bases iii) DNA bulky adduct removal. All DNA repair pathways are mediated by two mechanisms; high fidelity homologous DNA template recombination and non-homologous DNA end to end joining (Tubbs and Nussenzweig 2017).

5.3.5 Assay procedure.

Cryopreserved single sponge cells from polluted sites in the Niger Delta Nigeria, were prepared as previously described (Section 2.8.1) and were cultured in sterile synthetic sponge cell media in sterile T25 culture flasks. Sponge cells were cultured for aggregates formation and DNA repairs were monitored over five different time points of 8, 24, 48, 72 hours and 7days using the comet assay. Aggregates were processed for comet assay after each time points and DNA strand breaks were measured and statistically compared across all time points.

To ascertain the quality of water from Tenby bay castle beach, assay swap with samples from both locations (clean and polluted) was performed. Using previously described procedures, cryopreserved single *H perlevis* (Figure 2.1a) sponge cells from Tenby bay castle beach were cultured for 12 hours in water sample from the most polluted site (as shown by slightly higher level of DNA strand break) among seven Niger delta locations investigated. Samples of *A kalibiama* (Figure 2.1b) were also cultured in water sample from Tenby bay. For control, processed cryopreserved *H perlevis* was cultured simultaneously in synthetic sea water (sponge media). All aggregates were processed for comet assay analysis of DNA strand breaks, and the test results compared with the control.

5.3.6 Sample Digestion for metal measurement

Four sample digestion and metal extraction methods were adopted to compare efficiency and metal yield following established methods (Cebrian et al. 2007; Pan et al. 2011; Rao et al. 2009). Metal extraction methods used were:

1) EPA method 3052 Acid digestion according the method described by Mangum (2009)
2) Aqua-Regia digestion methods (Sastre et al. 2002)
3) HF/HC1O_4 digestion and Fusion Metaborate digestion method

5.3.7 Buffers:

The Buffers used for all the different digestion methods were; 16.7M HNO_3, 12.3M HCL and 12.7M H_2O_2 (EPA 3052 Acid digestion), 3:1 HCL:HNO and 12.7M H_2O_2 (Aqua-Regia digestion method) and Aristar 48% m/m hydrofluoric acid (HF), Aristar 60% m/m perchloric acid (HClO4) and Aristar 68-71% m/m nitric acid (HNO_3) for HF/HC1O_4 digestion.

5.3.8 Method Procedures.

Using EPA 3052 method, both sponge and sediment samples from the 7 sites were digested for metal extraction using microwave assisted acid digestion (CEM MARS 5 CORPOATION). Briefly, approximately 0.5g of samples from each site were weighed in triplicate and placed into the Teflon lined microwave reactor vessel (tube), 10mL of ultra-pure HNO_3, 500µL of ultra-pure HCL and 660µL H_2O_2 were added (3052-method) and 5mL of Aqua-Regia (Aqua-Regia method). Vessel contents were allowed to react for a minimum of 10 minutes without the lid in a chemical fume cupboard. Reaction tubes were thereafter

sealed and transferred into the microwave rotor and then placed in the microwave. Samples were digested for 20 minutes (10 minutes run time and 10 minutes holding time) at 180^0C and 1200W. Digests were allowed to cool at room temperature and afterwards transferred into a 15mL Falcon tubes and stored at 4°C until needed.

For HF digestion, Sponge and sediment samples were dried overnight at 105°C and approximately 0.5g weighed into a 60ml Teflon beaker. Samples were moistened with 18MΩ ultra-pure high quality (UHQ) water, and 10ml of HF was carefully added, followed by 4ml of $HC1O_4$. Beaker was placed on a hotplate and temperature raised to 200° C, allowing sample decomposition until near dryness leaving a crystalline 'pulp-like' substance. 4ml of $HClO_4$ was added again to completely digested samples and then evaporated to near dryness. Afterwards, 10ml of 5M HNO_3 was added to the sample and was slowly digested until clear solution was obtained. Digests were allowed to cool at room temperature and was made up to 50mL UHQ water in a volumetric flask. All digests were transferred into 50mL Falcon tubes and stored at 4°C until when needed. All procedures were performed in a HF/$HC1O_4$ fume cupboard with wash down facilities.

5.3.9 Metal extraction procedures.

Water samples from all seven sites were analysed for Al, As, Cd, Cr, Cu, Hg, Ni and Pb using inductively coupled plasma optimal emission spectrometry (ICP-OES; Ultima (2) C). Sponge and Sediment digests were measured with either inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700 series) or ICP-OES. Using Rh as internal standard, 6 concentrations of a multi-element standards (VWR Prolab chemicals, USA) and 3 blanks. Metal extracts in both sediment and sponge samples were measured for the concentrations of Al, As, Cd, Cr, Cu, Hg, Ni and Pb. These metals were measured because of the prevalence in polluted environment from previous reports (Deb and Fukushima 1999; Förstner and

Wittmann 2012; Gentric et al. 2016; Sarkar 2002) including the Niger Delta were these samples were obtained (Babatunde et al. 2013; Inengite et al. 2010; Iwegbue et al. 2018; Onojake et al. 2017). Hg however, is not so commonly reported in commonly utilised matrixes in the Niger Delta for metal content. Metal recovery and accuracy of the analytical methods used were verified using certified reference materials (CRM – NIST1646a and SRM-NIST1566 Oysters). Metal extracts in both samples and CRM were simultaneously measured against calibration curves generated with the multi-element standard concentrations (0ppm, 0.1ppm, 0.5ppm, 2.5ppm, 5ppm and 10ppm). Final metal concentrations in all analytes were reported in μg/mg of sponge and sediment and μg/L for metal concentration in water samples.

5.3.10 Quality Assurance and Statistical Analysis.

All chemicals and reagents used were of ultrapure analytical grade. For all measurements, reagent blanks (without the samples) was prepared and analysed in triplicate as the samples. Reported values are blank corrected mean ± SEM (standard error of mean).

The following certified reference materials were analysed in triplicate following the same procedure for sample preparation for quality assurance and control; NIST 1646a, NIST 1566b, JLS, SGR, MAG, CCH, SCO and MON. Percentage recovery was between 90.5% and 100%.

All data in this chapter were analysed using two way ANOVA, Dunett's and Tukey multiple comparison test on IBM SPSS and Graph pad prison version 7.0.

5.4 Results

5.4.1 Sample Collection, Identification and processing

Sponge samples (Figure 5.1) collected from 6 sites in the Niger Delta, Nigeria were cryopreserved and air freighted on dry ice to the laboratory. These were identified by both systematic taxonomy and phylogenetic characterisation. Sequence data of both Cytochrome C Oxidase 1 gene and 28S both identified the sponge as *Amorphinopsis sp* (Figure 5.2 and 5.3); and are available in the NCBI gene bank with the accession numbers MG029167 and MF685333.

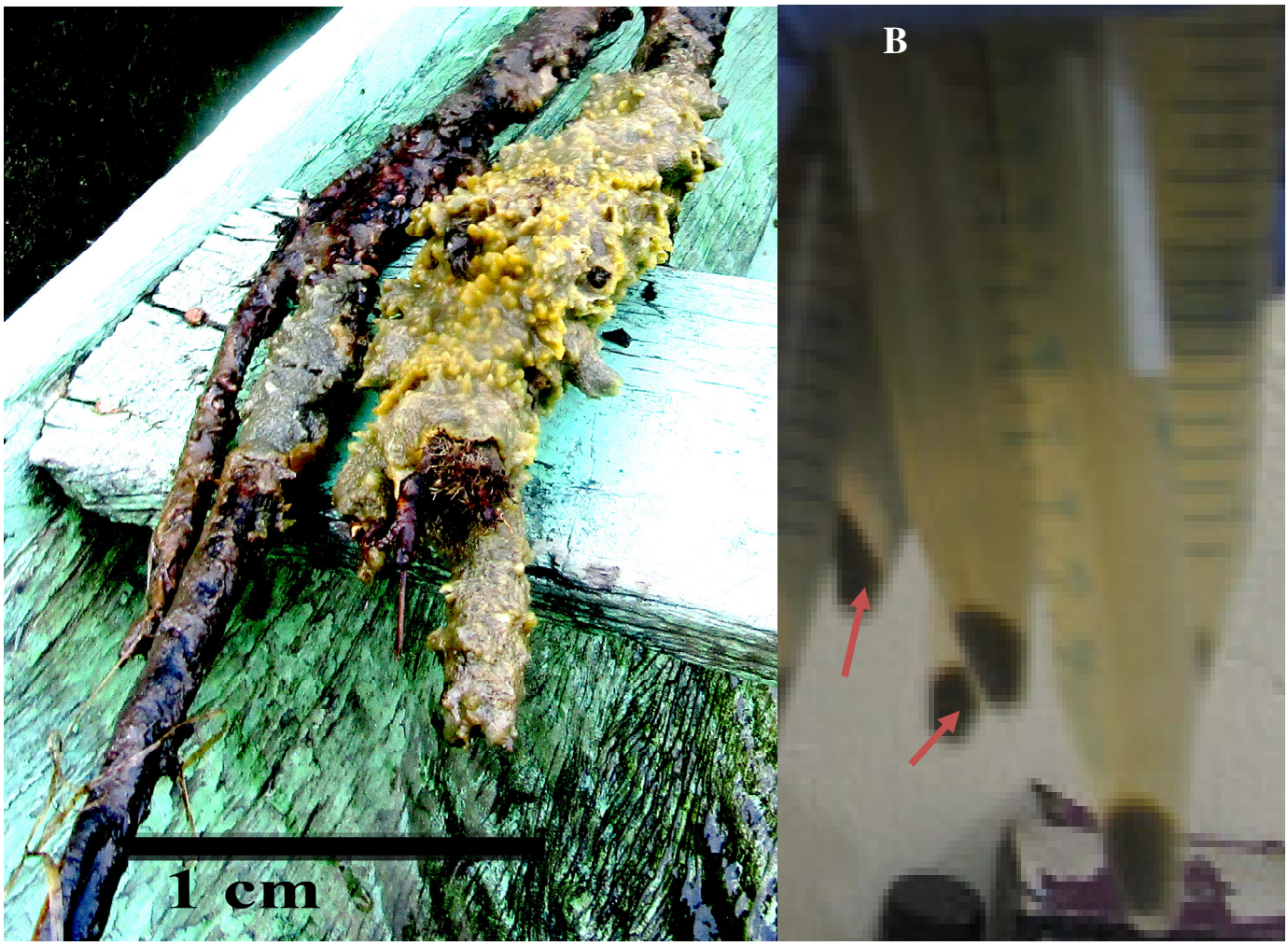


Figure 5.1 : Sponge cell collection and single cell prep a) Sample of *Amorphinopsis sp* attached to mangrove stomp b)Sponge cell pellet arrowed.

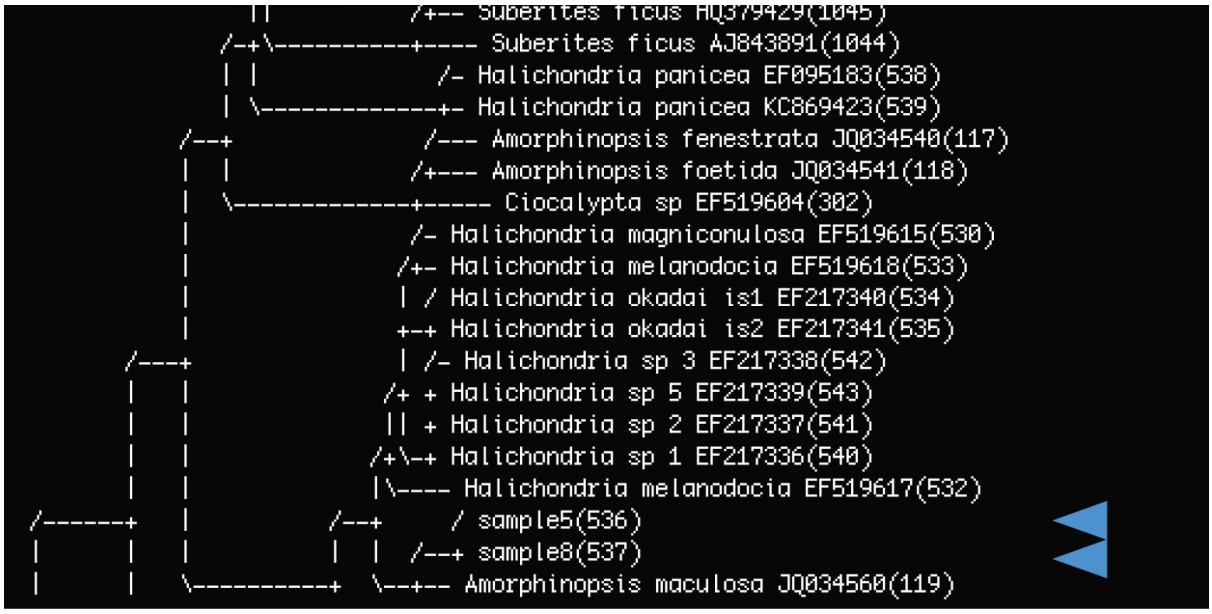


Figure 5.2 Cytochrome C Oxidase 1 gene sequence confirms sponge sample (arrowed) from the Niger Delta as Amorphinopsis sp.

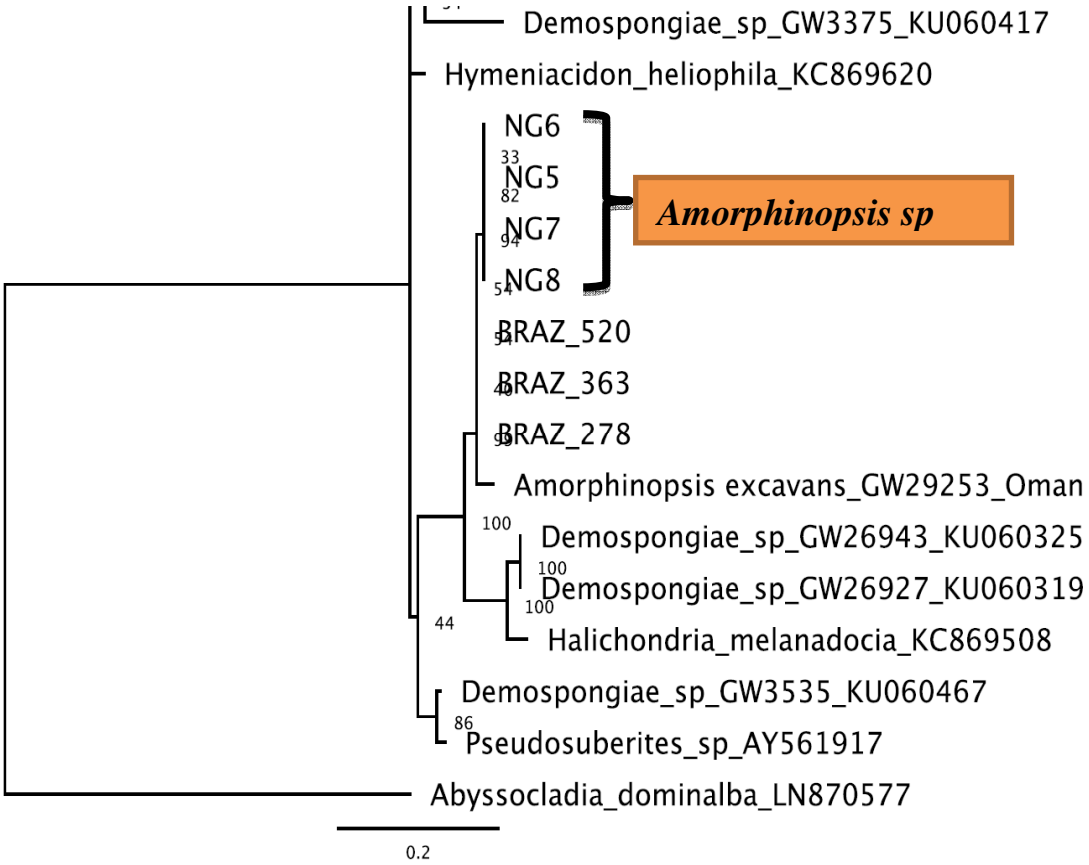


Figure 5.3 28S Phylogenetic cladogram of Amorphinopsis species: Sequence data aligned duplicate samples from the Niger Delta (NG6-8) with sibling species on the NCBI GenBank and further confirms the species as *Amorphinopsis sp.*

5.5 Cryopopreserved sponge cells from polluted sites are viable.

Single cells of the sponge prepared as previously described in the Chapter 2, formed aggregates (Figure 5.4 a) in culture that were viable for up to one week in culture media. Aggregate viability was further confirmed by MTT viability assay (Figure 5.4 b); comparison with untreated sponge cell sample of *Hymenicidon perlevis* from Tenby Bay (a near pristine site compared to the Niger Delta sample sites), showed no statistically significant difference in MTT reduction. Aggregate formation was monitored for up to 7 days, and result showed aggregates development in culture from loosely defined structure to a more compact structure with well-defined features (Figure 5.4a).

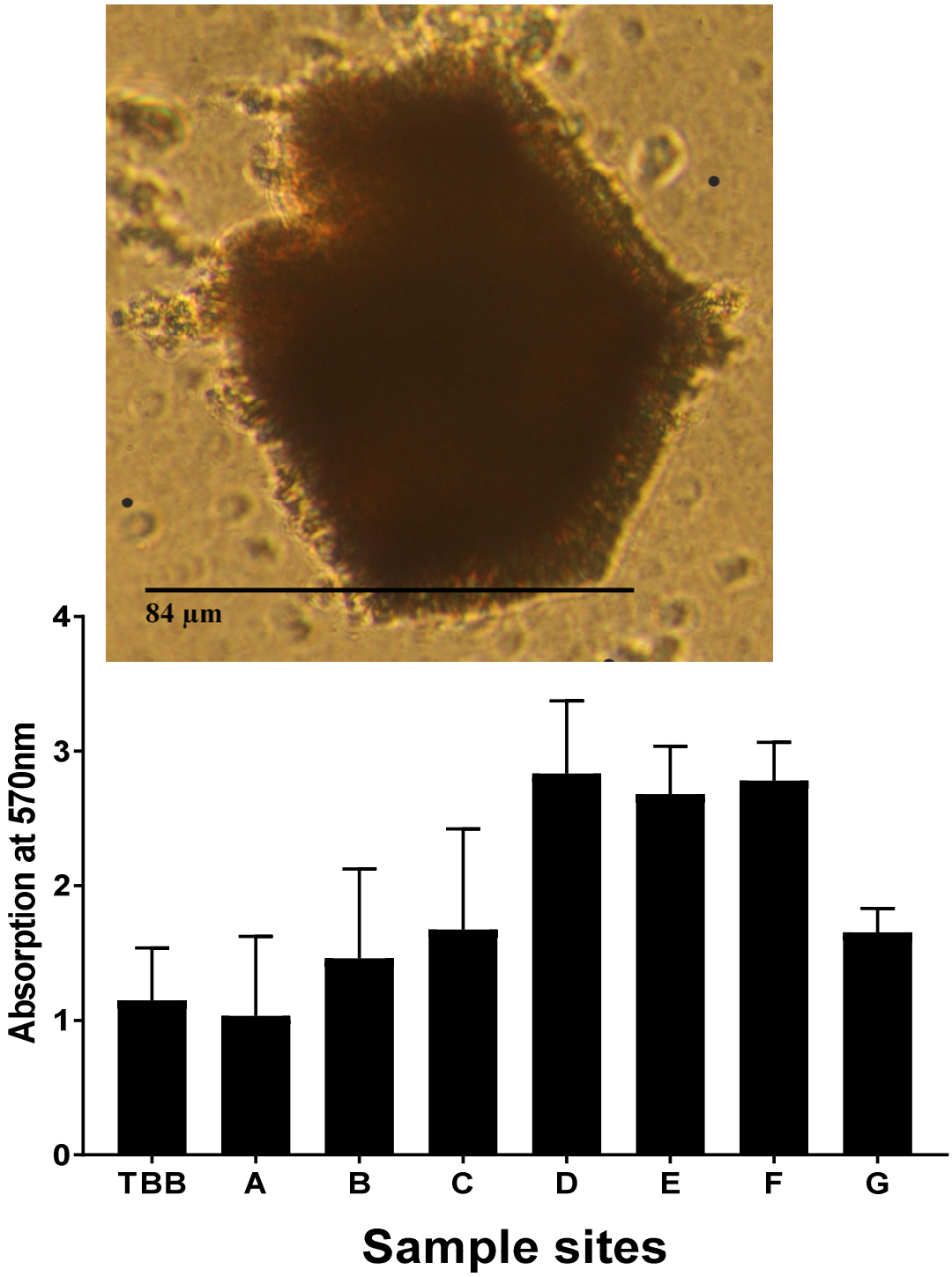


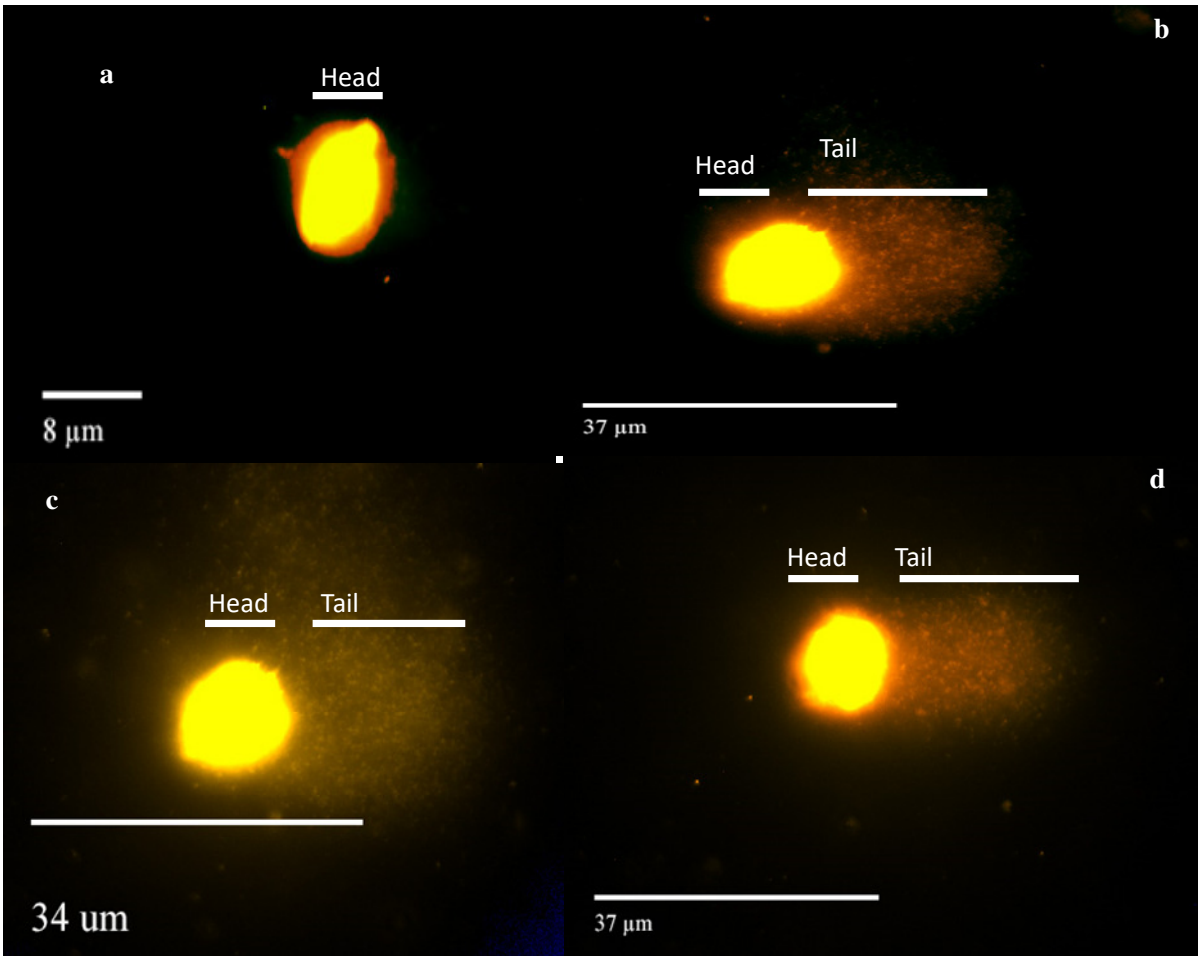
Figure 5.4 Cryopreserved sponge cells sponge cells: a) Sponge aggregate with well-defined feature showing viability b) MTT viability assay showed no statistically significant difference between cryopreserved sponge cells from the Niger Delta sites (A: Topo creek Badagry in Lagos state; B: Kalio-Ama river Okrika in Rivers State; C: Al-marine Ologbogbo, marine base Port Harcourt in Rivers state; D:Kalibiama creek, Bonny Island in Rivers state; E:Egbomu River, Andoni in Rivers state; F:Isaka creek Eagle island Port Harcourt in Rivers state, G: Pokokri Nembe/Brass Sea Bayelsa state) and sponge cells from Tenby Bay. Data displayed are mean ± SEM, n = 4.

5.6 Assessment of DNA strand breaks in cultured cryopreserved aggregates

Using the sponge in vivo exposure model developed in previous chapters; assessment of percentage DNA strand breaks in untreated sponge samples from all 6 sites in the Niger Delta and the control sites measured was statistically significant at $P < 0.05$ compared to control untreated sponge cell sample from Tenby bay castle beach (Figure 5.5). Sponge DNA with obvious comets (Figures 5.5A) were recorded from all sites including the control site A (Topo creek in Badagry, Lagos State). Strand breaks differed from sites to site with the highest level of DNA damage being observed with sponge samples from site E; Egbom, in Andoni. This site also had the highest concentration of all metals analysed (Section 5.7 below). Results shown were obtained over three technical repeats in duplicates, percentage DNA strand breaks were (mean ± SEM) 6.38 ± 1.56 in the control site A, 10.33 ± 3.27 for site B; 7.82 ±1.64 for site C; 10.83 ± 2.40 for site D; 11.63 ± 3.68 in samples from site E; 10.97 ± 1.42 for site F; and 11.13 ± 3.11 site G.

To investigate DNA repair process in sponge cells, samples from site E were monitored in a one week time point assay using the alkaline comet assay. Over time in culture, there was statistically significant reduction in DNA strand break in sponge cells at the different culture time points (Figure 5.6). Although, DNA repair process is a fairly quick process (Mitchelmore and Chipman 1998), repairs over time in these cells may have been influenced by the 'self-cleaning' (Strehlow et al. 2017) mechanism of sponge cells via water filtration in culture.

A



B

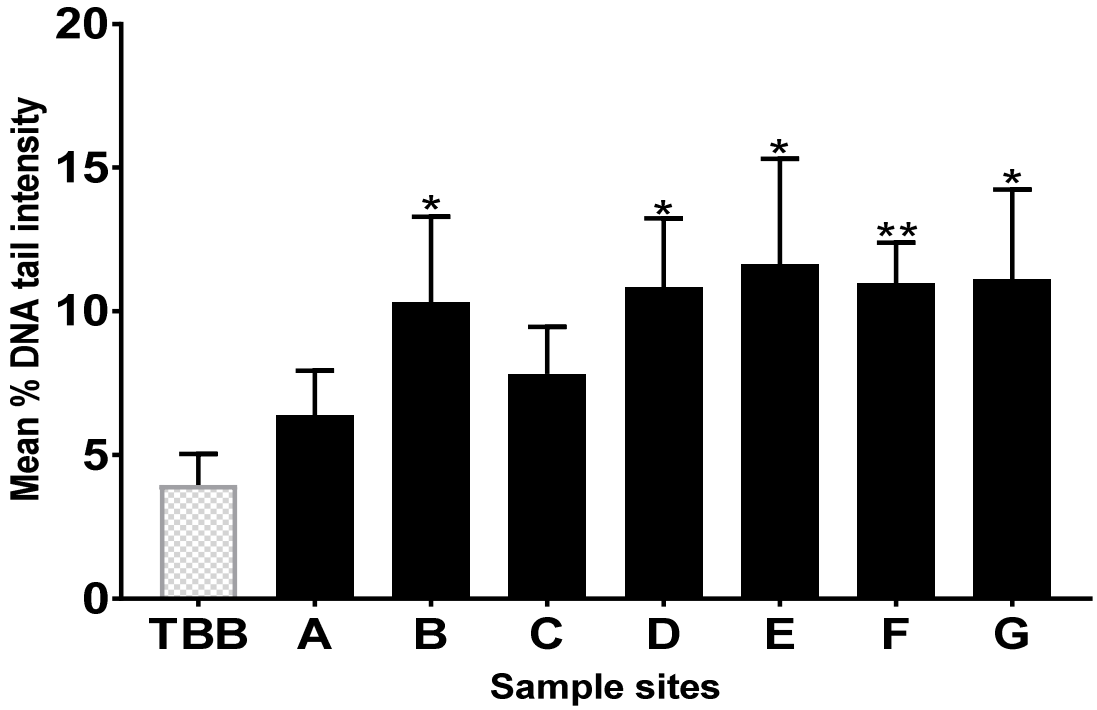


Figure 5.5 **Comet assay assessment of DNA strand breaks in untreated fields' samples obtained from polluted sites in the Niger Delta**. A) Representative comets a) undamaged control image b) Image from sample site F; c) Image from sample site E d) Image from sample site G. B) % DNA tail intensity for each site compared to sponge cells from Tenby bay castle beach. Data shows mean values of the percentage median tail intensity ± SEM P* < 0.05, n = 6. 1-way ANOVA with Bonferroni post-hoc multiple comparison test analysed using SPSS 22.0 shows a statistically significant difference between mean percentage DNA tail intensity of sponge cells from sites D, F, and G. Sample sites B and E were analysed using Kruskal-Wallis non-parametric test; P = 0.049 and 0.034 respectively.

5.6.1 DNA repair

Comparison of DNA damage in sponge cells from all 7 sites, shows that sponge cells from site E have slightly higher levels of damage than cells from all other sites. Thus, cryopreserved sponge cells from Ebgomu River (Site E) were cultured in synthetic sea water supplemented with PSG antibiotics for one week. DNA strand break repairs monitored in a time point assay of 12, 24, 48, 72 hours and 7 Days showed a statistically significant time dependent reduction in the level of DNA strand breaks. Subsequently, field sponge cells were cultured for 12- hours for aggregate formation before dissociating into single cell for downstream biochemical assays.

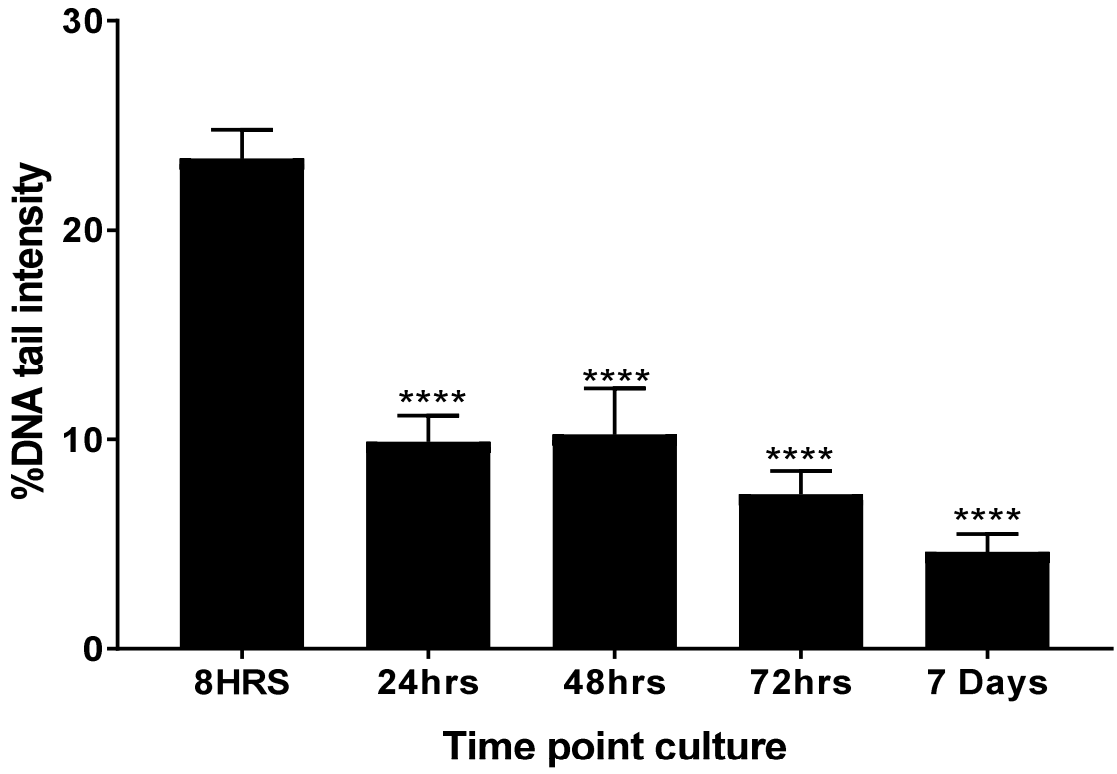


Figure 5.6 Measurement of DNA strand break repairs using the comet assay in sponge cells from polluted site over one week period. Displayed data a mean percentage DNA tail intensity ±SEM; P= 0.000 at P*<0.05, n= 6 (8hrs – 72hrs) and n=2(7days). 1 way ANOVA with Bonferroni post-hoc multiple comparison correction shows a statistically significant difference between 8hours time point and other time points (24hrs to 7days).

Furthermore, to investigate the effects of the water samples from the Niger Delta environment on sponges, single sponge cells of *H. perlevis* were cultured in filtered water samples from site with highest levels of contaminants, Egbomu (site E) in an Assay swap experiment. There was significant level of DNA damage by up to 2 fold (Figure 5.7) compared to control cells cultured in synthetic sea water under the same laboratory condition and sample preparation procedure.

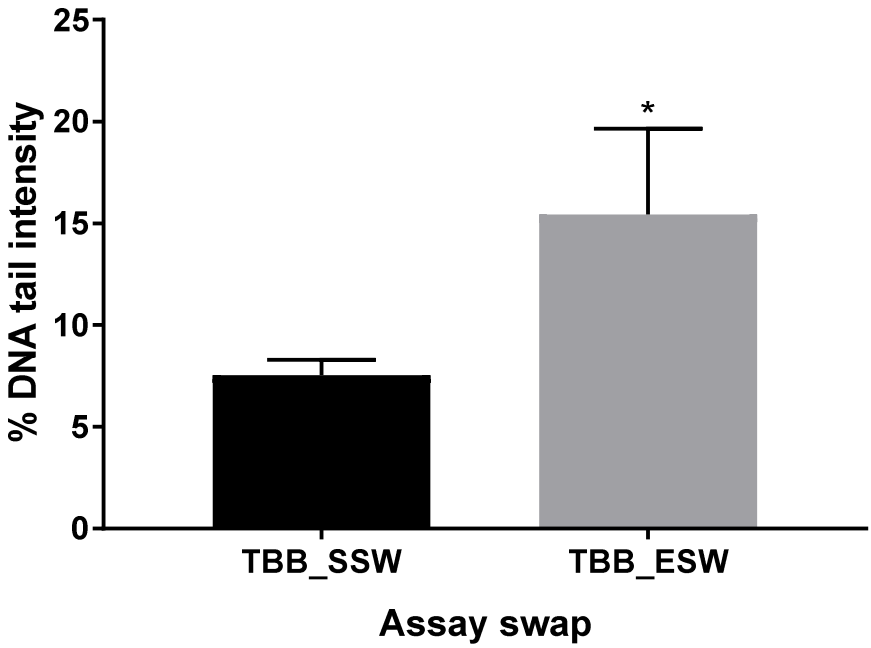


Figure 5.7 Assay swap: Sponge cells from Tenby bay (TBB) cultured in water sample from Egbomu (ESW) and a control culture of Tenby bay sample cultured in synthetic sea water (SSW). Up to two fold increase in the level of DNA damage compared to the control culture in synthetic sea water was observed and was statistically significant by 1 way ANOVA, on graph pad 7.0 at P<0. 05, mean values ± SEM, n=3.

5.7 Chemical Analysis of metal level in environmental samples.

Samples of environmental matrixes of sponge, sediment and sea water collected from seven sample sites (6 Niger Delta creeks and 1 creek from Badagry in Lagos) were independently analysed in three laboratories (Lab 1: Analytical Chemistry labs, Department of Chemistry Warwick University; Lab 2: Environmental analysis labs, School of Earth Science University of Birmingham; and Lab 3: Analytical chemistry labs, Kingston University, London). Metals analysed were Al, As, Cd, Cr, Cu, Ni and Pb using four metal digestion methods, described in section 2.12 and 2.13 in chapter 2. Most analytes in the result from the third laboratory were below the detection limit; hence they are not reported in this thesis, except for aluminium accumulation in sponge tissues correlated with the level of DNA strand breaks (Figure 5.11) and comparison of Aluminium levels in sponge tissues with Aqua-Regia digestion method (Figure 5.16a) both analysed using ICP-MS.

5.7.1 Bioaccumulation of metals in sponge tissues and DNA damage

Metal bioaccumulation in sponge tissues from all sample sites is summarised in Table 5.1, Figure 5.8 and Figure 5.9. Of all the metals analysed (Al, As, Cd, Cr, Cu, Hg, Ni and Pb), only Al, Cu and Pb were consistently detectable in sponge tissues digested by aqua-Regia method in all sample sites (Figures 5.8). Using acid digestion (EPA 3052) method, only aluminium was detected in all sites and trace amount of chromium in some of the sites (data not shown). However, sponge sample digestion using HF-fusion with lithium metaborate, detected As, Cd, Cu and Pb (Figure 5.9) in sponge samples from sites A, B, C,D,E, F and G. With site E being the only site with significant level of cadmium detection in sponge tissues and the site with highest level of DNA strand breaks. Al, As, Cd and Cu were highest in site E by both HF and Aqua-Regia digestion method (Table 5.5). Level of Aluminium was also

highest in Site E by Acid digestion from lab 1 (Data not shown); Pb however was highest in site F. Comparison of all consistently detected metals (Al, As, Cd, Cu, and Pb) in sponge tissues in all sample sites with % DNA strand break was statistically significant in both Sites E and G (Figure 5.10); these sites also had the highest level of DNA damage measured with comet assay (Figure 5.5). There was also good correlation between Aluminium levels in sponge from all sample locations versus % DNA strand break in all sample locations (Figure 5.11).

Table 5.1 Metal levels in sponge samples (mean ± SEM, µg/mg; n=3). HF metal extraction method, gave the highest total metal (As, Cd, Cu, and Pb) levels in sponge tissues. Aluminium detection was significant for all three extraction methods employed and the Aluminium data shown is a summary of the level in Acid Digestion method and ICP-MS analysis. Site E has the highest amount of most metals of Al, As, Cd, and Cu measured which was statistically significant compared to levels in other sites at P^{*} <0.05 and was the site with highest level of DNA strand breaks in sponge cells. Pb was highest in site F and was also significant at P^{*}< 0.05.

Sampling Locations	Al	As	Cd	Cu	Pb
A: Topo creek, Badagry/Lagos	0.34 ± 0.11	0.003 ± 2.2E-05	1.3E-04 ± 1.4E-06	6.0E-03 ± 4.2E-05	2.0E-03 ± 9.5E-06
B: Kalio-Ama Creek, Okrika	0.60 ± 0.19	0.003 ± 1.3E-05	4.0E-05 ± 1.2E-05	4.1E-03 ± 1.2E-04	6.8E-04 ± 1.8E-05
C: Al-Marine Creek, Ologbogbo	0.44 ± 0.18	0.002 ± 3.8E-05	3.4E-05 ± 4.2E-06	9.7E-03 ± 8.5E-05	1.2E-03 ± 1.4E-05
D: Kalibiama Creek, Bonny Sea	0.35 ± 0.05	0.003 ± 9.9E-06	2.0E-05 ± 5.3E-06	2.3E-03 ± 1.4E-05	2.5E-04 ± 8.6E-06
E: Egbomu River, Andoni	*0.70 ± 0.10	*0.004 ± 5.6E-05	*1.5E-03 ± 4.6E-06	*0.02 ± 2.2E-04	1.3E-03 ± 1.3E-05
F: Isaka Creek, Eagle Island	0.22 ± 0.03	0.003 ± 6.0E-05	5.01E-05± 4.5E-06	6.0E-03 ± 1.1E-04	*2.0E-03 ± 1.4E-05
G: Pokokiri Creek, Bayelsa state	0.60 ± 0.15	0.003 ± 1.6E-05	1.1E-04 ± 1.2E-06	3.1E-03 ± 9.3E-06	7.9E-04 ± 3.9E-06

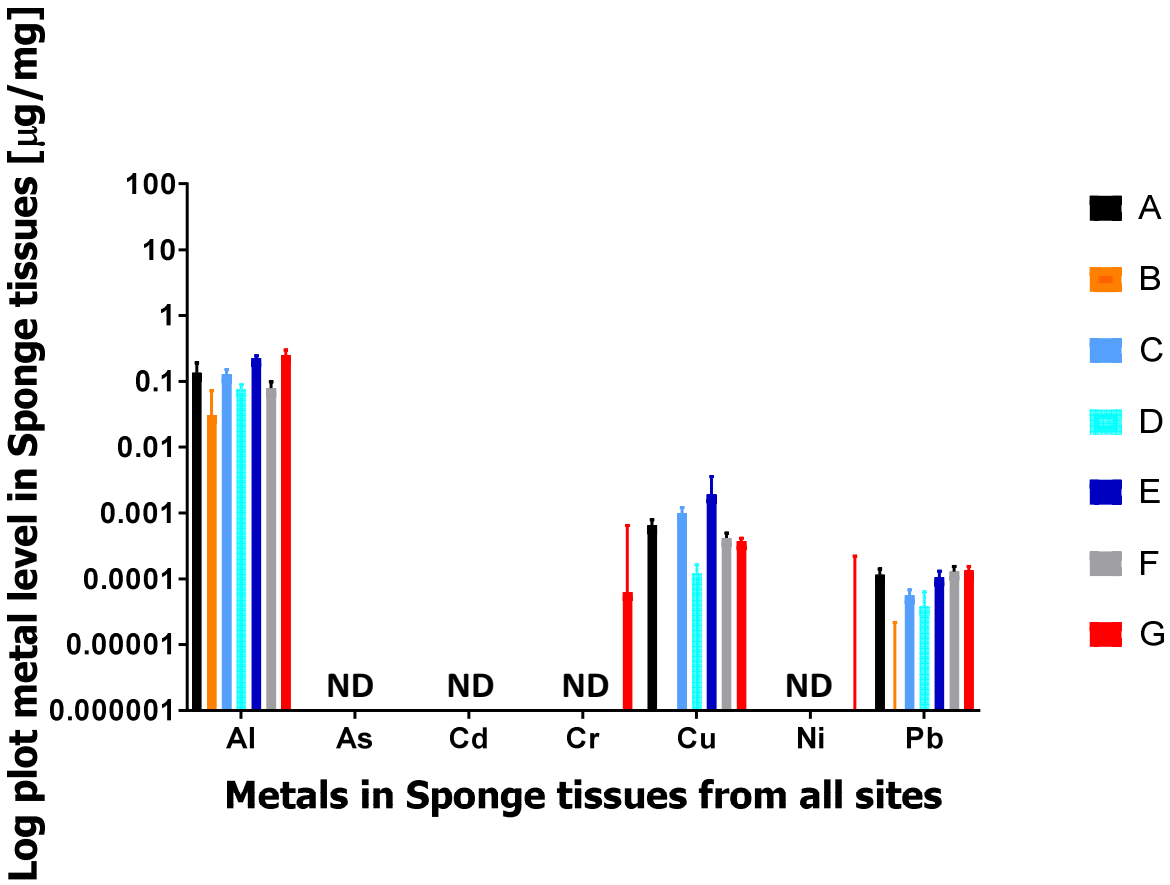


Figure 5.8 : Metal level in sponge tissues from all sample sites (Aqua-Regia extraction method) Displayed data are mean ± SEM; n=3; P<0.05. Two way analysis of variance with Dunnette's multiple comparison test showed a statistically significant difference between metal levels in all sites and % DNA intensities. ND*: not detected*

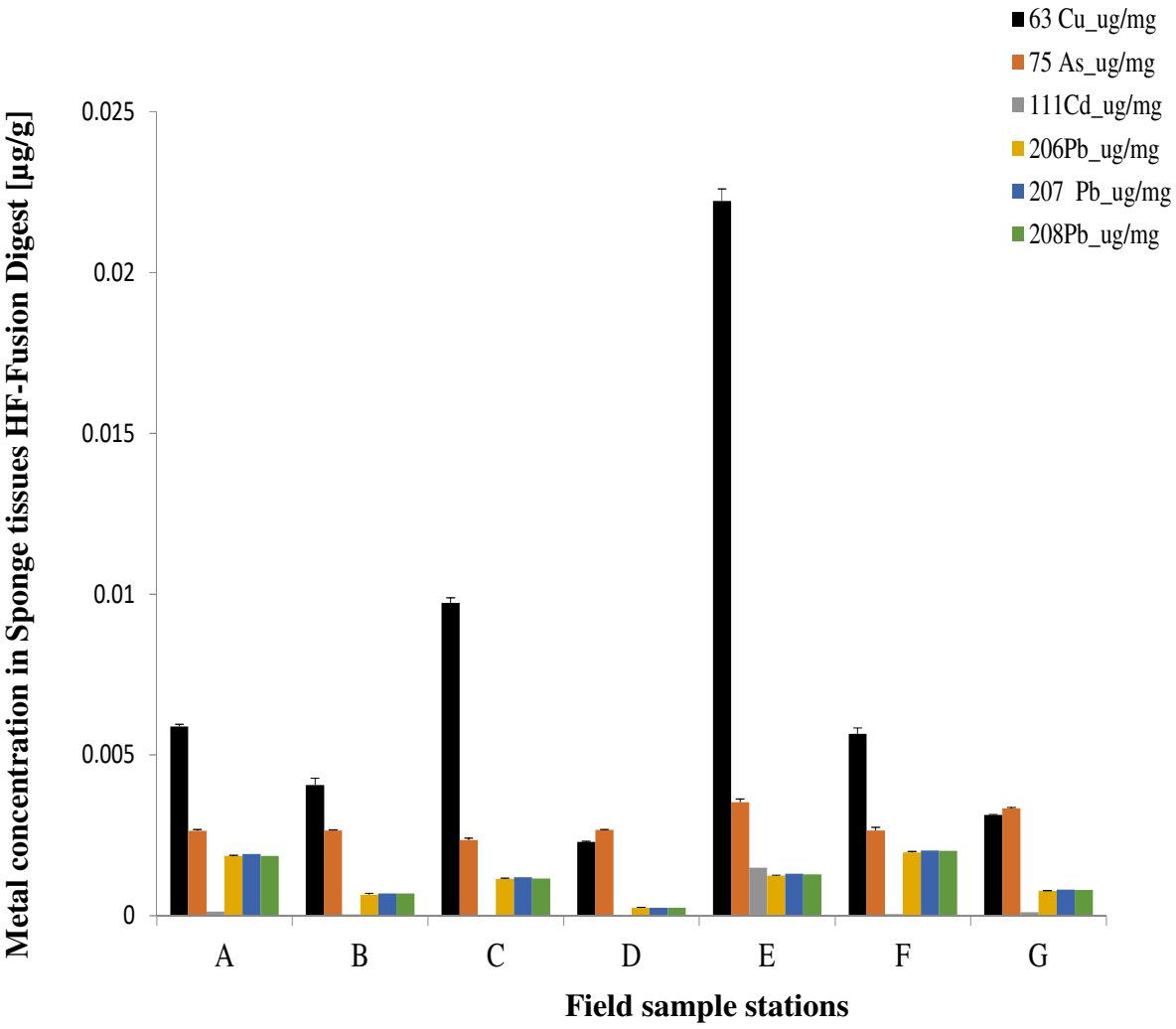


Figure 5.9 Metal levels in sponge tissues from all sample sites extracted by HF-fusion digestion methods. ICP-MS analysis of metal levels in HF digested sponge tissues detects significant amount of Cu in sponge tissues from all sample sites with highest amount detected in samples from site E.

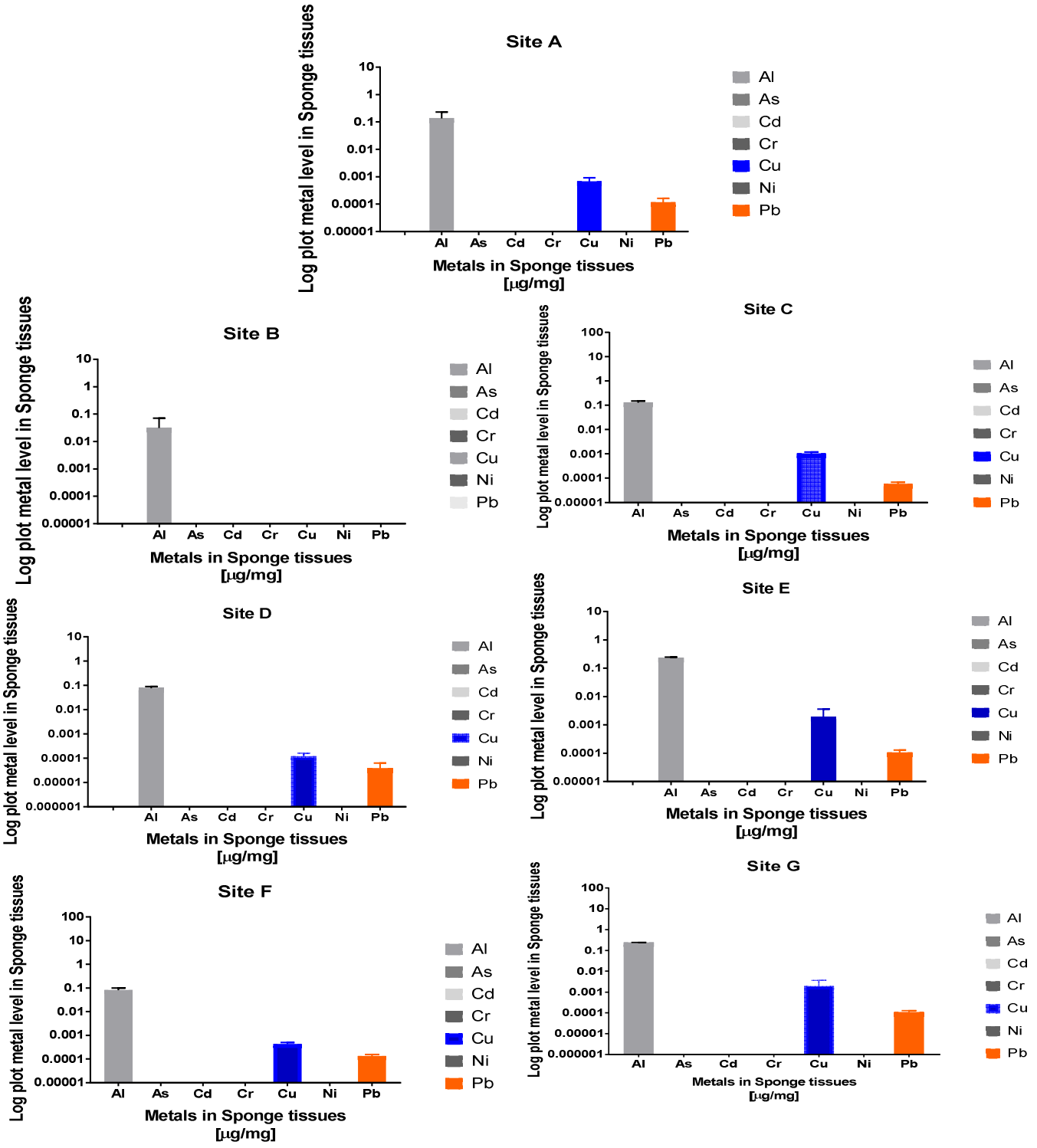


Figure 5.10 Metal level in sponge tissues extracted with Aqua-Regia digestion. Data shows comparison of the five consistently detectable metal (Al, Cu and Pb) levels in sponge tissues between sites. One way analysis of variance with Dunnett's multiple comparison test showed statistically significant difference in the levels of the metals in sites E and G compared to the control sites at $P < 0.05$.. Data shows Mean ± SEM values, n=3,

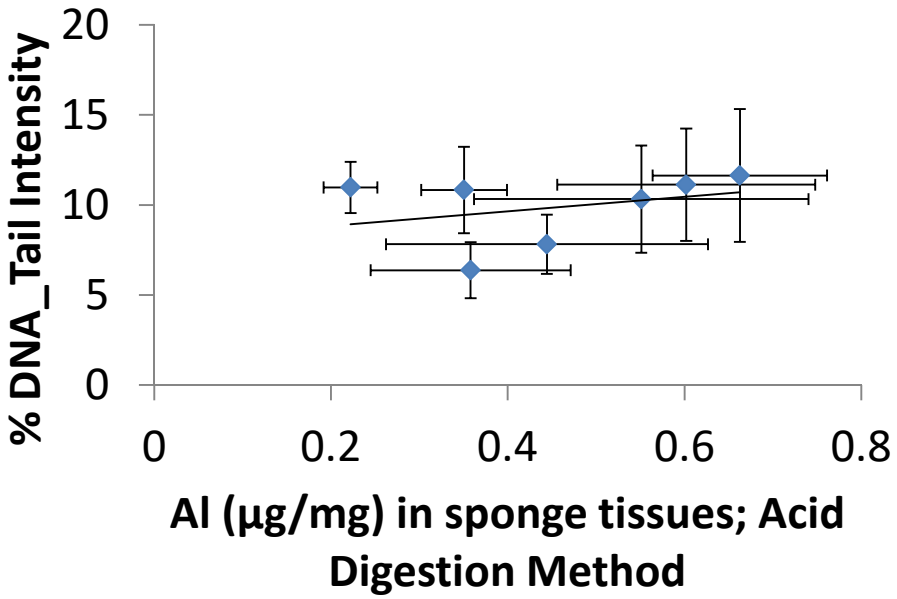


Figure 5.11 Correlation between % DNA strand break in sponge cells from all sample locations and Aluminium levels in sponge tissues from all sample location. R^2= 0.1028; Displayed data is mean ± SEM, n= 3 P=0.05

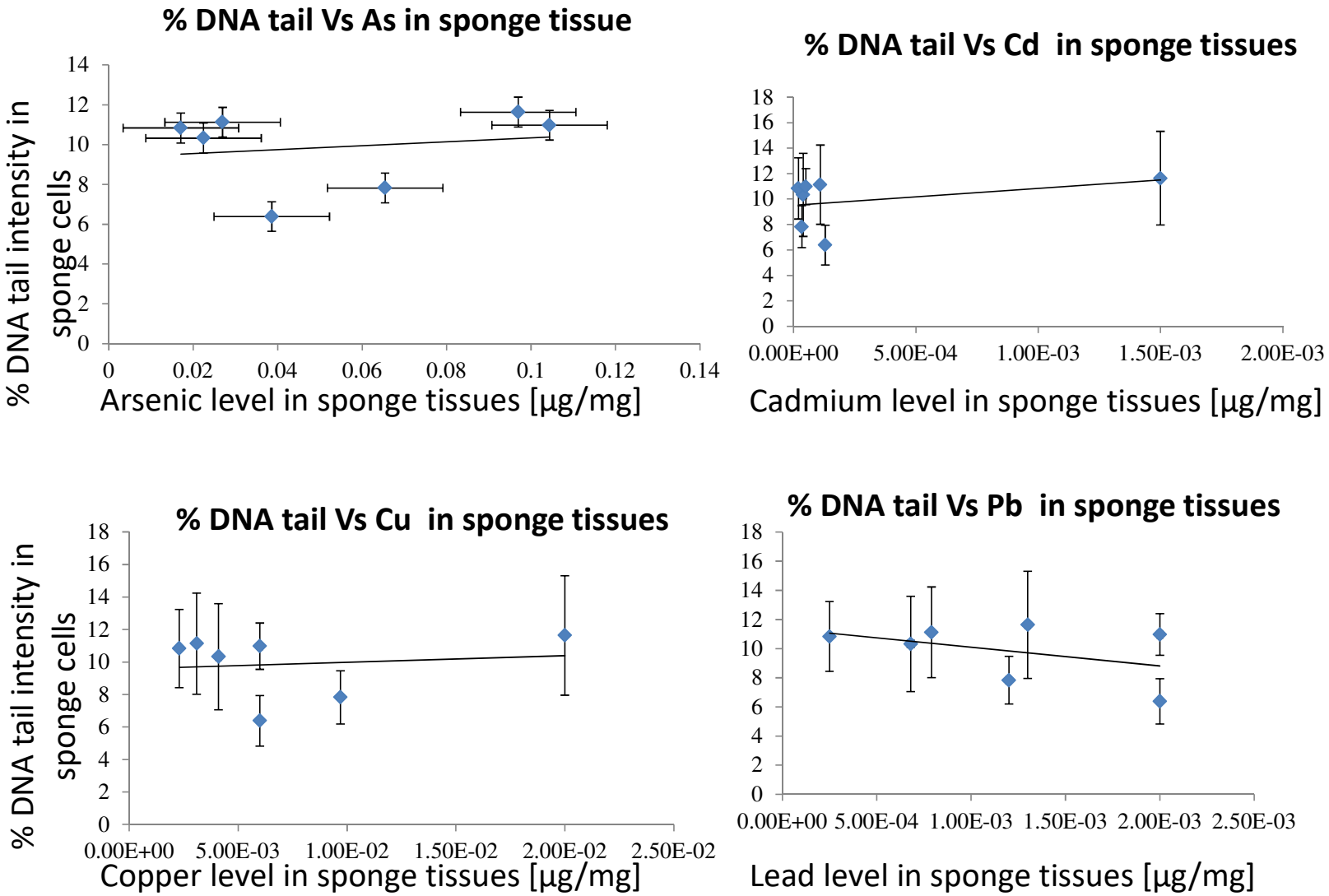


Figure 5.12 Lack of correlation between As, Cd, Cu, and Pb in sponge tissues and detected DNA strand breaks. Result showed little or no correlation between levels of As, Cd , Cu and Pb and DNA damage in sponge cells. Displayed data is mean± SEM, n = 3, R^2 Values , As =0.0329; Cd =0.133; Cu = 0.0157; Pb =0.1879.

5.7.2 Metal Accumulation in sediment and % DNA strand break in sponge cells

Table 5.2 shows the summary of metal levels in sediment across all sites extracted by HF digestion method and acid digestion methods for aluminium data. Metal levels detected by ICP-OES analysis were compared with sediment quality guidelines (SQG) (Table 5.3) and Department of Petroleum resources (DPR) standards for metals in licheate (DPR 2002). By aqua-regia method, Al, Cr, Ni, Cu, As and Pb were detected in all sites (Figure 5.13 b), while HF digestion method recovered Hg in the 6 Niger Delta sites and Cadmium in site E (Figure 5.13a). Metal levels in sediment compared with percentage DNA strand break were statistically significant in sites C, D, E and F (Figure 5.14).

Table 5.2 Metal concentration in Sediment samples digested by Aqua-Regia method and analysed with ICP-MS. Data shown represent mean ±SEM; mg/kg, n=3. Results are compared with standard sediment quality guidelines (Table 5.3).

Sample Locations	Al	Cr	Ni	Cu	As	Pb
A Topo creek,Badagry/Lagos	0.89 ± 0.11	0.09±0.005	0.04 ± 0.004	0.01 ± 0.001	0.001 ± 0.03	0.002 ± 0.0002
C Al-Marine Creek, Ologbogbo	62.09±7.87	0.17±0.02	0.07 ±0.006	0.05 ± 0.003	0.27 ± 0.01	0.04 ± 0.002
D Kalibiama creek, Bonny sea	73.33±2.24	0.19±0.005	0.08 ±0.002	0.05 ± 0.002	0.30 ± 0.01	0.04 ±0.002
E Egbomu River, Andoni	33.45±2.48	0.12±0.01	0.05 ±0.006	0.02 ± 0.002	0.23 ± 0.02	0.02 ± 0.001
F Isaka creek, Eagle Island	64.25±4.74	0.20±0.01	0.08 ± 0.004	0.06 ± 0.001	0.15 ± 0.01	0.06 ± 0.005
G Pokori creek/Nembe/Brass	55.39±13.39	0.19±0.05	0.07 ± 0.01	0.04 ± 0.006	0.18 ± 0.03	0.03 ± 0.004

Table 5.3 Sediment quality guideline values (µg/g =ppm=mg/L= mg/kg)

Metals	DPR[a]	TEL[b]	ERL[c]	ERM[c]	LEL[d]	SEL[d]
Al	nd	nd	Nd	nd	nd	nd
As	5.0	nd	8.2	70	6.0	33
Cd	nd	0.68	1.2	9.6	0.6	10
Cr	5.0	52.3	81	370	26	110
Cu	nd	18.7	34	270	16	110
Ni	nd	15.3	29.9	51.6	16	75
Pb	5.0	30.2	46.7	218	31	250

DPR: Department for Petroleum Resources, Nigeria; TEL: Threshold effect level; ERL: Effect range level; ERM: Effect range medium; LEL: Lowest effect level; SEL: Severe effect level. [a](DPR 2002) [b](Iwegbue et al. 2018); [c](Long et al. 1995) & [c](Fletcher et al. 2008)

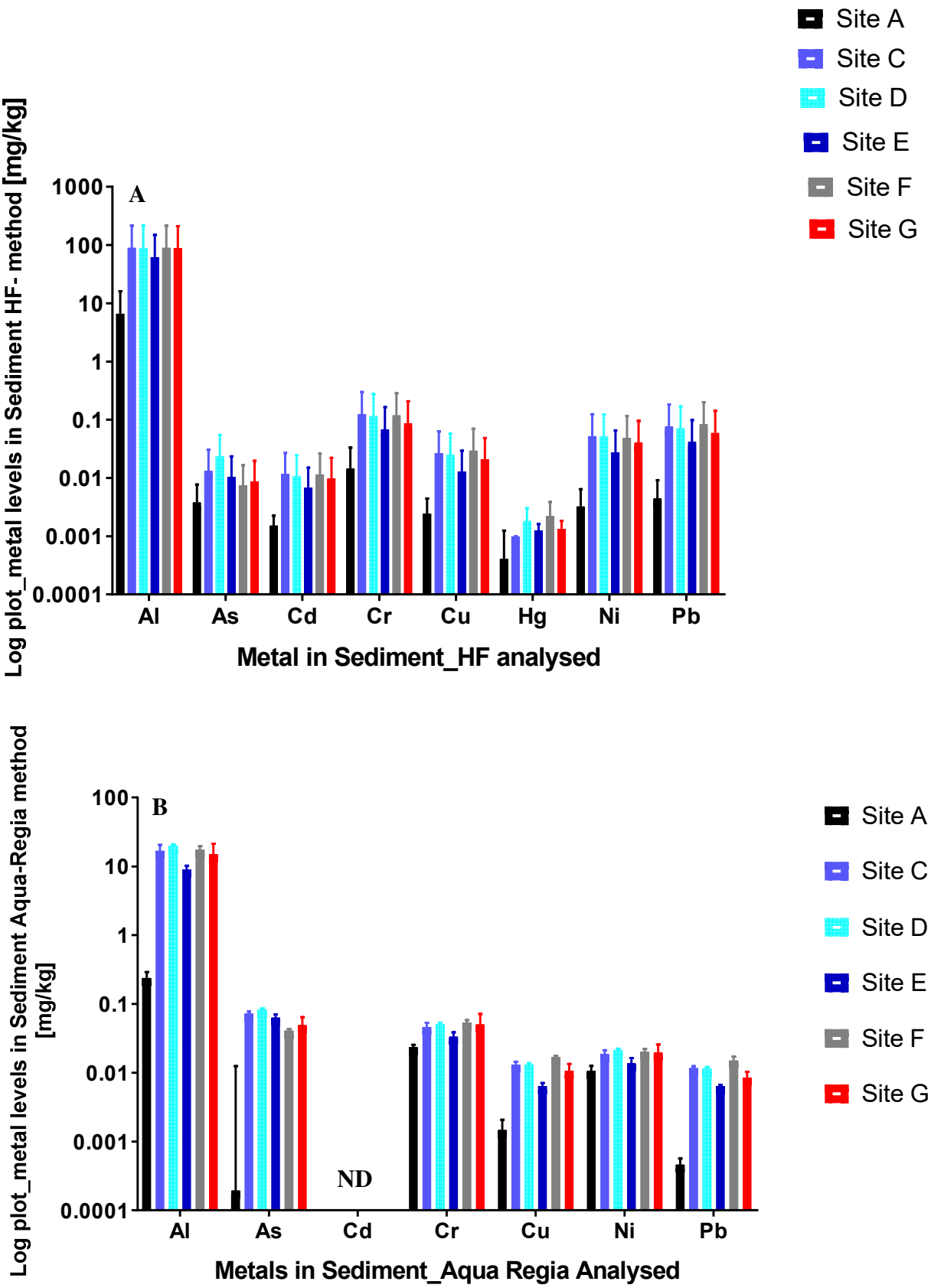


Figure 5.13 Metal levels in sediment, extracted with A) Hydrofluoric acid and B) Aqua- Regia. Data shows mean ± SD, n= 3. Cadmium was not detected by Aqua-regia method in all sample sites, but was detected by HF method. There was a statistically significant difference in metal levels between sample sites at P <0.05 Graphpad 7.0; Turkey multiple comparison test.

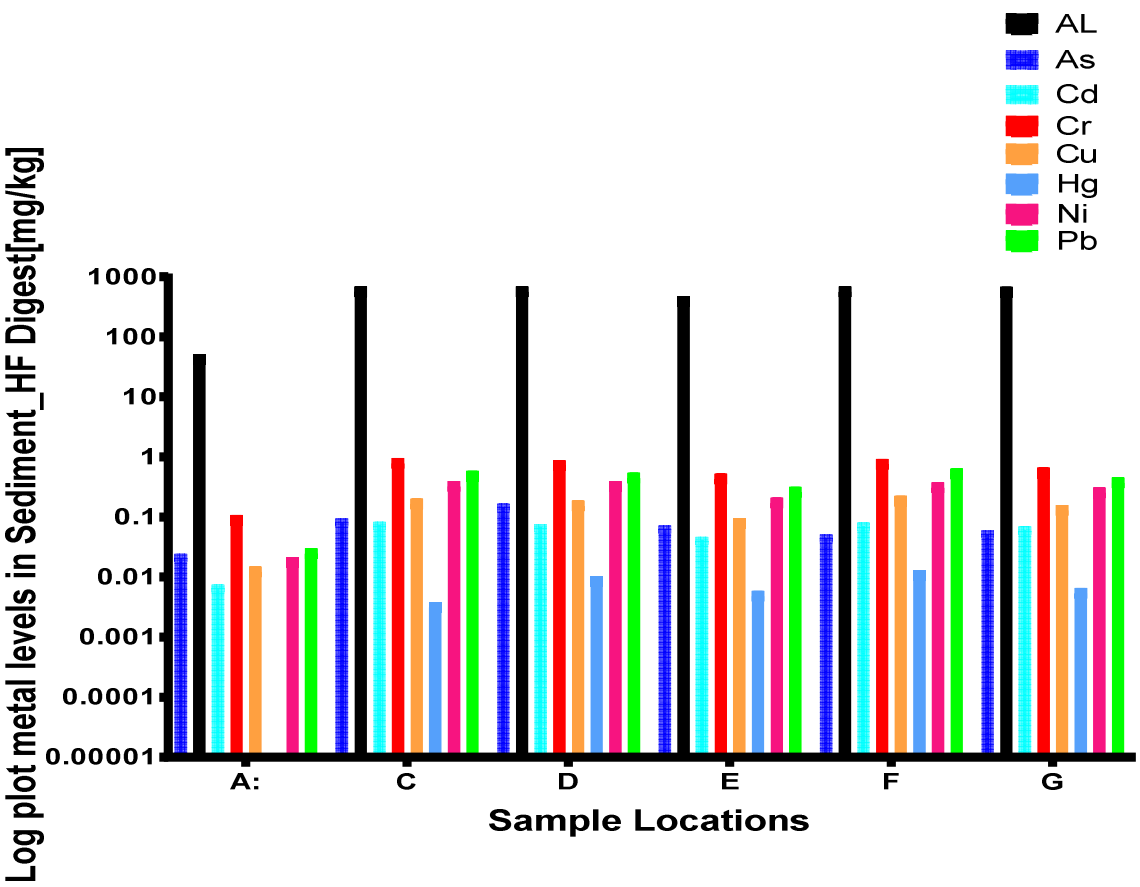


Figure 5.14 **Metal levels in sediment (Locations A-G details in Table 2.0.1).** Two way ANOVA at p< 0.05 and Dunnett's multiple comparison test showed a statistically significant difference between metal levels in sediment from sites C(P<0.001), Site D(P<0.0001), Site F (P<0.0001) and Site E (P= 0.0029).

5.7.3 Metal concentration in water column and % DNA strand break in sponge

Metal level in water column was semi quantitatively analysed in lab 3. Result showed relatively low metal concentration across all sample sites compared with sponge and sediment (Table 5.5). In contrast with measurement in sponge and sediment, Aluminium and Cadmium levels were highest in sample site B. There was a statistically significant difference between Aluminium level in water column in all sample sites and percentage DNA strand break in sponge cells from all sample site (Figure 5.15).

Table 5.4 Semi Quantitative analysis of metal concentrations in water column from all seven sample sites (mg/L water)

	Al	Cd	Cr	Cu	Ni	Pb
A	0.06	0.003	0.01	0.02	0.01	0.01
B	0.92	0.01	0.004	0.01	ND	ND
C	0.26	0.004	0.001	0.01	0.02	0.001
D	0.65	0.004	ND	0.01	ND	0.002
E	0.24	0.001	ND	0.01	0.003	ND
F	0.33	0.002	0.003	0.01	0.002	ND
G	0.25	0.002	ND	0.01	ND	0.003

ND: Not Detected

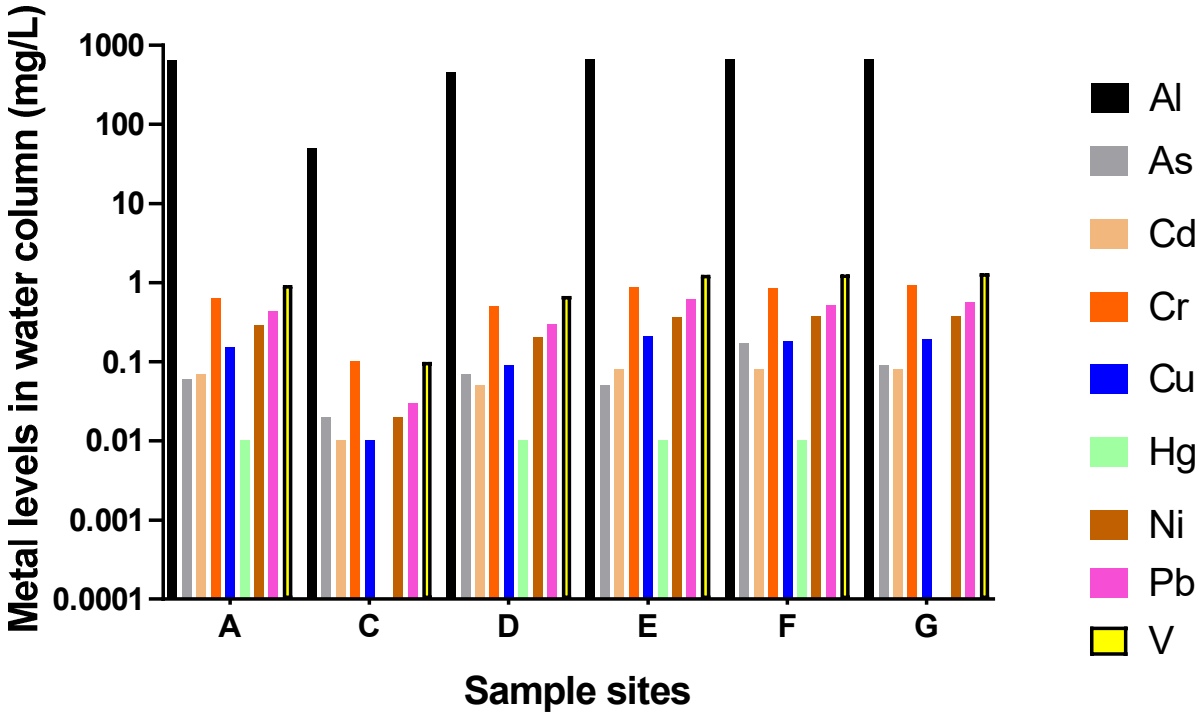


Figure 5.15 Metal concentrations in water column. Two way analysis of variance with graph pad prism 7.04 showed a statistically significant difference between Aluminium level in water and % DNA strand breaks at P <0.001, no significant difference was observed between DNA strand breaks and all other metals in the water column.

5.7.4 Comparison of metal detection: between matrixes and between sample digestion methods.

Table 5.5 shows metal levels in sponge tissues and sediment digested by both Aqua-Regia metal extraction method and HF digestion method. Metal accumulation in sponge relative to sediment is displayed in table 5.6 as fold increase with up to 200-fold metal accumulation in sponge. In sample site A, up to 108fold increase in As bioaccumulated in sponge tissues while accumulation in site D was 15fold. In site E 31 fold increase in the concentration of bioaccumulated cadmium in sponge tissues was observed. Overall metal level fold increase was highest in sample site A and least in sample site D. HF method gave the highest total metal load in both sponge and sediment, however metal levels in all matrices analysed by HF was well correlated with levels analysed by Aqua- Regia method (Figure 5.17). Generally, there is a good correlation between metal levels in sediment between labs (Earth Science and Kingston University), good correlation between ICP-MS analysed data and ICP-OES data, little correlation between metal levels in sediment and in sponge and also little correlation between metal levels in sponge tissues and in water. However, correlations between metal levels in sponge and levels in water was higher that correlation between sponge tissues and levels in sediment (Data in the appendix 2).

Table 5.5 Comparison of metal accumulation in sponge tissues and Sediment using two metal extraction methods. Aqua-Regia digestion methods extracted highest level of Arsenic in sediment samples. Although Pb is consistently highest in site F, in both Sediment and Sponge tissues, and Al and Cu in sediment by HF- extraction method, there was no correlation between Pb nor Cu and DNA strand break and site F was the only site with no correlation between DNA strand break and Aluminium. This explains the

Sponge tissues (µg/g)_Aqua-Regia method

	Al	As	Cd	Cr	Cu	Hg	Ni	Pb
A	134.35	0.07	ND	ND	0.25	na	ND	0.12
B	30.83	0.08	ND	ND	0.28	na	ND	ND
C	127.32	0.10	ND	ND	0.36	na	ND	0.06
D	76.65	0.02	ND	ND	0.07	na	ND	0.04
E	236.40	0.80	ND	ND	2.88	na	ND	0.12
F	79.50	0.04	ND	ND	0.13	na	ND	0.13
G	246.35	0.02	ND	0.064	0.07	na	ND	0.13

Sponge (µg/g)_HF method

	Al	As	Cd	Cr	Cu	Hg	Ni	Pb
A	Na	2.64	0.13	na	5.89	na	na	1.88
B	na	2.65	0.04	na	4.07	na	na	0.68
C	na	2.35	0.034	na	9.74	na	na	1.17
D	na	2.66	0.02	na	2.30	na	na	0.25
E	na	3.53	1.5	na	22.23	na	na	1.28
F	na	2.65	0.05	na	5.65	na	na	2.0
G	na	3.34	0.11	na	3.13	na	na	0.80

Sediment (mg/g)_Aqua-Regia method

	Al	As	Cd	Cr	Cu	Hg	Ni	Pb
A	0.88	0.0007	ND	0.09	0.005	na	0.04	0.002
C	62.08	0.30	ND	0.17	0.05	na	0.07	0.04
D	73.33	0.30	ND	0.19	0.05	na	0.08	0.042
E	33.45	0.23	ND	0.12	0.02	na	0.05	0.023
F	64.25	0.15	ND	0.20	0.06	na	0.08	0.06
G	55.40	0.18	ND	0.19	0.04	na	0.07	0.03

Sediment (mg/g)_HF method

	Al	As	Cd	Cr	Cu	Hg	Ni	Pb
A	49.00	0.024	0.01	0.10	0.014	0.020	0.03	ND
C	658.15	0.10	0.08	0.91	0.19	0.004	0.38	0.56
D	659.52	0.17	0.08	0.84	0.18	0.01	0.38	0.52
E	455.53	0.07	0.05	0.50	0.09	0.01	0.20	0.30
***F**	658.30	0.051	0.08	0.87	0.21	0.01	0.36	0.61
G	647.30	0.06	0.07	0.63	0.15	0.01	0.29	0.43

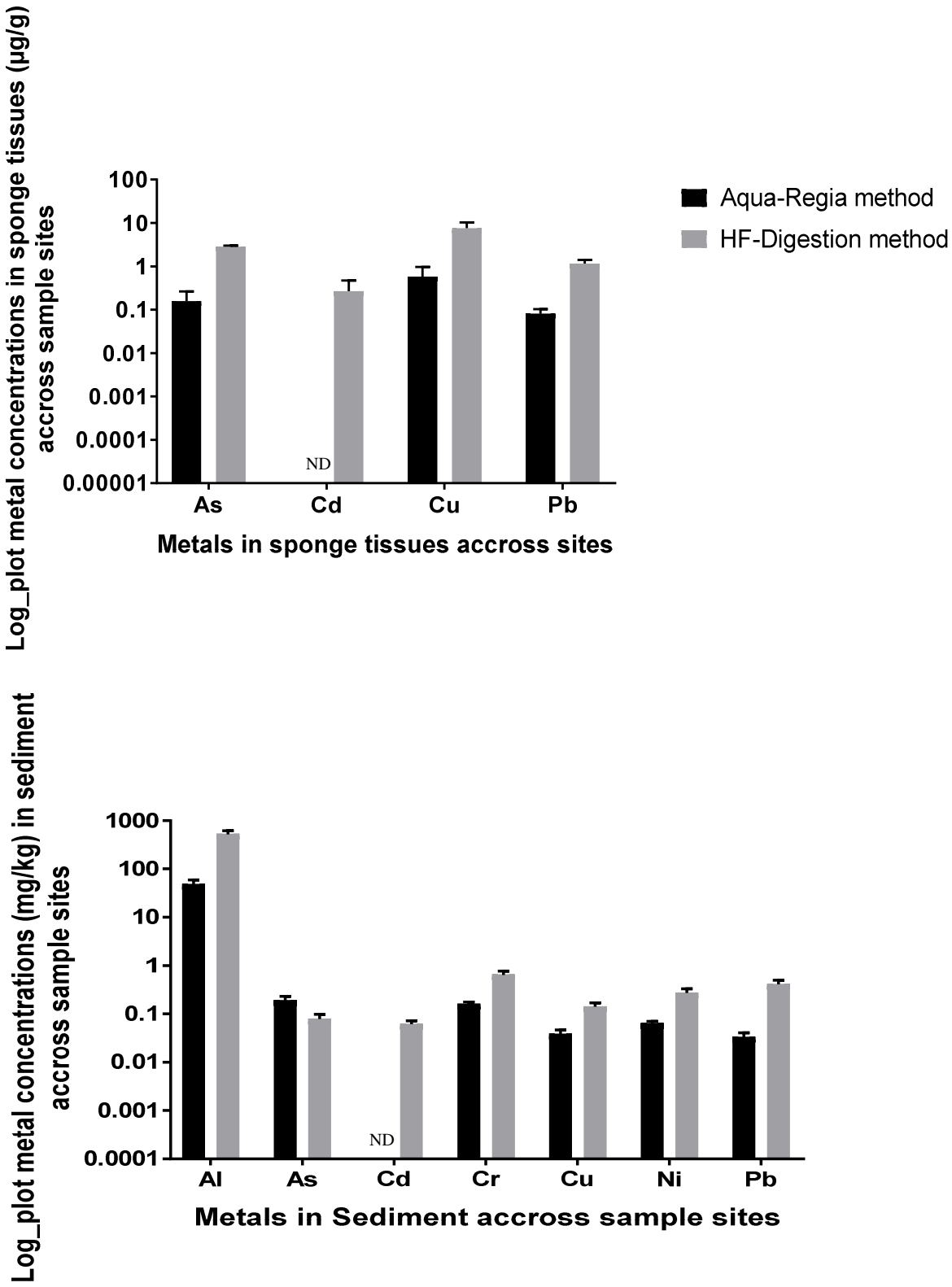


Figure 5.17 : Comparison of metal extraction methods based on metal levels across all sample sites. Data displayed represent mean ± SEM, P<0.05. Two way ANOVA on Graph pad prison version 7.04. a) Metal concentrations in sediment. Result shows a statistically significant difference P<0.0001 between Aluminium in sediment extracted by HF-Digestion method and all other metals analysed across all sites. No statistically significant difference was observed between the metals in sediment extracted by Aqua-Regia digestion method. b)Uptake in sponge tissue between metal extraction methods. Statistically significant difference observed between As & Cd; Cd & Cu; and between Cd & Pb in sponge tissues analysed by Aqua-Regia method. Differences between metal levels in sponge tissues extracted by HF were not statistically significant across all the sites.

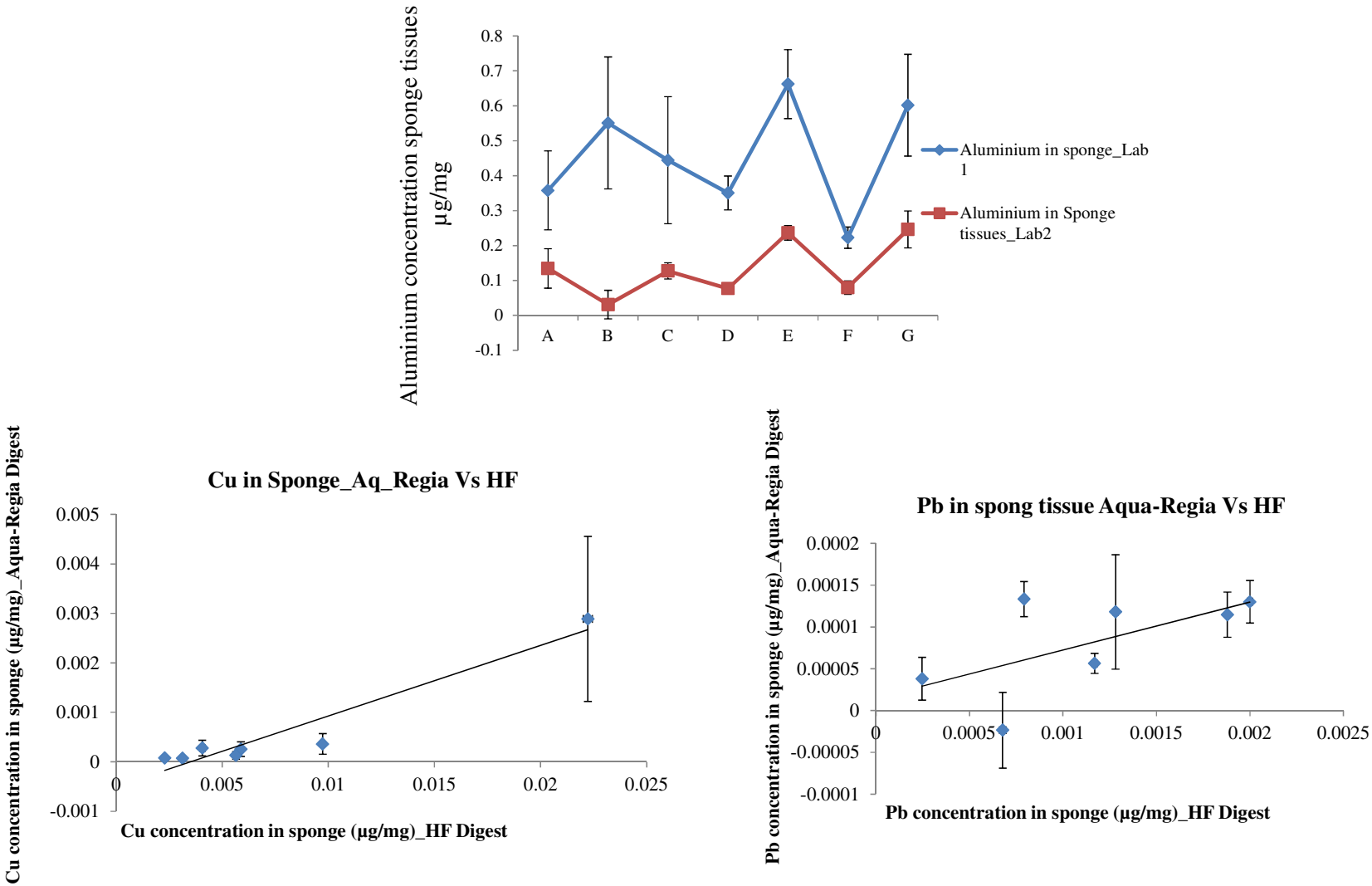


Figure 5.18 Comparison of all three reliably detected metals (Al, Cu and Pb) in sponge tissues between labs and metal between extraction methods a) Aluminium levels in sponge tissues independently analysed in lab 1 (Warwick university_Acid digestion method) and lab 2 (Earth Science University of Birmingham_ Aqua- Regia digestion method); b) Strong positive correlation in Cu levels in sponge tissues from all samples sites between lab 2 and lab 3 (Kingston University_HF-fusion digestion), $R^2 = 0.9236$; c) Little correlation in Pb levels in sponge tissues from all sample sites between labs 2 and 3, $R^2 = 0.3831$.

Table 5.6 Fold increase of Bioaccumulated metal in sponge tissues compared to Sediment

Al	As	Cd	Cu	Pb	
A	**152.2**	**108.3**	**17.0**	**413.0**	**65.3**
C	**2.1**	**24.8**	**0.4**	**50.3**	**2.1**
D	**1.1**	**15.9**	**0.3**	**12.9**	**0.5**
E	**7.1**	**48.7**	**31.9**	**245.7**	**4.2**
F	**1.2**	**51.5**	**0.6**	**26.4**	**3.3**
G	**4.5**	**55.0**	**1.6**	**20.9**	**1.8**

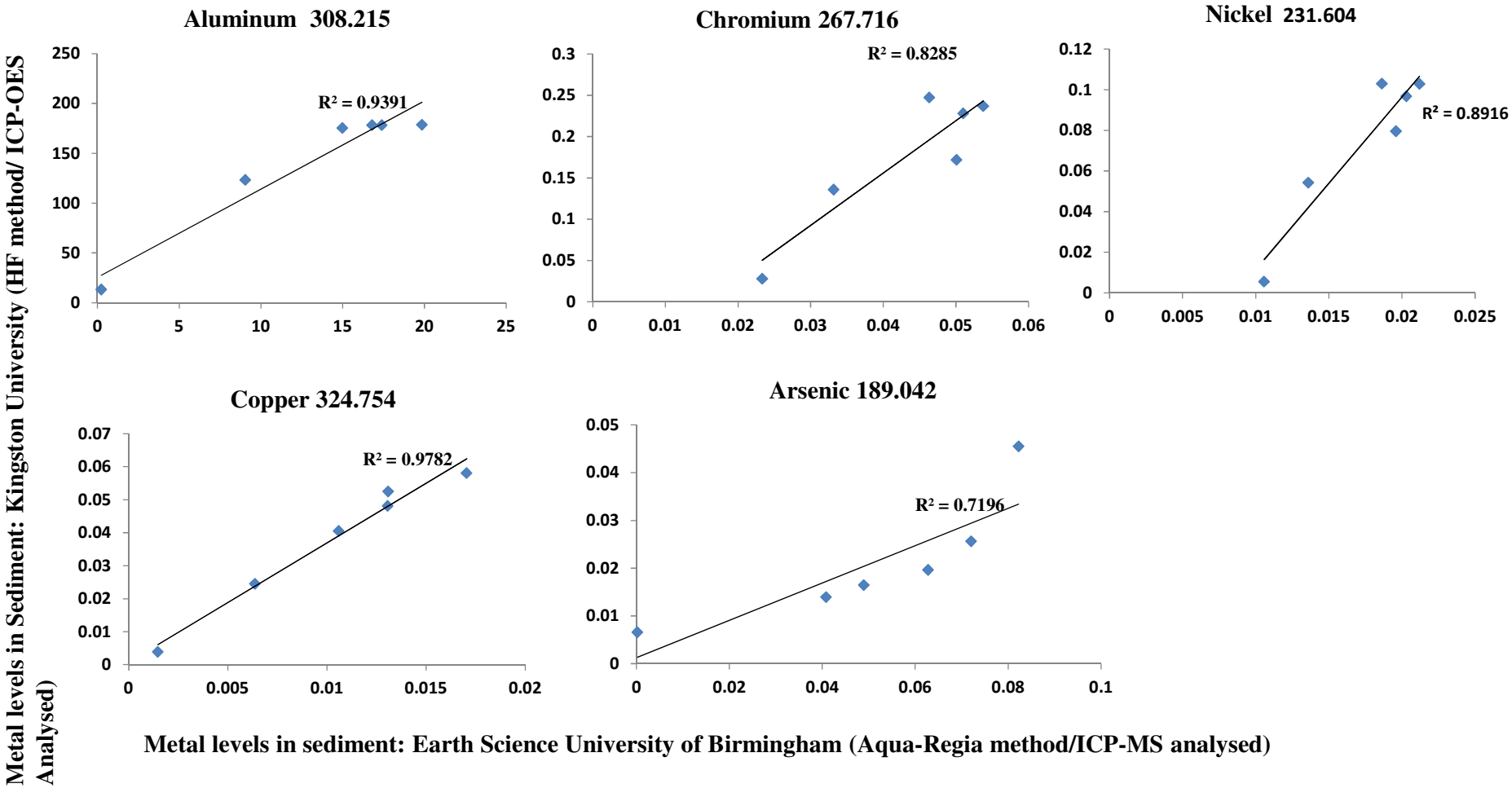


Figure 5.19 Correlation of metal levels in sediment from all sample location; HF digestion method Vs Aqua-Regia digestion method. Generally good correlation between metals in sediment and between labs (Earth Science and Kingston University). Between digestion method (HF and Aqua-Regia digestion methods) and between analytical methods (ICP-MS analysed data and ICP-OES data). R^2 = 0.9391-Al; 0.7196-As; 0.2512-Cd; 0.8285-Cr; 0.9782-Cu; 0.08916-Ni and 0.9696-Pb.

5.8 Discussions

Pollutants in the aquatic system exist as complex mixtures and act in synergy with one another (EPA 2018; Förstner and Wittmann 2012; Gentric et al. 2016). Hence evaluation of the levels of individual pollutants alone is not sufficient to predict the cumulative toxicity of aquatic pollutants in both humans and animals. Bioassays and biomarker data are therefore required to determine the complex toxic effects of the mixture of chemical compounds in aquatic media (EPA 2018). The comet assay is particularly useful in this respect because specificity to the individual pollutants is not required but the collective adverse effects following exposure can be quantified. Thus Environmental risk assessment (ERA) in the aquatic ecosystem involves the utilization of the combined data of biological effects and chemical analysis (EPA 2018; van der Merwe et al. 2010). As a result therefore, biomarkers expressing biological effects from exposure to environmental pollutants have become indispensable in ERA. This is because they provide key information on the bioavailable content of pollutants and serve as an early warning system in monitoring programmes. Biomarkers are applied in both effect-focused and dose–dependent responses to environmental stressors resulting from either of or all of environmental exposures to toxic chemicals, occupational exposure or via food consumption (Rainbow 1993; Van der Oost et al. 2003). The relationship between bioaccummulated metals, Bioavailable metal and toxicity is a major distinguishing factor between lab based toxicity testing and experimental design and organism exposure in situ. In laboratory toxicity testing, more metals are biologically available than in the bioaccumulated form, hence toxicity concentration can easily be determined (Rainbow 2002). Thus, the aim of this investigation is to apply biological effect

assay-the comet assay, developed in lab based toxicity testing in sponge cells in Chapter 4 and sediment chemistry to establish this threefold relationship in field sponge samples.

In this study, the sea sponge *A. kalibiama* a shallow water sponge was identified for the first time in the Niger Delta marine environment. Both cytochrome C oxidase 1 gene and 28S gene sequence data confirmed systematic taxonomic identification of the species as a new undescribed species (Figures 5.1, 5.2 and 5.3). For the first time, this species was deployed to measure the genotoxic effect of metal pollution in Niger Delta marine environment using the alkaline comet assay. Data was compared with bioaccumulated metal content, chemical analysis of metals in sponge tissues, sediment and water column. The application of the comet assay to measure DNA strand breaks sponges as a biomarker of metal genotoxicity is presented here as a potential tool for ERA and would serve as an early warning signal in environmental biomonitoring in the aquatic system.

DNA strand breaks was assessed using the alkaline comet assay in untreated sponge cells of *A. kalibiama;* compared with strand breaks in untreated samples of *H. perlevis* (sponge species used for method development and lab based toxicity testing in chapter 3 and 4) from Tenby bay castle beach was statistically significant in all sites (Figure 5.5). This indicated sample location with highest and least expression of biological effects of metal pollution. To evaluate metal pollution in the Niger Delta and assess potential ecological risks to both animals and humans in the region, several comparisons based on bioaccumulation of metals in sponge tissues, DNA strand breaks in sample sites, metal accumulation in sample sites and metal concentration in water column were made.

To assess bioaccumulation of metal pollutants in situ in sponge tissues, sponge samples of *A. kalibiama* were sampled from 6 Niger Delta marine environment and one control site from

Badagry in Lagos state. All seven sites were analysed for Al, As, Cd, Cr, Cu, Hg, Ni and Pb in sponge, sediment and water (sediment from sample site B was missing). Using different metal extraction methods (Acid digestion, Aqua Regia digestion and Hydrofluoric acid (HF) digestion) and different analytical techniques (Inductively coupled plasma mass spectrometry-(ICP-MS), Inductively coupled plasma optical emission spectrometry-(ICP-OES)) Al (Aluminium), Cu (Copper) and Pb (Lead) were consistently and reliably detected in all sponge samples from all sites. This is in agreement with the report of Cebrian et al. (2007) where out of almost 380 sponge samples analysed for Cu, Pb, Cd, Hg and V; only the concentrations of Cu and Pb were reliably detectable in three sites at levels (Cu; site 1 (3.4±1.2-47±10.3), site 2 (5.7± 0.8 – 82.0 ±3.0), site 3 (11.0 ±0.8 -299.3 ± 82.10) (Pb; site 1 (0.4 ± 0.2 – 6.2 ± 2.8), site 2 (0.3 ± 0.2 – 13.2 ± 1.5) and site 3 (0.4 ± 0.2) – (32.5 ± 6.1). HF digestion method, however also yielded in addition, significant level of Cadmium (Cd), and Arsenic (As) in sponge tissues from all sample sites (Figure 5.9). Comparison of percentage DNA strand breaks in sponge cells between sites tends to be slightly in sponge samples from site E (Egbomu in Andoni, Rivers state) (Figure 5.5) which also tends to be the site with highest bioaccumulated concentration of Aluminium (Al), Arsenic (As), Cadmium (Cd) and Copper (Cu). Lead (Pb) was highest in sponge tissues from site F analysed by HF digestion method. In sediment samples however, Al, Cu and Pb were highest in site F. Bioaccumulated metal load in site E and sediment chemistry data in site F compared with percentage DNA strand breaks in all sample sites using the comet assay was significant (Figure 5.4). The relationship observed between metal levels, in sponge, sediment and percentage DNA strand breaks also indicates the important relationship between Bioaccumulation, Bioavailability and toxicity. Hence even though some metals where highest in sediment samples from site F as stated above, yet more DNA damage were observed in sponge samples from sites E (As, Cd,

Cu – in sponge by both HF and Aqua-Regia digestion methods; and Al by acid digestion); G(highest Al in sediment- by Aqua-Regia digestion method); B(with highest level of Al and Cd in water column) and D(highest level of Al and As in sediment – Aqua-Regia). Also, considering the reoccurring detection of Aluminium in almost all sites and matrixes, correlation of percentage DNA strand break with Aluminium in all sample sites, was not significant in site F (Figure 5.16 a and 5.11). Result agrees with EPA report on the synergy of aquatic pollutants in the induction of toxic effects, rather than on an individual pollutant levels (EPA 2018). Thus, bioassays and biomarkers are useful for easy identification of contaminated sites or environment (Steinert et al. 1998).

Aluminium concentration in sponge tissue ranged from 662.6µg/g (Site E) and 222.2µg/g (site F) by acid digestion method. With Aqua-Regia digestion method, Al level was between the ranges of 246µg/g (site G) and 30.8µg/g (site B). In sediment, Aluminium ranged from659.5 µg/g (Site D) to 48.9µg/g (site A). Aluminium levels were consistently lowest in site A in both sediment and water column and highest in sight E only in sponge tissues analysed by acid digestion. However in all sponge samples analysed by both by HF and Aqua-Regia digestion, As, and Cu where consistently highest in site E. HF digestion of sponge tissues also yielded highest cadmium concentration in site E. DNA % strand breaks ranged from 30.4 ± 4.76 (Site E) and 13.9 ± 0.20 (site A). Strong correlation (R^2=0.9823) between Aluminium level in all sample sites (except site F) and DNA strand breaks. Although Al was lease in sediment analytes using both Aqua-Regia and HF digestion methods, concentration in sponge tissues compared with levels in sediment was 152-fold higher while comparison of Aluminium concentration in sponge tissues in site A in compared to concentration in water was as high as 2,233fold. Similar Aluminium burden was reported in sediment samples from Forcados River Bed in the Niger Delta, Nigeria. Aluminium range

between 9,560-25,000 mg/kg extracted by Aqua-Regia method was reported (Iwegbue et al. 2018). Ipeaiyeda et al. (2012) also reported very high level of Aluminium (1830- 2170µ/g) in surface soil close to an aluminium smelting plant in Ikot Abasi city in Nigeria. However, Aluminium level in the sponge *Amorphinopsis kalibiama* in this study contrasted with the comparatively low aluminium level of 33.19 ± 3.15 and 6.37±0.56 µg/g in the marine sponge *Sigmadocia fibulata* sampled from polluted sites in the Gulf of Manner India (Rao et al. 2007). Other studies have however reported very high concentration of Aluminium in the marine sponge *Hymeniacidon heliophila* (564 -1465µg/g) and a calcarean marine sponge *Paraleucilla magna* (68-691µg/g) sampled from heavily polluted sites and mildly polluted sites in Guanabara Bay and Rio de Janeiro in Brazil, respectively (Batista et al. 2014).

Copper (Cu) was also reliably detected in all matrixes and digestion methods investigated. Concentration ranges between 22.22µg/g and 1.29µg/g were reported in sponge tissues in sites E and D respectively, by HF digestion method. With Aqua-Regia digestion methods, sponge tissue copper burden ranged from 0.07µg/g to 2.887µg/g in sites E and G respectively. In sediments, copper concentration in HF digests ranged from 0.01µg/g to 0.21µg/g in sites F and A respectively. Consistently lowest amount of copper was recorded in sediment from site A by both Aqua-Regia and HF digestion and highest in sites F in same matrix and both digestion method. Very low levels, < 0.1mg/L were observed in water column. There was no correlation between copper concentration in all matrixes analysed and DNA % strand break. However, there was strong positive correlation (R^2= 0.9236) between copper levels in sponge digested with HF and Aqua-Regia digestion of same sample matrix. Batista et al. (2014) also reported lower copper concentration in the two sites above as 2.0-19µg/g and 0.2-3.9µg/g in *Hymeniacidon heliophila* and *Paraleucilla magna,* respectively (Batista et al. 2014) . Cebrian et al. (2007) also reported varying and significant copper levels in the tissues of the four

sponge species; *Chondrosia reniformis, Crambe crambe, Phorbas tenacior and Dysidea avara* across three sites in Catalonia in Spain. Cu levels ranginging from 8.2-299.3µg/g were detected by ICP-MS.

Lead (Pb) and Arsenic (As) were also detected in trace amount in all matrixes and digestion methods investigated. Highest level of As in sponge samples extracted by HF was detected in sponge samples from Site E, and level ranged from 2.3 µg/g (site C) to 3.5 µg/g (site E). Sponge As load in Aqua Regia digest was 0.02µg/g (site G) and 0.78µg/g (site E). In sediment samples digested using HF, the range was 0.02µg/g (site A) to 0.17µg/g (site C) and 0.0007µg/g (site A) and 0.3µg/g (site D). Pb was highest in site F in sediment digested by both HF and Aqua Regia with ranges such 0.056 - 0.61µg/g and 0.13-2.00µg/g, respectively. These levels were similar to those reported in (Cebrian et al. 2007) 0.3-2.1µg/g across sponge samples and location. There were no correlations between As level, Pb and % DNA damage in all matrixes.

Although heavy metals are readily bioaccumulated in aquatic biota, body load unlike bioaccumulation of organic pollutants do not however imply toxicity of metals (Phillips and Rainbow 1993); Rainbow and Luoma (2011); (Rainbow 1993; Van der Oost et al. 2003). This is because bioaccumulated metals are either removed by excretory processes or stored up in nontoxic forms, as such would not be biologically available to the organism and would not necessarily illicit any adverse effect (Phillips and Rainbow 1993; Rainbow 2002). However, metals become biologically available in living organism by passive adsorption through active sites on the semi-permeable membrane. Within the cell, they metabolically interact with cellular biomolecules and metabolic processes undermining and interfering with cellular macromolecules and biological pathways. Metal toxicity therefore ensues when bioavailable

metal contents reaches a threshold concentration within an organisms, and can only be measured using biomarkers (Phillips and Rainbow 1993; Rainbow 2007; Sarkar 2002; Van der Oost et al. 2003).

In conclusion, results show that metal concentrations in sediment and water column are below sediment quality standard in all the sites investigated; there are no standards for aluminium. However, in sponge tissues concentrations of copper and cadmium are significantly higher than the permissible standards in sediment which also coincides with DNA strand breaks in single sponge cells. This confirms the potential of sea sponges to bioaccumulate metal pollutants in levels higher than the ambient environmental concentration (De Mestre et al. 2012; Gentric et al. 2016) and the suitability of *Amorphinopsis kalibiama* for application in aquatic biomonitoring and environmental risk assessments.

Also, results shows that DNA damage in marine sponges measured with the comet assay technique is an important biomarker for the investigation of genotoxic effect of environmental pollutants. And can be used to determine sample locations with highest expression of biological effects from metal pollution, which would save time and money involved in chemical monitoring of individual pollutants (Steinert et al. 1998). This information is also vital for pollution biomonitoring and Environmental risk assessment.

Chapter 6 – General Discussion

6.1 General Discussion

Environmental monitoring (EM) involves scheduled or repeated observation of an environment for the presence of toxic substances and also verifying their potential to cause adverse health effects on life forms following interactions or exposure (Martins and Costa 2014; Van der Oost et al. 2003). Biomarker data in combination with chemical monitoring and bioaccumulation data are vital tools commonly employed in environmental risk assessment (ERA) in the aquatic ecosystems (Martins and Costa 2014; Steinert et al. 1998; Van der Oost et al. 2003). In coastal environments, benthic aquatic invertebrates because they express biomarkers of pollution similar to those found in higher metazoans have in gained significant attention in environmental toxicology (Martins and Costa 2014; Moriarty 1988; Rainbow 2017; Roberts et al. 2008; Van der Oost et al. 2003). Furthermore, as resident species, sessile invertebrates have the potential to bioaccumulate pollutants in their immediate environment. As a result they are useful for profiling the health status of their environment. In this respect, marine sponges because of their ability to accumulate both ionic and lipophilic pollutants in both suspended and dissolved phases in the aquatic system are considered suitable alternatives and in some cases an appropriate replacement for already established aquatic sentinels and invertebrate surrogates to higher organisms, such as mussels and other bivalves (Batista et al. 2013; Perez et al. 2003).

Among biomarkers employed to investigate pollution in the aquatic environment, genotoxicity is commonly utilised, because of the close link between genetic toxicity and aquatic pollution (Hose 1994; Sarkar et al. 2015). DNA strand breaks, particularly single

strand breaks are a commonly reported biomarker of genotoxicity in aquatic organisms (Ferrante et al. 2018; Lee and Steinert 2003b; Martins and Costa 2014; Mitchelmore et al. 1998b; Nacci et al. 1996; Schröder et al. 1999) and can be measured both in intact cells with all cytoplasmic content present and in lysed cells with all cytoplasmic materials removed (Whitaker et al. 1991). However, to assess the purest form of DNA, completely lysed cells are utilised. DNA damage is assessed by either determining changes in DNA mass following centrifugation or differences in the length of DNA following electrophoresis (Ahnström 1988; Whitaker et al. 1991). DNA damage is measured as either double strand break in neutral electrophoretic medium or as single strand breaks using alkaline electrophoretic media using assays such as; alkaline gel electrophoresis assay (the comet assay), nucleoid sedimentation assay, DNA precipitation assay and alkaline unwinding assay (Ahnström 1988; Olive 1988; Schröder et al. 1999; Whitaker et al. 1991). Other assays for assessing genotoxic effects in cells includes; micro nucleus assay, ^{32}P-postlabelling assay, Ame's test, and sister chromatid assay. Most of these assays and methods are however, cost and time intensive and require large amount of cells (Bernstein and Bernstein 1991; Steinert et al. 1998). The alkaline comet assay introduced by Ostling and Johanson (1984) and modified by Singh et al. (1988) is however extensively utilised in assessing DNA strand breaks in individual cells (Table 1.3, chapter 1), it is a straight forward assay and very economical to run.

The comet assay is reliably utilised in ERA, because of its sensitivity to environmental, mechanical and thermal stressors in single cells (Martins and Costa 2014). Results of the assay are analysed both visually and by measurement of a varying range of toxicity endpoints, it also requires fewer number of cells than other genotoxicity and DNA strand break assays (mentioned above) to run (Collins 2004; Cotelle and Ferard 1999; Koppen et al. 2017;

Steinert et al. 1998). In this thesis, DNA strand break in sponge cells was investigated using the alkaline comet assay as a biomarker of genotoxic effects in both lab-based toxicity testing and field collected samples. To the best of my knowledge, this would be the first study that has adapted the comet assay to investigate pollutants induced DNA strand breaks marine sponges. This is surprising, because the alkaline comet assay since its development has been widely used to determine different types of DNA damages in almost any system capable of indicating exposure to environmental pollution (See table 1.3, chapter 1).

Sea sponges being important members of the aquatic system and coral reef community (Alcolado 2007), have been extensively utilised in bioaccumulation studies because of the sessile life style, potential to filter large amount of water, ability to readily bioaccumulate pollutants even at low concentration, the ability to retain pollutants for an extensive period with the expression biomarkers of pollution (Batista et al. 2013; Berthet et al. 2005a; Cebrian et al. 2007; De Mestre et al. 2012; Hill et al. 2002; Pan et al. 2011). The results in chapter 3 showed that sponge cells are suitable for downstream biochemical assays including the alkaline comet assay. Using cryopreserved single sponge cell suspension, an 'in vivo' exposure sponge model was developed for lab-based toxicity testing. Preliminary exposure of the model to cadmium chloride showed a concentration dependent increase in the level of DNA strand breaks measured with the comet assay. However, treatment with 1mg/L cadmium for 12 hours resulted in reduced toxicity (decrease in the level of DNA strand breaks) compared to lower concentrations of 0-0.1mg/L. This is possibly due to the activities of DNA repair genes in sponges or programmed cell death with selective removal of damaged cells. In a study assessing the genotoxicity of Aluminium in human lymphocytes, 25µg/mL Aluminium exposure was reported to result in decline in the level of DNA damage compared with lower concentrations of 1-10 µg/mL (Lankoff et al. 2006). In our data, sponge cells

treated with 1mg/L cadmium, showed nuclei with mostly diffused tails and individual cells with higher responses were seen as mostly 'hedge-hogs' (Lorenzo et al. 2013) compared to cells exposed to lower concentration. Schröder et al. (1999) investigated cadmium induced DNA damage using microplate alkaline elusion assay and observed the maximum amount of DNA strand breaks expressed as strand scission factor (SSF) after 12 hours exposure of the marine sponge *Suberites domunculai* to 1mg/L cadmium; they also reported decline in DNA strand breaks beyond 12 hours exposures.

A number of studies have utilised the alkaline comet assay in environmental monitoring (Cotelle and Ferard 1999; Martins and Costa 2014; McKelvey-Martin et al. 1993; Mitchelmore and Chipman 1998) and also the application of sea sponges in biomonitoring of aquatic pollution (Berthet et al. 2005a; Ferrante et al. 2018; Hansen et al. 1995; Olesen and Weeks 1994), In this thesis we present the use of the comet assay in sponge cells (suspension made from dissociated cultured cryopreserved aggregates) combined with both bioaccumulation and sediment chemistry as a reliable tool for aquatic pollution monitoring and application in environmental risk assessment in coastal marine environment. In this thesis, investigation of cadmium uptake in cultured sponge aggregates showed up to 3-fold increase following exposure to 0.01-1mg/L cadmium chloride (Section 3.2.9; chapter 3). This is in agreement with other bioaccumulation studies. For example up to 1520µg/g chromium accumulation in the sponge species *Spirastrella cuspidifera,* has been reported Patel et al. (1985) . Also preferential bioaccumulation of polychorobiphenyl congeners in the *Spongia officinale* has been reported by Perez et al. (2003) compared with bivalves. Gentric et al. (2016) also reported preferential chromium accumulation in the sea sponge *H perlevis* and *Raspaila ramose* in a comparative study with oyster. Other reports on extensive metal and

organic bioaccumulation in sponges are those of (Cebrian et al. 2007; Ferrante et al. 2017); Pan et al. (2011).

Although sea sponges have all the features and potentials required for serving as biomonitors and bioindicators of aquatic pollution (Section 1.11.1 chapter 1); till date however, there are no commercially available sponge cell lines for sustainable in vitro and lab-based toxicity testing of aquatic pollutants. Hence the limited application of sponge cells in in vitro assessment of the toxicity of model aquatic pollutants (Rinkevich 2005). The use of cryopreserved sponge cells and the development of functional sponge cell aggregates (Primorphs) have provided flexibility in sponge cell culture and opportunity for mechanistic studies using sponge cells. Data in chapter 3 shows that cryopreserved sponge cells remain viable after freezing and thawing and are very suitable for toxicity assessment of aquatic pollutants and any other bioassay (Figures 3.2, chapter 3). In a previous study, Mussino et al. (2013) reported high sponge cell viability following freezing and thawing, however they were not able to produce primorphs from single sponge cells after thawing cryopreserved cells. In contrast, we have demonstrated in this thesis extensive primorph formation in culture following thawing of cryopreserved sponge cells (Figure 3.3 chapter 3). The primorph system is the future of sponge cell research because it provides opportunity for sustainable production of sponge bioactive metabolites; it serves as model for environmental biomonitoring, and provides opportunity for prolonged sponge cell culture (Müller et al. 2000a; Pomponi and Willoughby 1994; Pomponi and Willoughby 2000). Sea sponges serve as host to several aquatic endosymbionts, which means that in sponge cell extract there are other symbionts that might be misinterpreted as sponge cells. According to Rinkevich (2005), sponge cell culture for primorph formation is a purifying system that provides an axenic sponge cell population in culture and with the use of appropriate antibiotics, an aseptic sponge cell culture is possible.

This was demonstrated in chapter three, sections 3.2.5 and figure 3.6. Thus, for all bioassays in this thesis, sponge primorphs were developed from cryopreserved single sponge cells as a novel 'in vivo' exposure model and a method for disaggregating them back into single cells with ethylenediaminetetraacetic acid (EDTA) containing synthetic sea water was established. In other to assess the genotoxic impacts aquatic genotoxicants on sponge cells in the aquatic system, model and environmentally relevant relevant pollutants were assayed using sponge cells to measure their potential for inducing toxic effects and the mechanisms of toxicity.

In chapter four, the primorph system was utilised for testing the genotoxic effects of model aquatic pollutants as well as the potential of the test concentrations to induce oxidative stress in sponge cells, using the comet assay and H_2DCF-DA assay respectively. Our data shows a concentration dependent increase in both % DNA strand breaks and the amount of reactive oxygen species (ROS) production. These findings also agrees with the report of Peskin et al. (1998), who showed increase ROS formation in untreated whole sponge species *Sycon sp.* Metal induced DNA strand breaks was also demonstrated in both laboratory cultures of sponge cubes and in situ exposures of *Suberites domuncula* (Schröder et al. 2006; Schröder et al. 1999). Our data showed that cadmium concentrations higher than 0.4mg/L maybe cytotoxic in sponge cells or might induce the expression of DNA repair genes as seen in preliminary cadmium exposure to 1mg/L in chapter 3. A strong positive correlation between DNA damage and ROS in cadmium exposed sponge cells shows that reactive oxygen radicals are likely to be a contributor to cadmium-induced DNA strand breaks and toxicity (Stohs and Bagchi 1995). This thesis also investigated the genotoxic effects of hexavalent chromium in sea sponges. Chromium is a toxic heavy metal (Table 1.1 and 1.2, in chapter 1) that is easily bioaccumulated in aquatic animals, Cr(VI) is an established human carcinogen and as such priority contaminant for environmental regulators (IARC 1990). Data presented in chapter

four shows that chromium concentration between 0.1-0.4 mg/L is not cytotoxic in sponge cells but an increase to 5.2mg/L (100μM) resulted in significant cytotoxic effects in single sponge cells. Our data however showed that non-cytotoxic chromium concentrations caused significant DNA damage and reactive oxygen species generation in sponge cell culture compared to control untreated cells in a concentration-dependent manner. The genotoxic impact of chromium is well studied in aquatic organisms (Ahmed et al. 2013; Hansen et al. 1995; Nussey 2000; Wise et al. 2016). Unlike cadmium, production of reactive oxygen radicals is an alternative pathway for the induction of DNA strand break, main mechanism being the reduction of hexavalent chromium to Chromium III via a Fenton-like reaction which then results in the induction of DNA damage (Henkler et al. 2010). In this thesis a better correlation was observed between cadmium induced DNA damage and ROS formation (Figure 4.5, chapter 4) than those of chromium induced DNA damage versus ROS formation (Figure 4.9). In a previous study the DNA strand break induction by cadmium and chromium was measured using the comet assay technique (Ahmed et al. 2013; Ahmed et al. 2010). Other environmentally relevant pollutants investigated in this thesis for their genotoxic impacts are Nickel, Aluminium and Benzo[a] Pyrene. Nickel is a well-known human allergens as well as a human carcinogen. The toxicity of nickel is also mainly linked to ROS formation with other alternative pathways for the induction of DNA damage including DNA hypermethylation and inhibition of DNA repair (Chakrabarti et al. 2001; Henkler et al. 2010; Xie et al. 2007). Exposure of single sponge cells to nickel in chapter four produced a concentration-dependent increase in DNA strand breaks, and was strongly correlated with ROS formation. Although questions still exist as to the mechanism of Nickel toxicity, it is however reported that in addition to the ROS formation and other pathways previously mentioned Nickel works in synergy with other ionizing (UV radiation, X-rays and gamma radiations) genotoxicants

(Schillack and Buisson 2005). Aluminium is an interesting metal, although it is the third most abundant element on the earth crust and the most abundant metal, its toxic effects in both animals and humans have been largely ignored (Zatta et al. 2002). In this thesis, results in Chapter 5 shows the chemical analysis of both sediment chemistry and bioaccumulation of metals in sponge tissues for metals, identified aluminium as the major contaminant in sponge, sediment and water column, these results was well correlated with percentage DNA strand break as a biomarker of genotoxicity in untreated sponge cells from the same sites using the alkaline comet assay. Toxicity of Aluminium was further confirmed by lab-based toxicity testing of a range of concentrations 0-0.4mg/L. Exposure of sponge cells to these concentration for 12 hours at room temperature produced a statistically significant concentration-dependent increase in percentage DNA strand break (Figure 4.15, chapter 4). A number of studies have confirmed Aluminium toxicity, however the mechanism of its toxicity is still unclear (Ipeaiyeda et al. 2012; Lankoff et al. 2006; Yousef 2004; Zatta et al. 2002). In a previous study, comet assay assessment of the level DNA damage in human lymphocyte cells exposed to 1,2,5,10 and 25µg/mL Aluminium for 72 also showed a concentration-dependent increase in DNA damage (Lankoff et al. 2006). At 25µg/mL however, they reported increased cell apoptosis, decrease in the level of DNA damage and DNA repair impairment. Indicating that, the DNA repair inhibition might be possibly involved in Aluminium toxicity. Our data in chapter four however, showed a statistically significant dose dependent increase in the amount of Reactive oxygen species formation using H_2DCF-DA assay in sponge cells exposed to similar concentrations as those of the comet assay. This data strongly and positively correlated with percentage DNA damage with an $R^2 = 0.9974$ (Figure 4.16, chapter 4) indicating that reactive oxygen species have a significant contribution in aluminium toxicity (Henkler et al. 2010). In another study also, Aluminium toxicity was

investigated in ZF4 zebra fish cell lines, exposure to concentrations between 1-250μM (0.027 – 6.75mg/L) resulted in increased DNA strand break by the alkaline comet assay (Majer et al. 2014). In contrast with our result however, while 100μM Aluminium was non-cytotoxic to sponge cells with no statistical difference between lower concentrations of 0.1-0.4mg/L and 100 μM (Figure 4.17 and Table 4.1), exposure of ZF4 to aluminium concentration of 100μM and over, showed marked cytotoxic effect (Majer et al. 2014).

The field study data presented in chapter 5 showed significant DNA strand breaks in cryopreserved single cell samples of *Amorphinopsis kalibiama*. This is the first identification and characterisation of this sponge species and it is also the first identification of the genera in West Africa. Interestingly, this is also the first entry of any sponge data from West Africa in the sponge Barcoding project (Erpenbeck et al. 2016; Vargas et al. 2012).

Until now biological effect data in Niger Delta polluted area has only focused on bioaccumulation compared with sediment chemistry and analysis of pollutants in water column (Babatunde et al. 2013; Ezemonye 2005; Fatoba et al. 2016; Iwegbue et al. 2018; Ogamba et al. 2015; Ogbeibu et al. 2014; Onojake et al. 2017; Otitoju and Otitoju 2013; Wogu and Okaka 2011). This thesis present for the first time effect based data using DNA strand break analysed with the alkaline comet assay as a biomarker of xenobiotic pollution in Niger Delta aquatic environment. These data were compared with chemical analysis of sponge tissue metal burden, sediment metal load and metal concentrations in water column.

Compared with untreated control sample of *Hymeniacidon perlevis*, percentage DNA damage was significant in sponge cells from all field sites studied, our data showed a varying range of DNA damage in sponge cells across all sites; mean % DNA tail intensity ± SEM for all sites was; Site A (6.38±1.56), Site B (10.33 ± 3.27), site C (7.82±1.64), site

D(10.83±2.40), site E(11.63±3.68), site F(10.97 ± 1.42) , and site G(11.13 ± 3.11). These data compared with chemical analysis of bioaccumulated metals were significant for Al, As, Cu and Pb levels in sponge tissue from all sample sites extracted with HF-digestion method. Comparison of % DNA strand break in sediment samples in all sample locations was also significant for Al, As, Cd, Cu, Hg and Pb.

In this thesis, the genotoxic effect of Aluminium is reliably confirmed in agreement with previous studies. Of all 8 metals (Al, As, Cd, Cr, Cu, Hg, Ni and Pb) analysed in the digests, only Al, Cu and Pb were repeatedly detected in digests from all metal extraction method used, although trace amount of Cd (HF digests) and Cr (Acid digestion method) were detected in sponge sample from most sites, these were not significant compared to DNA strand breaks. Correlation of Al, Cu and Pb with percentage DNA strand break in untreated sponge cells from the 7 field locations was only significant between Aluminium correlation and DNA damage (Figure 5.11). Pearson's correlation of Cu data with percentage DNA strand breaks, showed very little correlation between Cu concentration sponge and DNA damage. Correlation of Pb data with DNA strand break was however a negative correlation.

Pollution in the aquatic medium is made of complex mixture of toxic substances, that work in synergy one with another to cause adverse health effects to aquatic lives and to human through sea food consumption (EPA 2018). This then means that the high concentration of an individual pollutant may not necessarily mean pollution in that environment. Thus, in ERA, an environment is only considered polluted if the levels of the pollutants presents are high enough to cause adverse health effect (Chapman 2007), which means that data on pollutant concentration in the environment must be accompanied by biological effect data for it to be relevant in ERA (Martins and Costa 2014). Environmental data for risk assessment therefore are not to be based on just one single line of evidence (LOE). Results in the field study data in

this thesis, very high level of some contaminant had no correlation with the comet assay assessed DNA strand breaks. For example the concentration of copper in sponge tissues in site A , was up to 413 fold higher than sediment content (Table 5.6, chapter 5), but copper was not correlated with DNA damage and in site E, which is the site with the highest amount of percentage DNA tail intensity, copper bioaccumulation was also 245 fold higher in sponge tissues. However Aluminium which is the highest occurring metal in all field sites investigated, compared to measured concentrations in sediment was only 7.07- fold higher than sediment concentration in site E. Aluminium bioaccumulation in sponge tissues from site A, however was 152-fold higher than levels in sediment from same site and was well correlated with DNA strand break. Our data show that apart from Aluminium, fold increase of all metals bioaccumulation was highest in site A, however the lowest amount of DNA damage in sponge cells was recorded in this site. Interestingly, sponge cells from site E had the highest DNA damaged measured by the alkaline comet assay, it was also the site with the highest concentration of Aluminium in sponge tissues, and the only site with the reliable cadmium detection in both sponge tissues and sediment. Thus, it could be speculated that Al, Cd, Cu, As and Pb and likely aquatic organic pollutants are all working in synergy to induce the high level of DNA strand break in sponge cells from site E. In a previous study Steinert et al. (1998) compared the relationship between biological effects bioaccumulation and DNA damage measured with the comet assay technique) and sediment chemistry. They reported that comet assay assessed DNA damage was well correlated with contaminants in all sample sites investigated.

Using the comet assay technique water quality assessment was performed. Untreated control cells of *Hymeniacidon perlevis* were cultured in filtered sea water from site E for 12 hours and processed for comet assay as previously described. Significant DNA damage was

observed compared to the control (*H. perlevis* cells in synthetic sea water) Figure 5.8. This shows that sponges can be used to assess the quality of a water bodies, using the comet assay.

In a previous study in southern Ontario water quality of creeks and ponds were assessed by quantifying DNA damage in the erythrocytes of two tadpole species; *Rana clamitans* and *Rana pipiens* using the comet assay. Compared to control data obtained with samples from near the French River, significant level of DNA damage was reported (Ralph and Petras 1997).

Also, DNA repair was investigated in our field sponge cells. Sponge cells from Site E were monitored for DNA strand breaks for up to one week in culture. Decrease in the amount of % DNA strand break was significant with increased time (Figure 5.6, chapter 5). Thus, these data shows a time dependent decrease in DNA strand breaks. This is also demonstrates a 'self-cleaning' mechanism in sponge as most of the contaminants were possibly filtered off each time the culture media was changed. DNA repair process is however not very well understood in aquatic organisms (Buma et al. 2003; Chipman et al. 1992; Valavanidis et al. 2006). The persistence of DNA adducts in fish and unscheduled DNA synthesis are both indicators to poor DNA repair mechanism in aquatic organisms (Bailey et al. 1996; Chipman et al. 1992; Maccubbin 1994).

Data presented in chapter 5 also compared metal bioaccumulation in sponge tissues (other biological effects) obtained from polluted Niger Delta sites and accumulation in sediment samples from same sites. Results showed marked concentration increase of all metals investigated in sponge tissues relative to sediment and even higher fold increase compared water column. Our result confirmed that sea sponges as a useful tool for aquatic pollutant biomonitoring. According the standard sediment guidelined data used for comparison showed

that pollution in the Niger Delta from As, Cd, Cu and Pb is not significant. However, the high level of DNA strand breaks in sponge cells from all sites investigated, provides a safeguarding warning and justifies the need for continues biomonitoring of pollutants in this region. Aluminium data was however not listed in the guidelines used (Fletcher et al. 2008; Long et al. 1995). Metal levels in the analysis of all matrixes in this thesis was lowest in water column, this is in agreement with the data in (Cebrian and Uriz 2007b; De Mestre et al. 2012; Pan et al. 2011). Combination of information on pollutants biomarkers as well as data on bioaccumulation (biological effects) and chemically analysed pollutant levels (in sediment and water) are all required to reliably determine the potential deleterious effects of pollutants on the aquatic biota (Livingstone et al. 2000; Van der Oost et al. 2003).

In this thesis the toxicity of Benzo[a]pyrene as an important organic pollutant and the possible activity of P40 CYP1A1 enzyme in sponge cells were also investigated. B[a]P is a model and commonly reported organic pollutants in the aquatic ecosystem and its toxicity is widely investigated both in vivo and in vitro (Bo et al. 2014; Zahn et al. 1982) with fish being the most commonly investigated aquatic organism and some reports on mussels (Van der Oost et al. 2003). The mechanism of B[a]P toxicity involves the induction of oxidative DNA damage via DNA adducts formation and production of ROS (Mitchelmore et al. 1998a; Thompson et al. 2010). Data in chapter 4 shows that non-cytotoxic concentrations of B[a]P as assed By MTT viability assay (Table 4.1, Figure 4.19 chapter 4) causes significant DNA strand break induction in sponge cells. In the aquatic system fish and bivalves are commonly utilised for investigation of B[a]P toxicity is mainly (Bo et al. 2014; Mitchelmore et al. 1998a; Thompson et al. 2010; Van der Oost et al. 2003). In Chapter 4, we showed that exposure to B[a]P concentrations between 0.1µM (0.02mg/L), 0.5µM (0.1mg/L), 2.5 µM (0.6mg/L) and 10µM (2.3mg/L), compared 1% DMSO (Dimethyl sulfoxide) had no statistically significant

cytotoxic effects on sponge cells, however these concentrations caused significant concentration-dependent increase in the level of DNA strand breaks. In a previous study with mussel, B[a]P concentrations of 0.4nM, 7.9Nm, and 40nM was genotoxic reported to be genotoxic in Zebra mussel (Binelli et al. 2008), however increasing dose dependent increase in DNA damage was observed with concentrations from 0.8nmol/g and above. Also comet assay assessment of DNA damage in the sea anemone *Anthopleura elegantissima* exposed to 50,100 and 200µM B[a]P showed that cnidarians are responsive to B[a]P, which suggests the possibility of P450 metabolism in these organisms (Mitchelmore and Hyatt 2004). In chapter 4, CYP1A1-P450 metabolism in sponge cell 'Microsomes' using 2x NADPH regenerating system was also investigated with rat liver S9 as control. This preliminary attempt produced a promising result in comparison with Rat liver S9 used as positive control (Figure 4.18). Hence this result and the marked DNA damage observed with the comet assay toxicity assessment, suggest the possibility of P450 activity in sponges.

In the aquatic system, B[a]P activates the expression of P450 enzymes mainly the CYP1A1 subfamilies (Mitchelmore et al. 1998a). Metabolism of B[a]P by CYP1A1 enzymes produces toxic metabolites and the ultimate BDPE (7,8-dihydrodiol-9, 10-epoxide (BPDE) metabolites, which interacts with genomic DNA and forms a BDPE-DNA complex. This complex formation results in DNA adducts and DNA cross linkages formation resulting in B[a]P induced genotoxicity (Thompson et al. 2010). B[a]P also induces the production of reactive oxygen species radical which also results in B[a]P induced DNA strand break , hence B[a]P toxicity is via reactive oxygen formation which induces DNA damage and formation of DNA adducts and cross linkages. Considering the prevalence of organic pollutants in the aquatic system (Kotelevtsev et al. 2009; Livingstone 1998; Livingstone et al. 2000; Livingstone 1991)

organic biomonitoring studies in the aquatic system mainly utilizes the induction P450 enzymes as biomarkers of organic pollution (Michel et al. 2001; Stegeman and Hahn 1994).

Pollution in coastal environment and in the general aquatic system is mainly a function of human activities, with minor contributions from natural sources exist (Roberts et al. 2008). It is therefore important to regularly monitor the aquatic environment to ensure its safety for animals and humans. It is particularly important to monitor these systems for the genotoxic effects of environmentally relevant pollutants, because of their contribution to adverse health outcome such as cancer, mutation and reproductive impairment. If unmonitored, pollution in the Aquatic system could reach a record high level with significant genotoxic damage to sex gametes, developmental stages and sudden death following acute exposure (Kotelevtsev et al. 2009).

In summary, data provided in this thesis confirmed DNA strand breaks in sea sponges measured by the comet assay technique as an efficient tool for aquatic biomonitoring of pollutants and application in Environmental risk assessment. Successfully, sea sponges have been developed as model for investigating xenobiotic induced DNA strand break with the alkaline comet assay and we have shown that sponge cell model is suitable for both toxicity testing of bioavailable pollutants and measurement of biological effects of field contaminants. The thesis has also successfully shown that cryopreserved sponge cell aggregates remain viable for primorph formation and for downstream biochemical assays for environmental monitoring. This is particularly important to sponge research as cryopreservation and primorph formation is the future for sustainable production of sponge bioactive metabolites and use of sponges for environmental impact assessment. This thesis has also reliably contributed information on a newly described sponge species to the sponge Barcoding project and NCBI gene bank. We also report the application of the comet assay and reactive oxygen

species formation in *Hymeniacidon perlevis* for the first time. Finally, our preliminary data on P450 expression, shows the activity of AhR, which is also a first to the best our my knowledge.

6.2 Future Work

6.2.1 Immediate possible future work post PhD

Specifically, the following approaches would be exploited to further advance the findings of my research:

- Networking and collaborations with senior researchers who are interested in environmental fate of chemicals, development of test guidelines, and assay development for regulatory purposes. Specific steps would be to reach out to very senior scientists involved in the DEFRA –Academic Stakeholder group on OECD.

- Attendance and presentation of my research findings at Environmental toxicology meetings such as the forth coming BTS-UKEMS annual congress, 2019 and possibly SETAC Latin America Biennial meeting in September, 2019.

- Grant applications for further research and validation of other assays in my sponge systems. Proposed studies are assessment of organic chemical effects in sponges, P450 and AhR activities in sponges and possibly attempt to clone CYP1A1 homologous genes in my Niger Delta sponge species.

6.2.2 Other Possible future work

The Comet assay data presented in Chapter 3 and 4 could be extended to the modified enzyme version. Using formamidopyrimidine DNA-glycosylase (FPg) in sponge cells, specific oxidative DNA damage dependent on FPg could be detected. The Data in chapter 3 on preliminary cadmium exposure reveals the possibility of DNA repair gene in the sponge

Hymeniacidon perlevis, hence gene expressions could be measured in the two sponge species investigated.

The work in chapter four could be replicated in the field sponge sample (*Amorphinopsis kalibiama*) from polluted Niger Delta sites. *Amorphinopsis kalibiama* cryopreserved cells could be treated with varying concentrations of environmentally relevant pollutants in the laboratory and then tested for stress response. This would compare stress response between sponge cells from contaminated sites and those from fairly clean sites. Metallothioneins induction is major metal removal pathway in invertebrates including sea sponges. The work in chapter 4 could be extended to measure metal detoxification in both sponge species which would be useful in comparing tolerance to pollutants between the species. Multiple xenobiotic resistances have been reported in other sponge species as a mechanism for detoxification, these need to be measured in *Hemeniacidon perlevis* and *Amorphinopsis kalibiama* as an extension of the work in chapter 4 and 5.

Assessment of alternative biomarkers in sponge cells in addition to DNA damage ROS production is required. Alternative assays such as Glutathione GSH tripeptide antioxidant assay, assays for measurement of thiols such 4'4-Dithiodipyridine (DTP) assay, and lipid peroxidation assays (TBARS) are all important assays that are required to understand the range of stress response in toxicity testing. These should be measured in sponge cells.

Preliminary data on the assessment of P450 (CYP1A1) enzyme expression in sponge was significant compared to rat liver S9 fraction. CYP1A1 induction is an important early warning biomarker of aquatic organic pollutants, however there are no data on CYP activities in sponges until now, hence the work on P450 metabolic activity in sponge in chapter 4 should

be expanded include more technical repeats and assessment of a range of organic contaminants both in field samples and in laboratory cultures.

Finally, in the aquatic environment, organic pollutants bioaccumulate in sessile organisms, hence investigation of their biological effects combined with chemical analysis of their levels in aquatic organism is a vital tool for environmental risks assessment. The results in chapter 5 should be extended to include the measurement of organic chemical contaminants in sponge tissues, sediment and water.

Chapter 7 -References

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Appendices

Appendix 1 Publications

Appendix 1.1 Abstract

Akpiri RU, Konya RS, Hodges NJ (2017) Application of the comet assay to detect metal-induced DNA strand breaks in cultures of the marine sponge *Hymeniacidon perlevis* and *Amorphinopsis kalibiama.* Mutagenesis 32, 6
https://academic.oup.com/mutage/article/32/6/e1/4844756

Appendix 1.2 Publications

Akpiri RU, Konya RS, Hodges NJ (2017) Development of cultures of the marine sponge Hymeniacidon perleve for genotoxicity assessment using the alkaline comet assay. Environmental toxicology and chemistry. *Society of Environmental Toxicology and Chemistry (SETAC) Journal*, **https://doi.org/10.1002/etc.3907**

Akpiri RU, Eduardo H, Erpenbeck D, Soest Van R, Hodges NJ (*In draft)* Systematic and molecular description of a new *Amorphinopsis* species (Porifera, Demospongiae, Suberitida) from West African South-Atlantic Ocean.

Akpiri RU, Konya RS, Hodges NJ (*In draft*) Comet assay assessment of Aluminium induced DNA damage and H_2DCF-DA measurement of Reactive Oxygen species formation in cryopreserved marine sponge cells.

Appendix 1.3 Platform Presentations

Environmental Omics Group Seminar, school of Biosciences, March 2016.

Biosciences Graduate Research School (BGRS) Symposium July, 2017. School of Biosciences University of Birmingham, UK.

12th International Comet Assay Workshop, Pamplona 2017 (Skype presentation).

Appendix 1.4 Poster Presentations

University of Birmingham Research Poster Conference, June 2015

British Toxicology Society (BTS) Annual Congress Manchester , March 2016

European Environmental Mutagenesis and Genomics Society (EEMGS) annual meeting, Copenhagen 2016.

7th SETAC World Congress, 37th Annual North America meeting Orlando, November 2016

British Toxicology Society Annual Congress Manchester, April 2017.

Appendix 1.5 Awards

Commonwealth Scholarship Commission in the UK, 2014

Best Poster Award, EEMGS Copenhagen 2016.

BTS travel grant and Bursary, Annual Congress 2017.

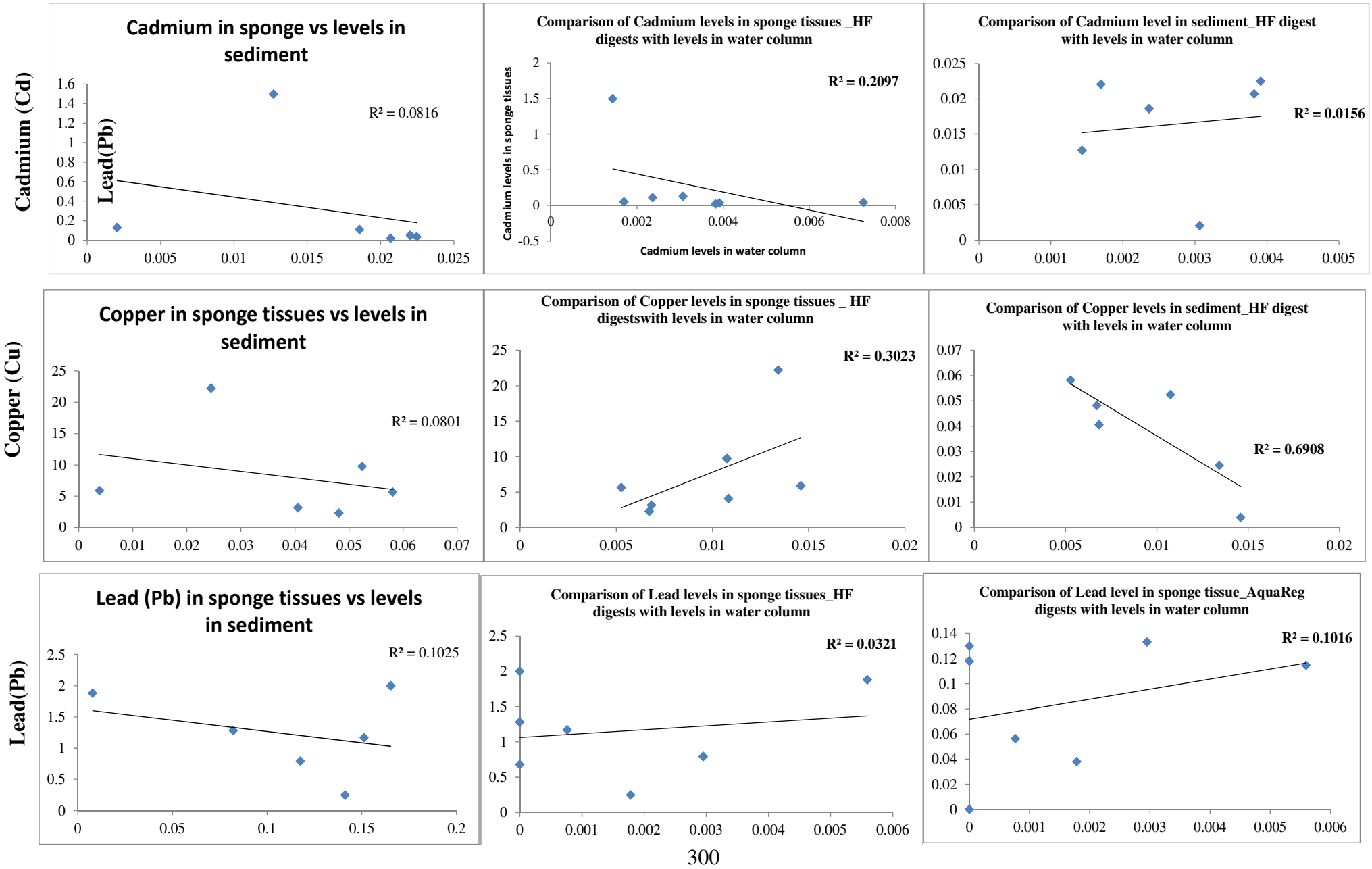
Appendix 1.6 Scientific Discovery of a new sponge species

https://www.birmingham.ac.uk/schools/biosciences/news/2017/10Jul-new-species-of-marine-sponge-discovered.aspx (School of Biosciences News editorial)

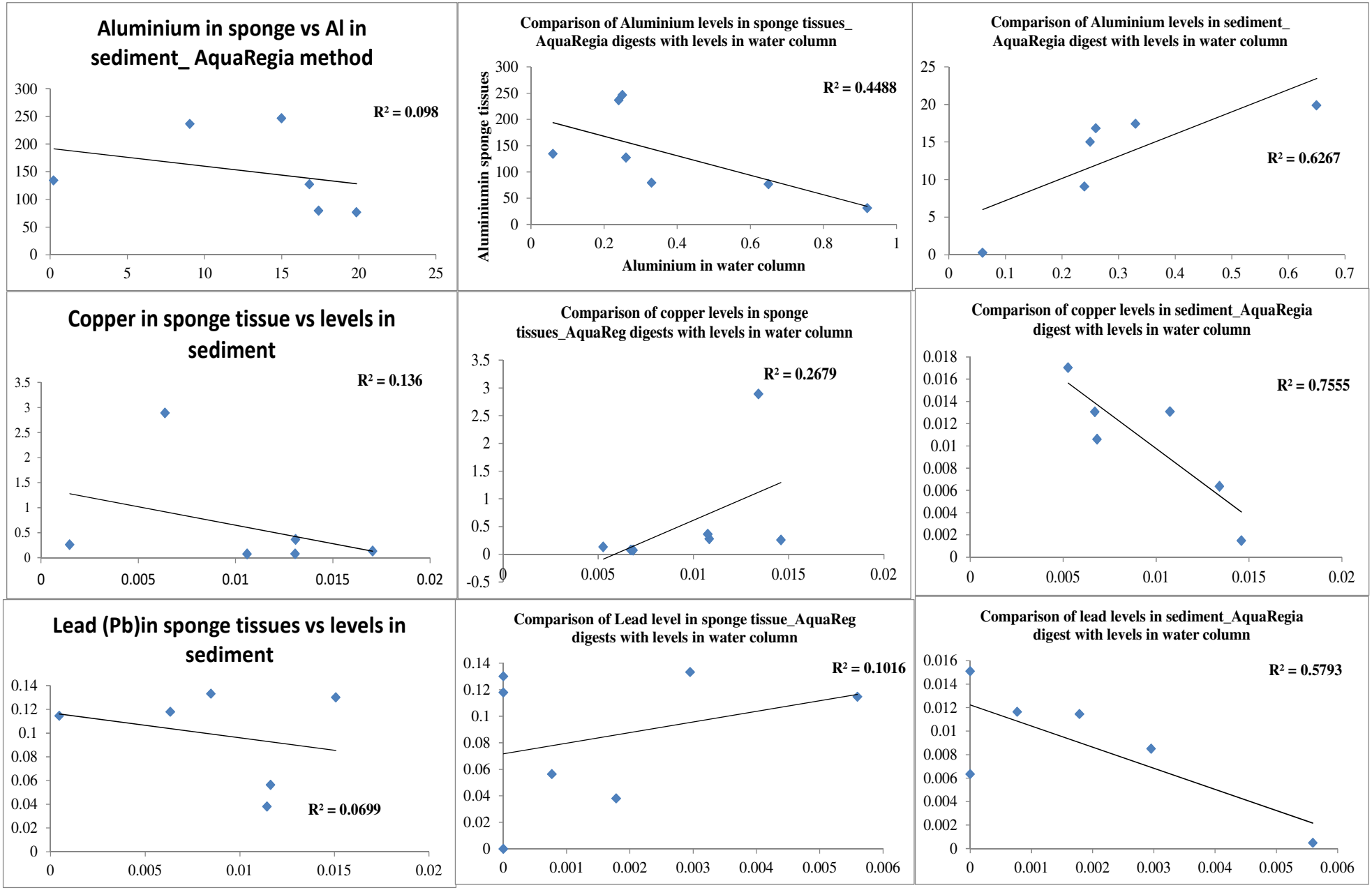
https://www.ncbi.nlm.nih.gov/nuccore/?term=Amorphinopsis (Genebank sequence link)

http://cscuk.dfid.gov.uk/common-knowledge/issue-5 Page 17 (Commonwealth Scholarship commission News editorial)

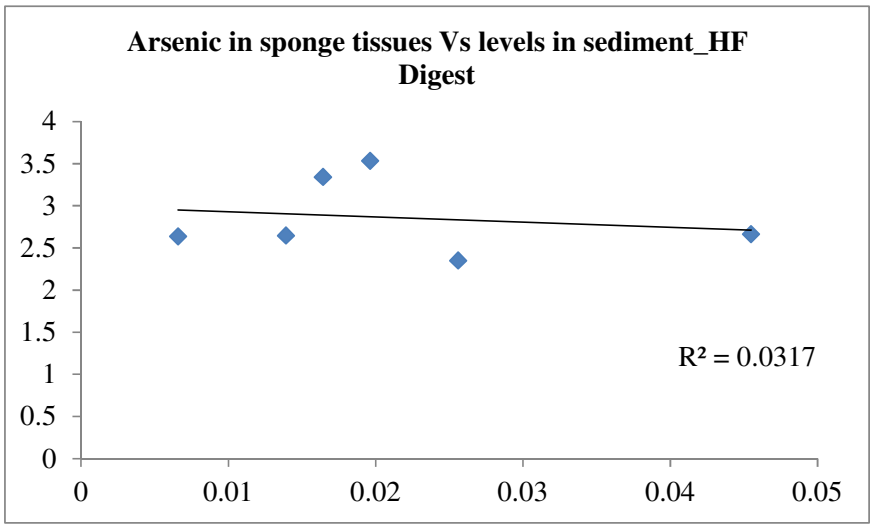
Appendix 2 Correlation of metal accumulation in environmental matrixes

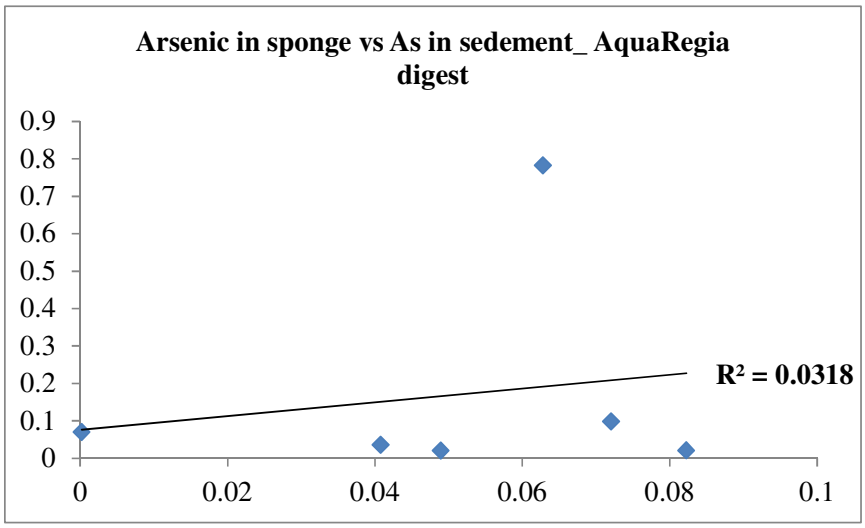


Appendix 2.1 Comparison of metal levels in all three environmental matrixes (Sponge, sediment and water) extracted by HF digestion methods.



Appendix 2.2 Comparison of metal levels in all three environmental matrixes (Sponge, sediment and water) extracted by Aqua-Regia digestion methods.





Appendix 2.3 Arsenic In environmental matrixes

Appendix 3 Metal recovery in certified reference materials

	Al 308.2		As 189.0		Cd 226.5		Cr 267.7		Cu 324.7		Ni 231.6		Pb 220.3	
	mg/l	ppb	mg/l	ppb	mg/l	ppb	mg/l	ppb	mg/l	ppb	mg/l	ppb	mg/l	ppb
BLANK	0.01	100	0.01	5.8	0.00	0.2	0.00	2.2	0.00	3.9	0.00	3.4	0.00	1.2
BLK FUSS	0.09	92.7	0.01	9.7	0.00	1.3	0.01	5.0	0.00	3.7	0.01	8.9	0.00	ND
NIST														
NIST Oyster														
JLS FUSION	0.3	268.8	0.01	5.8	0.00	0.4	0.00	3.7	0.00	4.7	0.01	7.1	0.00	ND
JSL FUSION	77.8	77778.8	0.01	5.9	0.00	2.5	0.06	57.4	0.03	33.8	0.04	39.6	0.03	34.4
SGR FUSION	27.9	27851.6	0.01	7.7	0.00	4.6	0.03	34.6	0.05	50.4	0.03	27.0	0.01	8.9
MAG FUSION	72.5	72501.4	0.00	3.4	0.01	6.3	0.10	101.2	0.02	15.9	0.06	61.8	0.02	21.7
CCH FUSION	1.24	1243.0	0.00	4.0	0.00	0.3	0.01	8.8	0.01	5.3	0 .02	16.5	0.00	ND
SCO FUSION	60.4	60442.0	0.01	5.85	0.01	10.4	0.07	70.0	0.02	24.8	0.05	47.0	0.03	26.0
MON FUSION	27.8	27879.6	0.00	0.19	0.00	4.6	0.04	36.4	0.05	51.9	0.04	39.4	0.01	10.1
blank HF	0.05	45.9	0.0	ND	0.0	ND	0.0	4.72	0.00	4.0	0.01	11.3	0.00	ND
NIST HF	196.6	196579.5	0.03	27.3	0.03	32.1	0.37	365.6	0.07	71.6	0.21	210.3	0.18	175.1
NIST Oyster														
JLS Mon HF	0.99	993.6	0.00	4.0	0.00	3.0	0.03	27.5	0.02	16.2	0.00	ND	0.00	ND
JSL HF	662.3	662265.0	0.09	87.1	0.08	83.4	0.6	564.6	0.3	327.4	0.33	333.2	0.41	413.8
SGR HF	297.2	297164.8	0.32	324.0	0.04	35.7	0.3	288.3	0.5	524.5	0.27	271.5	0.44	442.1
MAG HF	621.9	621926.9	0.05	49.2	0.07	73.9	1.0	949.0	0.2	223.3	0.5	446.9	0.44	440.9
CCH HF	11.6	11610.4	0.01	6.6	0.00	2.0	0.1	56.7	0.04	35.5	0.06	63.9	0.04	38.7
SCO HF	615.0	614991.5	0.09	86.5	0.06	60.7	0.6	636.1	0.2	221.0	0.24	243.7	0.45	450.4
MON HF	1.00	1002.3	0.00	3.3	0.00	ND	0.02	24.5	0.01	14.8	0.01	12.8	0.00	ND
MON HF	1.00	1003.3	0.00	3.1	0.00	ND	0.03	26.0	0.02	15.3	0.01	5.2	ND	ND

Aqua-Regia

Sample Id	Al 27(ppb)	Cr 52(ppb)	Ni 60(ppb)	Cu 63(ppb)	Ge 74 (IS)	As 75(ppb)	Cd 111(ppb)	Pb 208(ppb)
blk1	ND	ND	ND	ND	1.18	66.5	ND	ND
Crm1-nist	7562.2	127.9	93.4	33.3	1.2	191.5	ND	24.7
Crm2	9668.1	191.2	121.8	40.7	1.2	224.2	ND	30.1
Crm3	9658.0	194.9	131.1	41.8	1.2	226.7	ND	41.6