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ARTICLE

Aluminium Induced DNA-damage and Oxidative Stress in Cultures of the Marine Sponge *Hymeniacidon perlevis*

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ABSTRACT

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. Although the potential genotoxic and carcinogenic effects of aluminium are established in mammalian and other model systems, there is however very limited information on aluminium genotoxicity in aquatic invertebrates. Mechanism of aluminium toxicity is also largely unclear. With a concentration range between 0.001– 0.05mg/L in near-neutral pH water, and up to 0.5-1mg/L in acidic water , aluminium poses a potential threat to the marine ecosystem, however, it is poorly studied. This study therefore presents for the first time, aluminium-induced DNA damage using the comet assay and Reactive Oxygen Species (ROS) formation using 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) assay as biomarkers of genotoxicity and oxidative stress in the inter-tidal marine sponge *Hymeniacidon perlevis*, respectively. *H. perlevis* is widely distributed in the British Isles, Mediterranean and the Arctic sea and has been reported as a model for environmental biomonitoring in aquatic ecosystem and as a suitable alternative to bivalves. In this study, cryopreserved single sponge cells of *H. perlevis* were cultured as viable aggregates and were thereafter treated with 0.1, 0.2, 0.3 and 0.4mg/L aluminium chloride (AlCl3) for 12 hours. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our results showed that non-cytotoxic concentrations of AlCl3 caused a statistically significant concentration-dependent increase in the level of DNA-strand break and reactive oxygen species formation single sponge cells of *H. perlevis*. There was also a statistically significant positive linear correlation between aluminium-induced DNA strand break and ROS formation suggesting the involvement of ROS in the causative mechanism of the aluminium induced DNA-strand breaks observed.

1. Introduction

Marine sponges are simple invertebrate animals with ecological importance in the aquatic ecosystem. A prominent feature of sponges that have advanced their ecological usefulness is their ability to pump large volumes of water through their body tissues during filter feeding. During this process, large amounts of particulate matter both in their dissolved and suspended phase, including xenobiotics are trapped within the

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sponge tissues[1,2]. These materials are mostly retained within the animal and constitute vital sources of information for biomonitoring and risk assessment of their immediate aquatic ecosystem. *Hymeniacidon. perlevis*, a demosponge belonging to the order suberitida, family halochondridae and genus *Hymeniacidon* is a commonly utilised sponge species for biomonitoring, bioremediation, bioactive compound analysis and water quality [3-6]. It is an intertidal species widely distributed on the British Isles, Mediterranean and the Arctic sea; with records of appearance in Belgium and France. 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). *H. perlevis* has a well-defined seasonal life cycle with four developmental stages; dormancy, resuscitation, bloom and decline stages which are useful for predicting environmental changes. Current sponge research is focused on the development and maintenance in a culture of functional sponge aggregates (primorphs), which is thought to be the future for sustainable production of sponge bioactive metabolites, investigation of response to environmental chemical exposure in the aquatic ecosystem, and genomic annotations [8-11]. Previous studies comparing *H. perlevis* with its counterpart sentinel bivalve neighbour; *Mytilus. edulis* (the blue mussel) as models for environmental biomonitoring, shows that *H. perlevis* accumulates certain in-situ pollutants up to 10-fold more [4]. Also, *Hymeniacidon* has been reported to be more sensitive in the detection of petrogenic and pyrogenic xenobiotics than the brown mussel: *P. perna* [7]. Several studies have utilised H. perlevis as a model for the investigation of important biochemical processes and chemical exposure. Recently, DNA damage induction following exposure of *H. perlevis* cells to environmentally relevant concentrations of Cadmium, Chromium, Nickel and Benzo[a] Pyrene showed a concentration-dependent increase in DNA damage [6,12] which is consistent with the findings in this present study. Hence, H. perlevis , therefore, is a suitable species for environmental risk assessment and biomonitoring pollutants in the aquatic medium and it is reliable sentinel representative for investigating the impact of environmental stressors on aquatic biota.

Among pollutants accumulated by sponges, heavy metals and organic pollutants have been extensively studied [2,12-16]. Heavy metals bioaccumulation in sponges are reported even when concentrations are very low and below the limit of detection in other environmental samples such as surrounding water and sediments [7,17-19]. Ferrante, Vassallo [1] have also demonstrated the potential of sea sponge to bioaccumulate a wide range of organic pollutants both *in vitro* and *in situ*. There is, however, limited information on the biological effects of aquatic pollutants and their biomarkers in marine sponges which are needed if these species are to be developed as reliable tools for risk assessment and biomonitoring. One such reliable biomarker of effect and exposure to environmental pollutants in both humans and animals is DNA damage and it has been widely applied in assessing the biological effects of environmental pollutants in established model systems [20-22]. Although the genotoxic potential of pollutants in marine sponges has been demonstrated previously [6,12,23] much less is known compared to other species and there is no information on the genotoxic effects of emerging pollutants like aluminium to these potentially important organisms.

The toxicity and mechanism of toxicity of most class B and borderline heavy metals (Cd, Cr, Cu, Ni, Pb, Hg, Co, Ag, Au,) are well established as is their deleterious effects on human health and involvement in the pathology of diseases including cancer [24-26]. In contrast, class A metals (Al, Be, Li, Ca, K etc) are often considered as essential metals with a strong affinity for oxygen [27,28], and were considered until recently as relatively non-toxic or having very low potential to exert adverse health effects [29]. Aluminium, for example, is the third most abundant element and the most abundant metal in the earth crust, making up to 8% of the earth crust and occurs as oxides, hydroxides or silicates of sodium or fluorides and as organic matter complexes with wide industrial application [29,30]. Major uses and applications are in construction companies, aircraft production, and automobile industries and as alloys [29]. However, some reports that have shown the toxic potential of aluminium in both humans and animals. Iron-induced reactive oxygen species formation and lipid peroxidation, protein phosphorylation, apoptosis, and interference with gene expression, have all been reported following exposure to aluminium [31-33]. Other studies have also demonstrated chromosome aberrations, induction of micronuclei, and sister chromatid exchange induced by aluminium exposure [31,34]. Furthermore, the involvement of aluminium in the aetiology of neurodegenerative disorders such as presenile Alzheimer's and Parkinson's disease has also been reported and studied [35]. Therefore, there are potential genotoxic and carcinogenic effects of aluminium [31,33,36,37], however, the mechanism of aluminium genotoxic and carcinogenic pathways remain largely unclear [31]. In the marine environment aluminium concentration in water varies depending on pH, for example, ranges such as 0.001– 0.05mg/L were recorded in near-neutral pH water while up to 0.5-1mg/L was recorded in acidic water [29] making it a potential but poorly studied threat to the marine environment. This study presents for the first time,

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aluminium-induced DNA damage and reactive oxygen Species (ROS) formation in cultured sponge cells exposed to non-cytotoxic concentrations of aluminium.

2. Materials and Methods

2.1 Sponge Collection and Preservation

Samples of the marine sponge *H perlevis* were collected from Tenby Bay castle beach in Pembrokeshire, South Wales, UK on exposed rock pools at low tide. These were immediately transported back to the laboratory in aerated seawater and processed into single cells and thereafter cryopreserved in vapour phase liquid nitrogen following a previously described protocol with modifications [38]. Briefly, single sponge cells were isolated from sponge tissues using ethylene diamine tetra acetic acid (EDTA) containing calcium magnesium-free seawater (CMFSW+E) and calcium magnesium-free seawater (CMFSW) prepared according to Cold Spring Harbor Laboratory Protocols [39]. 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).

Freshly collected sponge samples were carefully cleaned to remove debris and dirt, washed three times in filtered natural seawater and chopped into cubes approximately $1cm^3$ 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) and then placed on a rotor shaker (Rotator Labnet Orbit 1900). Tubes were 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 filtering the CMFSW+E soaked tissues into a 50 mL falcon tube and pellets obtained at 300 x g for 7 minutes and the supernatant discarded.

The resulting single sponge cell pellets were then washed three times with CMFSW and resuspended 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). 1 mL freezing re-suspended single cells were then aliquoted into corning cryogenic vial and stored overnight in a -80^0C freezer before been transferred to vapour phase liquid Nitrogen for long term storage until required.

2.2 Sponge Cell Culture and Aggregate Formation

Cryopreserved sponge cells were quickly thawed in a water bath at 37^oC and re-suspended in 5.5 mL sponge media; sponge cell pellets were obtained by centrifuging cell suspension for 7 minutes at 300 x g. Pellets were then resuspended in 6 mL and cell density and viability determined using a haemocytometer (Neubauer improved superior Marienfield, Germany) and trypan blue staining. For sponge cell culture, approximately, 20 x 10^6 cells/mL were placed in sterile T_{25} culture flasks and 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. Sponge cells rapidly formed aggregates that maintained viability for more than 1 week in culture (as shown by MTT viability assay: data not shown).

2.3 'In vivo' Exposure Sponge Cell Model

In a minimum of three independent experimental repeats, aggregates were exposed to 0 mg/L, 0.1 mg/L, 0.2m/L, 0.3 mg/L and 0.4 mg/L aluminium chloride for 12 hours. All experiments were conducted at room temperature in 12-well cell culture cluster flat bottom with lid plate (Corning) placed on a horizontal shaker set at 45 rpm.

2.4 3-(4, 5-dimethylethiazole-2-yl)-2,7-diphenyl-etetrazoliumbromide (MTT) Viability Assay

Following 12 hours laboratory exposures of *H perlevis* sponge cell aggregates to different concentrations of aluminium chloride, MTT viability assay was performed to investigate potential cytotoxic effects of the test concentrations on sponge cell aggregates. Dissociated single sponge pellets 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 suspensions were then transferred into 6 well plates and incubated at 37^oC for three hours. After incubation, well plate contents were transferred into 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.

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2.5 Comet Assay Procedure for the Assessment of DNA Strand Breaks

Briefly, aluminum treated sponge cell aggregates were dissociated into single cell and the suspensions 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. 15 µL of CMFSW suspended cells aliquots (in duplicates) were mixed with 150 µL of 0.5% w/v molten low melting point agarose (LMPA) in PBS and added to previously coated microscope slides (with 0.5% w/v normal melting point agarose in phosphate buffered saline (PBS). 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 gently slide off horizontally and slides transferred into previously chilled lysis buffer (made from 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 a Coplin jar and incubated for 1 hour. Afterwards, slides were transferred into a horizontal electrophoresis tank model: GSA/VA FisherBiotech™ Horizontal Electrophoresis Systems, containing electrophoresis buffer (80 mL 9 M NaOH, and 12 mL 200 mM EDTA, and 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, next 300 mA current at a voltage of 32V was applied to the electrophoresis setup for 30 minutes. Following electrophoresis, slides were washed 3x with 5mL neutralization buffer (0.4M Tris base in 500mL UHQ water, pH 7.5) and then stained with 50 µL 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 x40 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 [40].

2.6 DCFH-DA Assay for the Measurement of Reactive Oxygen Species (ROS)

To measure the fluorescent intensity of DCF proportional to the amount of ROS produced as a biomarker of oxidative stress induction from aluminium exposure, 100 µL of aluminium chloride treated single sponge cell suspension (obtained as in the comet assay procedure) was mixed with 1 mL aliquot of 3 µL 100 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 1 mL RPMI mixture in 6 well plate. Plate was wrapped in aluminium foil to protect from light and incubated at room temperature on the bench, after 1 hour incubation, sponge cells were digested in 100 µL 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.

2.7 Statistical Analysis

Results were analysed in duplicates in three technical repeats for all experiments. Using IBM SPSS version 22.0 and Graph pad prism version 7.0.2. All data were checked for normality and homogeneity of variance using Shapiro Wilk's test and Leven's test respectively. Differences between control measurements and test concentrations for all data were analysed using 1way ANOVA and Bonferroni multiple comparison Post Hoc test, at P= 0.05. Comparison between DNA damage and reactive oxygen species formation was evaluated with Pearson correlation coefficient.

3. Results

3.1 Sponge Cell Culture and Viability Assessment

Cryopreserved single sponge cells rapidly formed viable aggregates in cultures (Figure 2). Following treatments with $AlCl_3$ (0.1mg/L, 0.2mg/L, 0.3mg/L and 0.4mg/L) for 12 hours aggregates were assessed for viability using the MTT assay. The results obtained showed that the Al concentrations used in this study do not have any significant cytotoxic effect on *H. perlevis* aggregates (Figure 3). As a positive control, aggregates were also treated with known cytotoxic concentration of cadmium chloride, sodium dichromate and nickel chloride (Figure 4). Furthermore, concentrations of $AlCl_3$ up to 100µM (2.7mg/L) were also shown to be non-cytotoxic as assessed by the MTT assay (data not shown).

3.2 Aluminium-induced DNA Strand Breaks

Induction of DNA-strand breaks was assessed using the comet assay in sponge aggregates treated with non-cytotoxic concentrations of $AlCl_3$. Untreated sponge nucleoids were spherical with no evidence of DNA comets (Figure 5A), in contrast, following treatment with $AlCl_3$ clear comet tails were apparent (Figure 5B) and there was a concentration-dependent (1-Way ANOVA with Dunnett's multiple comparison tests, $P < 0.05$) increase in the levels of DNA-strand breaks as quantified by tail DNA % (Figure 5C). Mean ± SEM values of percentage median tail intensities, were 1.8 ± 0.5 (control/untreated samples), 4.9 ± 0.9 (0.1mg/L treatment), 9.0 ± 1.6 (0.2mg/l), 14.9 ± 2.7 (0.3mg/L) and 23.1 ± 3.6 (0.4mg/L treatment).

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3.3 Aluminium Induced Reactive Oxygen Species Correlates with DNA-strand Breaks

ROS formation as assessed by oxidation of DCF-DA following aluminium exposure was also statistically significant at $P^{*} < 0.05$ and $P^{****} < 0.0001$ (Figure 6) and concentration-dependent. 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. Furthermore, DNA strand breaks and levels of reactive oxygen species positively correlated (Figure 7).

4. Discussion

The toxicity of aluminium is largely associated with its solubility in water in an acidic pH, while aluminium in neutral pH is generally thought to be insoluble, non-bioavailable and thus, less toxic [30]. This study presents for the first time the application of the alkaline comet and H2DCF-DA assays to assess aluminium-induced DNA strand breaks and reactive oxygen species (ROS) formation in laboratory cultures of marine sponge cell aggregates, as biomarkers of effects and exposure to aluminium chloride. In the current study cryopreserved single sponge cells were cultured in synthetic seawater and consistent with previous work [6] rapidly formed viable aggregates that proved a useful "*in vivo*" exposure model that was also could be readily dissociated into single-cell suspension using calcium magnesium-free seawater containing EDTA for down-stream biochemical and toxicological investigations.

The comet assay's sensitivity among other advantages distinguishes it from other in vitro genotoxicity assays and it is widely employed in environmental risk assessment of model environmental pollutants in both humans and animals [21,31]. The alkaline comet assay detects alkaline labile sites and double and single-stranded DNA breaks induced by genotoxic agents [41,42]. Previous studies have demonstrated the mutagenic and genotoxic potential of aluminium in mammalian cells using assays such as micronucleus assay, Ames test, and sister chromatid exchange assay [43-45] but there is limited data on aluminium genotoxicity in marine species. In the current study using our novel *in vivo* exposure model, non-cytotoxic concentrations of aluminium caused a concentration-dependent increase in DNA strand breaks. This is similar to the report of [46]; strains of *Salvelinus fontinalis* (Brook trout) kept in a mimicked '*in situ* field exposure' condition showed greater sensitivity to the combined toxic effect of low pH and aluminium than free-swimming ones. Significant reduction in survival rate and fish weight was observed following exposure to aluminium concentration between 29 µg/L and 222 µg/L at pH less than 4.8. Although the concentrations used in the current study were relatively high, the toxic effects of aluminium observed suggests a potential for toxic effects following chronic exposure of sponges to lower concentrations. In addition, solid aluminium particles like other metals in the aquatic system are biologically available through cellular uptake pathways [47, 48] which are enhanced by increase aluminium levels in some marine environment and may result in high local intracellular concentrations.

In the aquatic environment, aluminium accumulates in invertebrates' body tissues and the gills of gill breathing animals with toxicity being directly linked with high concentration from acidification [49-52]. According to the report of Ingersoll, Gulley [46] however, more pronounced toxicity were observed in the gills of two strains of *Salvelinus fontinalis* (Brook trout) exposed to low pH and elevated aluminium than those exposed to only low pH. Although not widely considered an environmental pollutant, aluminium concentrations as high as 9560-25000mg/kg in sediment [53]; 1830-2170µg/g in water [54] and 222.2–662.6µg/g in marine sponge tissues taken from the Niger Delta environment (Akpiri et al., in draft). Aluminium toxicity in aquatic invertebrates has been previously reported. For example, sub-lethal toxicity of aluminium has been investigated in the freshwater crustacean crayfish: *Pacifastacus leniusculus*. Marked behavioural dysfunction was observed following exposure to 500µg/L of freshly neutralised aluminium for 5 days with over 70% aluminium accumulation mainly in the gills rather than the body [30]. Exley, Wicks [32] and Kádár, Salánki [55] have also demonstrated aluminium toxicity in fish and benthic mollusc. However, to the best of our knowledge, this is the first report on the toxicity of aluminium in marine sponges. There is no known biological function of aluminium; however, in humans, the involvement of aluminium in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease has been reported [24,56].

The mechanism of metal-induced DNA damage is often linked to the production of reactive oxygen species radicals [24,57]. For example, transition metals undergo redox cycling and are able to deplete levels of intracellular glutathione which results in the production of reactive oxygen radicals (H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, $^1O_2$), induction of lipid peroxides and oxidative DNA damage. To investigate the involvement of reactive oxygen species involvement in aluminium genotoxicity, we measured the amount of ROS produced following treatment with $AlCl_3$ and observed a concentration-dependent increase in levels of ROS following treatment. Similar induction of ROS following aluminium exposure has been previously reported in male

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rabbit [33,36]. Our data is the first on aluminium-induced reactive oxygen species in marine sponges and aluminium mediated oxidative stress in aquatic invertebrates. The results obtained demonstrated a statistically significant correlation (R^2 = 0.9974) between DNA damage and reactive oxygen species formation.

We conclude that Al is genotoxic even at non-cytotoxic concentrations with active induction of oxidative stress. The strong correlation observed between Aluminium induced oxidative DNA damage and reactive oxygen species formation, suggests the active involvement of reactive oxygen radicals in the mechanism of aluminium mediated toxicity in this experimental model. Further studies are required to reassess the potential toxicity of aluminium and the involvement of other biomarkers in the mechanistic pathways in the marine environment.

Appendixes

List of Figures

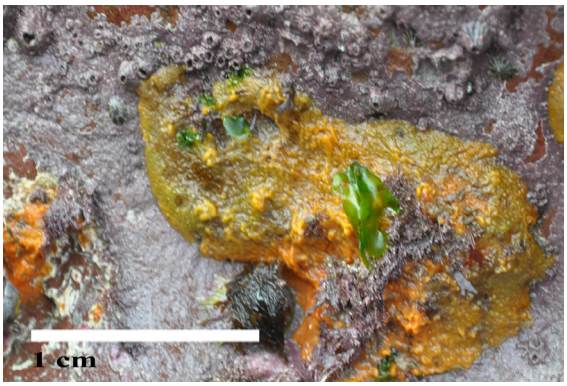

Figure 1. *Hymeniacidon* perlevis *in situ* on an exposed boulder at low tide in Tenby Bay Castle beach, Pembrokeshire

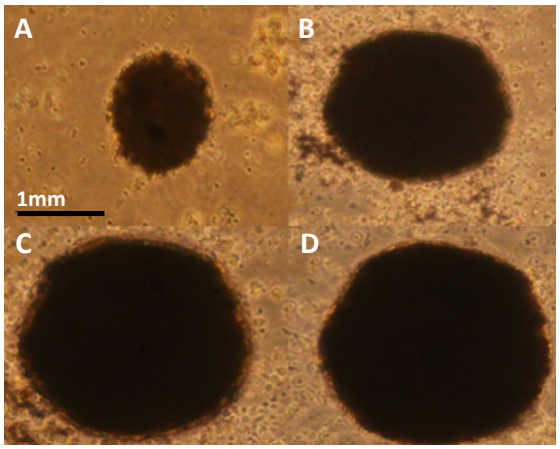

Figure 2. Representative examples of sponge cell aggregates formation after: A) 24hrs, B) 48hrs, C) 72hrs and D) 7 days in culture

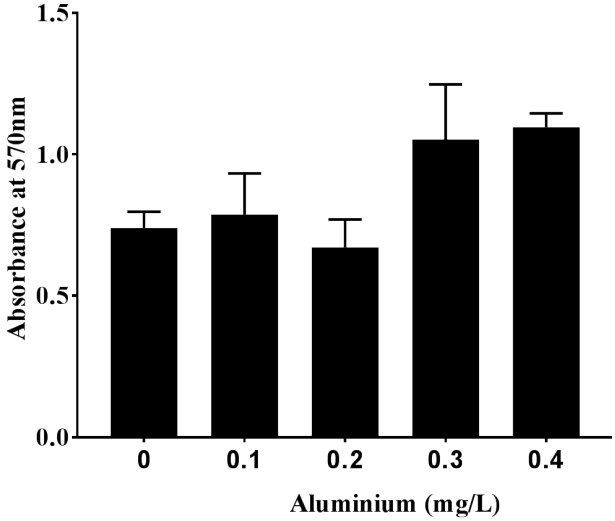

Figure 3. MTT viability assay of sponge cell aggregates exposed 12- hours in Aluminium III Chloride solution (0, 0.1, 0.2, 0.3 and 0.4mg/L)

Note: Data represent mean absorbance at 570 ± SEM, n =3. In all Aluminium exposures there was no cytotoxic effect on sponge cells by 1-Way ANOVA using Graph pad Prison version 7, P < 0.05

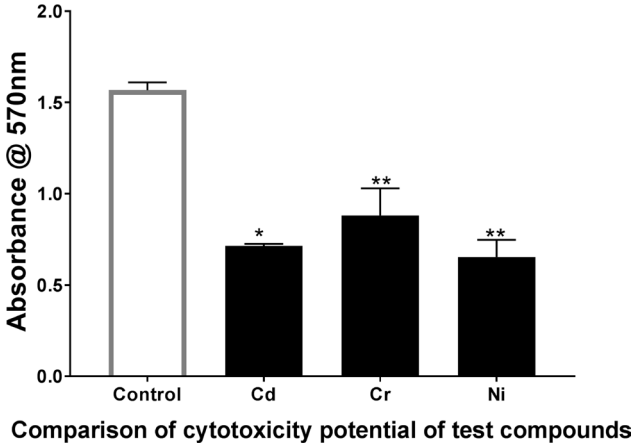

Figure 4. MTT viability assessment of positive control treatments with 100µM, Cd, Cr and Ni

Note: Mean absorbance ±SEM, n=3; P < 0.05 are presented. 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 control and treated sponge cell aggregates, * P<0.05 and ** P<0.01 respectively.

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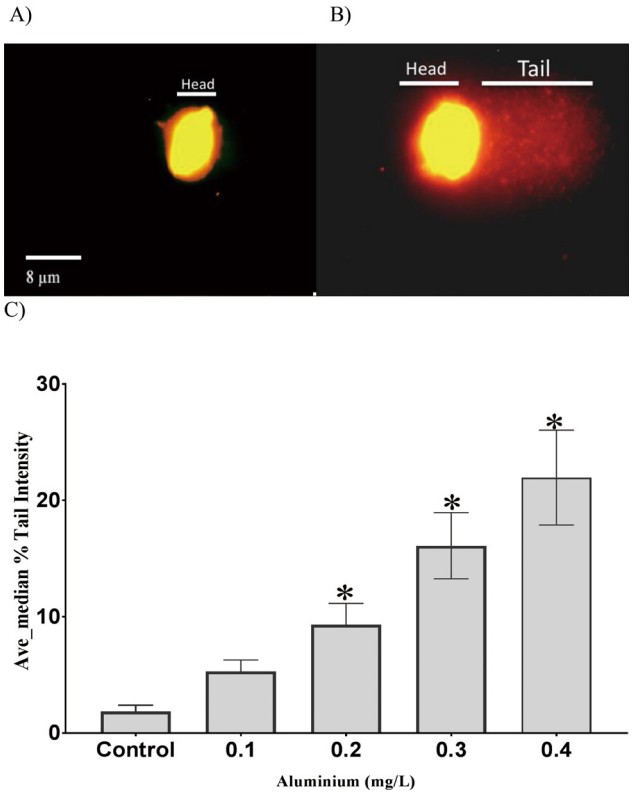

Figure 5. Representative comets images of sponge comet nucleoids, A) control B) 0.3mg/L $AlCl_3$. C) DNA strand breaks in sponge cell aggregate cultures expressed as % DNA tail intensity, following treatment with 0–0.4 mg/L aluminium chloride for 12 hours.

Note: Displayed data shows Mean values ± SEM, n= 3, $P^{**} < 0.05$ 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.

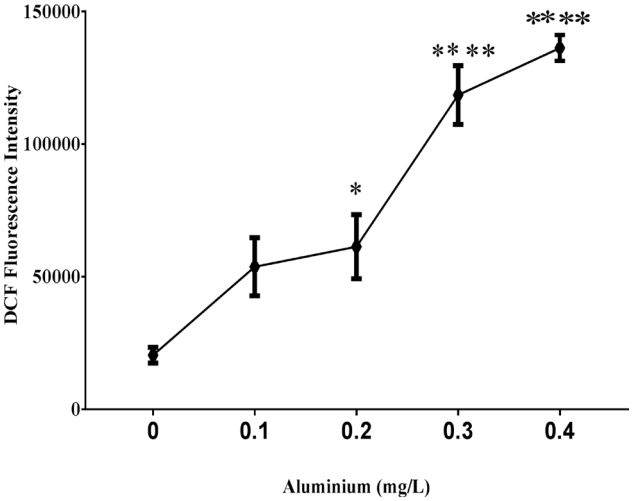

Figure 6. Aluminium Induced Oxidative stress in cultures of sponge cell aggregates

Note: 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.

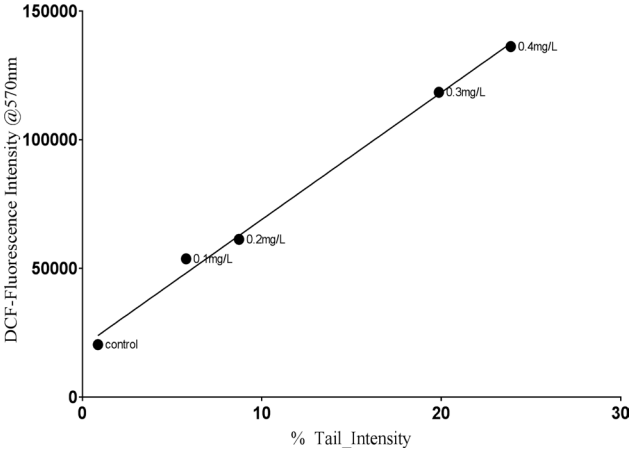

Figure 7. Correlation analysis of DCF- Fluorescent intensity versus Median % Tail Intensity in sponge cell aggregates exposed to 0, 0.2, 0.3 and 0.4mg/L aluminium chloride for 12 hours

Note: Result showed very significant correlation between ROS formation and DNA strand breaks in aluminium treated Sponge aggregates. $R^2 = 0.9974$.

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