Stress response of the coral *Stylophora pistillata* towards possible anthropogenic impacts in the Gulf of Aqaba, Red Sea

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Abstract

Coral deterioration is often linked with coastal pollution. This aimed to study biochemical stress responses in the common coral *Stylophora pistillata* collected and/or planted in coastal sites subject to pollution and sites without pollution in the Gulf of Aqaba. DNA damage and lipid peroxidation were analyzed to measure stress in corals. High DNA damage was found in natural corals from polluted sites, while higher lipid peroxidation was found in control site compared with polluted sites. Lipid peroxidation was higher in polluted sites after one-year of deployment. Corals' incubations with copper and lead produced high levels of DNA damage and lipid peroxidation compared with control samples. The results suggested that although corals are visually looking healthy, but they are suffering at subcellular levels. The consequences of such stress might affect the fecundity and growth rates of corals. The results suggest that biomarkers used are efficient tools for early stress detection in corals, though the cost of assessing DNA damage is relatively expensive compared with lipid peroxidation.

Keywords: Biomarker**;** comet assay; **c**oral; DNA damage; Gulf of Aqaba; lipid peroxidation.

1. Introduction

Coral reefs are globally significant ecosystems due to their ecological and socioeconomic importance to mankind (Burke *et al.,* 2012). Red Sea coral reefs are significant ecosystems at global scale (Gladstone *et al.,* 1999). The Gulf of Aqaba (GoA) is the only marine access of Jordan, where all sea-related human activities are concentrated (Al-Horani & Khalaf, 2013). Coral reefs are vulnerable to impacts from overlapping human activities, of which marine pollutants are major threat to them (Al-Horani *et al.,* 2011; Binelli & Provini, 2003).

When corals are impacted by anthropogenic stressors, their colonies display signs of stress like bleaching or tissue necrosis, which may result in their final death (Ginsburg *et al.,* 2001). Proper evaluation of the impacts of pollutants on corals is essential to avoid their death since sublethal status can be missed when visual inspection is used to check on their health status, where biomarkers are useful tools for such purposes (Wells *et al.,* 2001).

The DNA damage in living cells leads to the appearance of lesions and can cause various cell dysfunctions, genome mutations and gene instabilities. Therefore, there is an essential demand for assessments that can evaluate DNA damage (Boer *et al.,* 2002). The comet assay technique (single cell gel electrophoresis) is efficient for testing DNA damage and can be used as monitoring tool for environmental pollution (Wilson *et al.,* 1998; Collins, 2004). The method is reliable and sensitive to measure DNA strand breaks induced by various agents in eukaryotic cells using florescence microscopes.

Lipid peroxidation (LPO) is a process mediated by reactive oxygen species (ROS) and might lead to degradation of polyunsaturated fatty acids and cell membrane disorganization and can be used as oxidative damage bioindicator (Valavanidis & Vlachogianni, 2010; Vijayavel *et al.,* 2012). Levels of ROS might increase as a result of stress response mechanisms related to temperature, UV exposure, oxygen levels in addition to pollutants such as heavy metals (Lesser, 2011; Banc-Prandi, & Fine, 2019). The products of LPO indicate the levels of ROS in the cell, which in turn reflects the oxidative stress by internal and external factors (Girotti, 1998). Malondialdehyde (MDA) is one of the major end products of LPO that can be used as indicator to detect oxidative stress in biological objects (Yadav, 2010).

This study aims to assess stress responses of *Stylophora pistillata* towards marine pollutants in contaminated versus uncontaminated sites in GoA. The assessment examines DNA damage and LPO as biomarkers to measure stress in corals.

2. Methods Coral Samples

Stylophora pistillata colonies were collected from shallow reef slope (<10m) at the Marine Science Station (MSS) and were housed temporarily in coral wet laboratory. The mother colonies (ca. 10 mother colonies) were fragmented into small pieces (5cm long) and were glued to plastic tubes and placed in a flow-through seawater raceway for two weeks before deployment as described by Al-Horani (2013). Coral fragments were fixed on aluminum racks (80x40x60 cm) and were placed at 8m depth in front of the MSS. Three types of samples were collected; aged fragments (one year old), newly planted fragments (one week old), and natural colonies found in the site (4 replicates were collected from each site). From each treatment group, four replicates were collected by SCUBA divers.

3. Study Sites

Study sites included the Industrial Complex (IC) site, where various industries are concentrated, the Public Beach (PB) site, which is regularly exploited by the general public and the MSS site, located within the MPA and has no direct human impacts (used as control site) (Figure 1). Pollutants at the IC and PB sites may include oil spills, antifouling paints, organic and solid wastes including metal cans and plastics. Runoff during winter bring various pollutants to the sea in the three sites when it occurs.

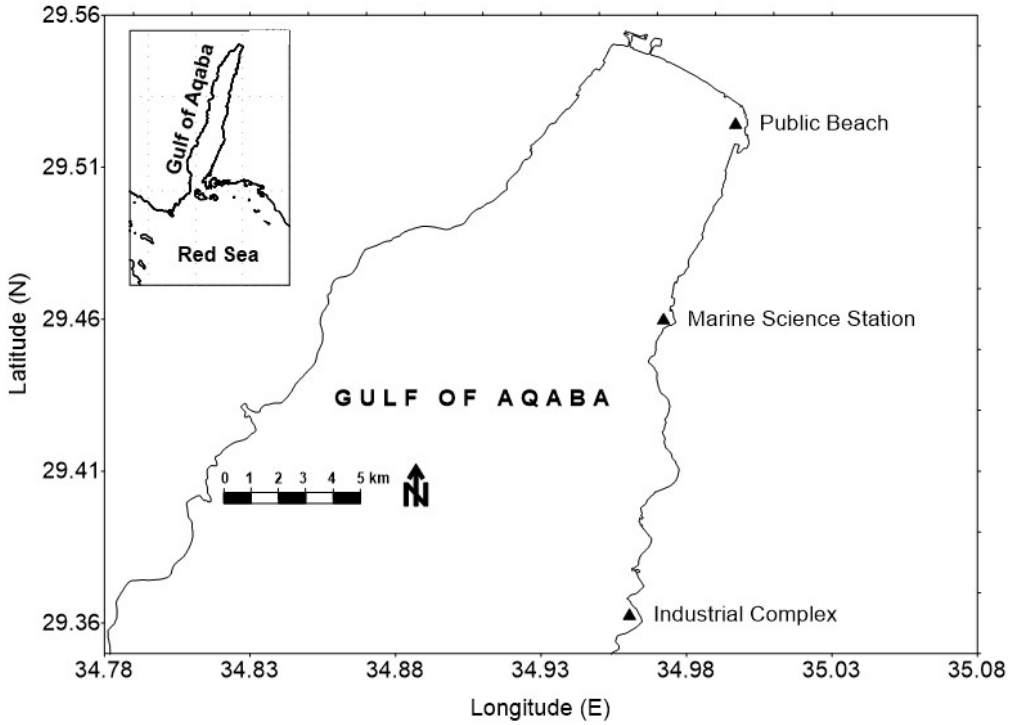


Fig. 1. GoA map with latitude (N) and longitude (E) drawn by surfer software.

4. Isolation and preparation of coral cells

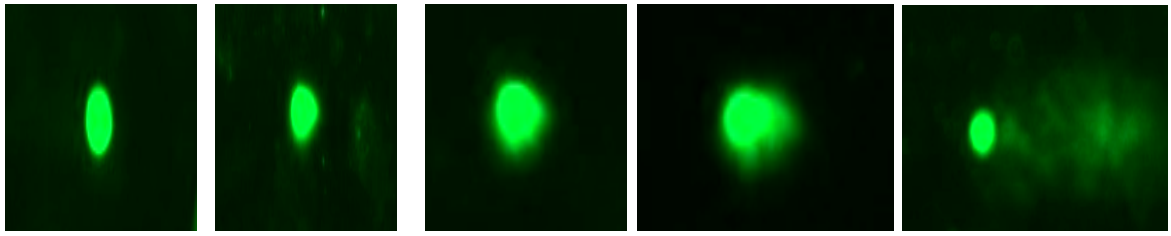
Artificial seawater free of Ca^{2+} and Mg^{2+} was used to dissociate the tissue from skeleton (Rinkevich *et a*l., 2005). Samples were immersed for 10min. at room temperature, followed by gentle shaking until the tissue was fully dissociated. The isolated tissue suspension was centrifuged at 4000rpm for 5min to remove zooxanthellae and the supernatant was then centrifuged for 15min at 4000rpm. The pellet was used for LPO analysis and the cells for the comet assay were resuspended in 500µL PBS until analysis.

5. Measurement of DNA damage

Trevigen® comet assay kit was used to measure DNA damage in coral cells according as described by Szeto *et al.,* (2012). In this method, coral cells were centrifuged at 10000rpm for 5min and the cell suspension (10µL) was mixed with LMA and 50µl/well was pipetted onto the comet slide and electrophoresis was run as described. Then, slides were examined by inverted fluorescence microscope (EVOS®) on 100x magnification (GFP filter, 470nm excitation, 525nm emission) to measure the migration of damaged DNA from the nuclei.

The formation of specific structures like comets in the gel indicates DNA inside the tissue is damaged. The comet tail intensity relative to head reflects the number of DNA breaks. For scoring of the DNA damage in cells, the damage levels were graded from 0 to 4 (Figure 2), where score 0 represents no damage and score 4 represents severe damage. The score of each sample was calculated by using the following equation:

$$\text{Score} = \frac{0*\text{N0}+1*\text{N1}+2*\text{N2}+3*\text{N3}+4*\text{N4}}{\text{total number of cells}}$$



Score 0 **Score 1** **Score 2** **Score 3** **Score 4**

Fig. 2. Comet assay score (0-4) showing DNA damage levels in *S. pistillata* cells.

6. Measurement of lipid peroxidation

Lipid peroxide was measured by analyzing MDA, an end product of lipid peroxidation, which reacts with thiobarbituric acid (TBA) as a reactive substance, and produces a red colored complex (MDA-TBA) that has a peak absorbance at 535nm (Wahsha *et al.,* 2012; Saeed *et al.,* 2020). The coral cells were prepared as described previously and the absorbance was measured at 532nm and 600nm with TBA-TCA mixture used as blank, and the lipid peroxides quantified by using Beer's law with an extension coefficient of $155mM^{-1}$ cm^{-1}. The results were expressed as M/g wet pelleted coral tissue weight.

7. Effects of addition of heavy metals on lipid peroxidation

Fragments of *S. pistillata* were glued in an upright position to a small (5x5 cm) ceramic plates using epoxy glue. The following incubations were carried out; the first included five plastic aquaria that were filled with 5L seawater with aeration. The first aquarium was used as negative control. Copper (Copper Chloride) was added to two aquaria at two different concentrations (1 and 5ppm), while the last two aquaria were supplemented with lead (Lead Nitrate) (1 and 5ppm). In the second incubation experiment, the concentrations of Cu and Pb were adjusted to 0.1 and 0.2ppm. In the first day (0 time), coral fragments from the five aquaria were taken and prepared for LPO test as described earlier. Samples were incubated for 10 days or until they died and were taken every two days for LPO analysis, and samples from the Cu and Pb 1 and 5ppm incubations were used for comet assay analysis.

8. Statistical analysis

Statistical analysis was performed using one- and two-ways ANOVA. Statistical significance was considered at p-value of 0.05 or less. Data was analyzed by using Sigma Stat software.

9. Results Comet assay *in situ* experiments

DNA damage in coral fragments incubated *in situ* at MSS site were not significantly different in the 1wk incubated, 1yr incubated and natural colonies, though a DNA damage was recorded in MSS samples. The 1wk incubated samples from PB and IC sites had similar levels of DNA damage to the MSS site. While, after 1yr of deployment in the sea, the DNA damage increased significantly by 22% and 24% compared to 1wk in PB and IC sample, respectively. High DNA damage scores was recorded in natural corals collected from PB and IC sites (Figure 3).

10. Comet assay for ex *situ* experiments

DNA damage scores was much higher in copper- and lead-incubated corals when compared to all field incubated-corals. The lead-incubated corals had average DNA damage scores of 2.35, regardless of the metal concentration. In the copper-incubated samples, DNA damage scores was lower than those incubated with lead (Figure 4), although copper-incubated samples suffered with severe tissue necrosis and died after four days of incubation.

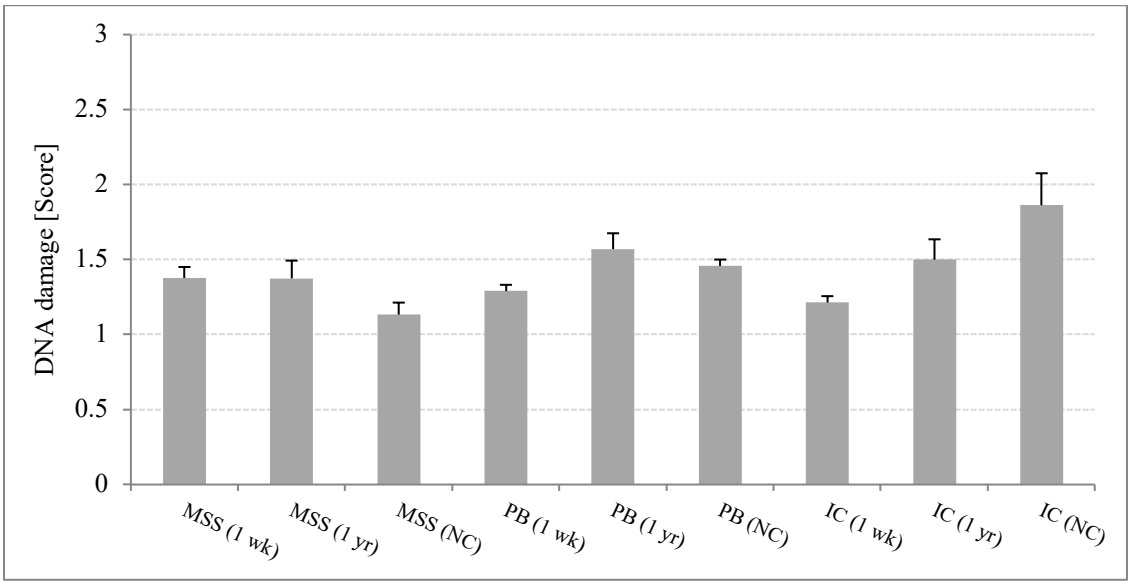


Fig. 3: Scores of DNA damage in corals at the 3 sites (wk; week, yr; year, NC; natural corals). Results presented as average±SD.

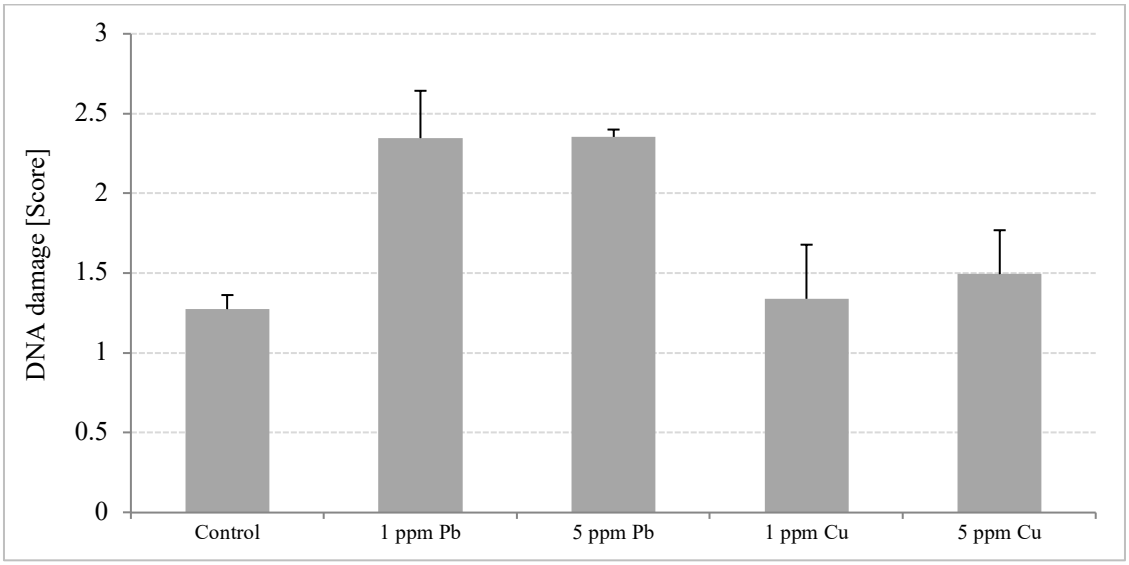


Fig. 4: Scores of DNA damage in corals incubated with Pb and Cu and control samples.

11. Lipid peroxidation *in situ* experiments

Analysis of LPO in field-incubated corals showed high levels of peroxidation in all samples except for the natural corals in IC site. There were no observed trends among the values obtained (Figure 5).

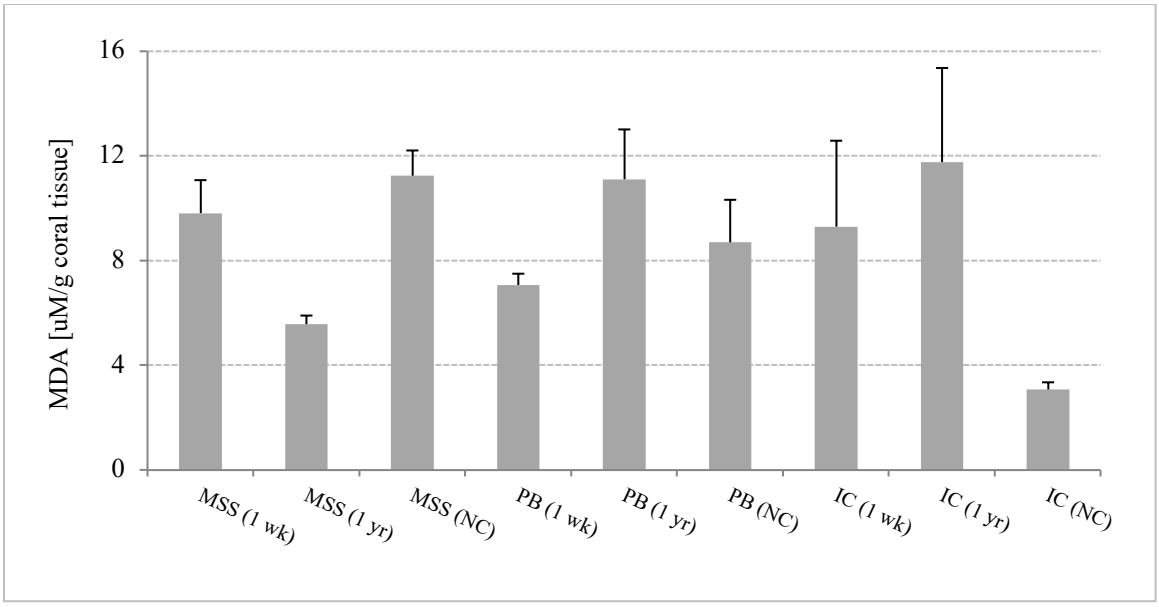


Fig. 5. MDA levels in corals incubated at MSS, IC and PB sites (wk; week, yr; year, NC; natural corals).

12. Lipid peroxidation ex *situ* experiments

The MDA concentrations in control samples showed low levels of LPO for the whole incubation period. When 0.1ppm Pb was added, the MDA concentration increased gradually to about 5µM after 10 days incubation. When 0.2ppm Pb was added, the MDA increased to about 11µM after 8 days, which then started to decrease as a result of tissue necrosis (Figure 6). Cu had more deleterious effects on corals, which showed primary increase in MDA levels, then started to suffer from tissue necrosis and died after 4 days of incubation with 0.1 and 0.2ppm concentrations (Figure 7).

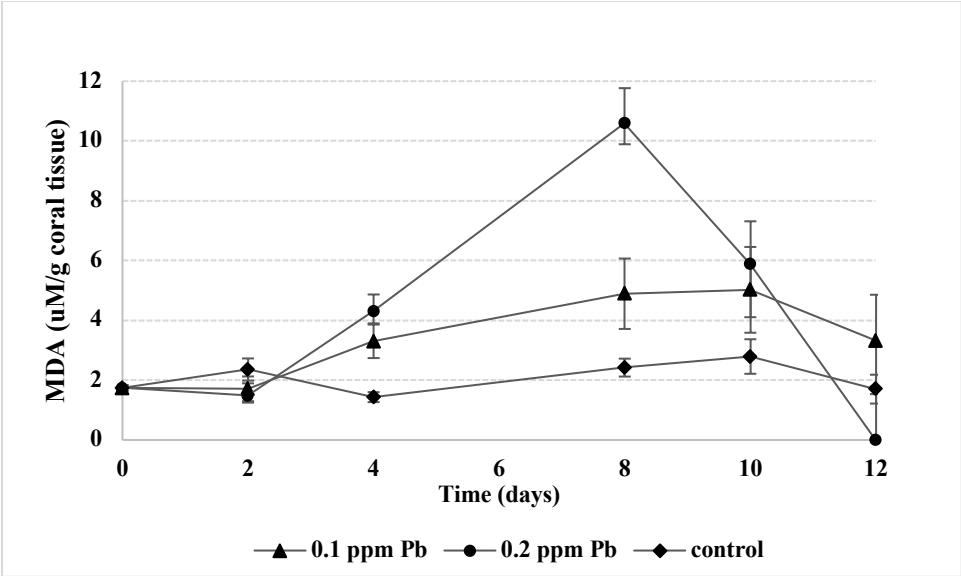


Fig. 6. MDA levels in *S. pistillata* incubated with 0.1 and 0.2ppm lead.

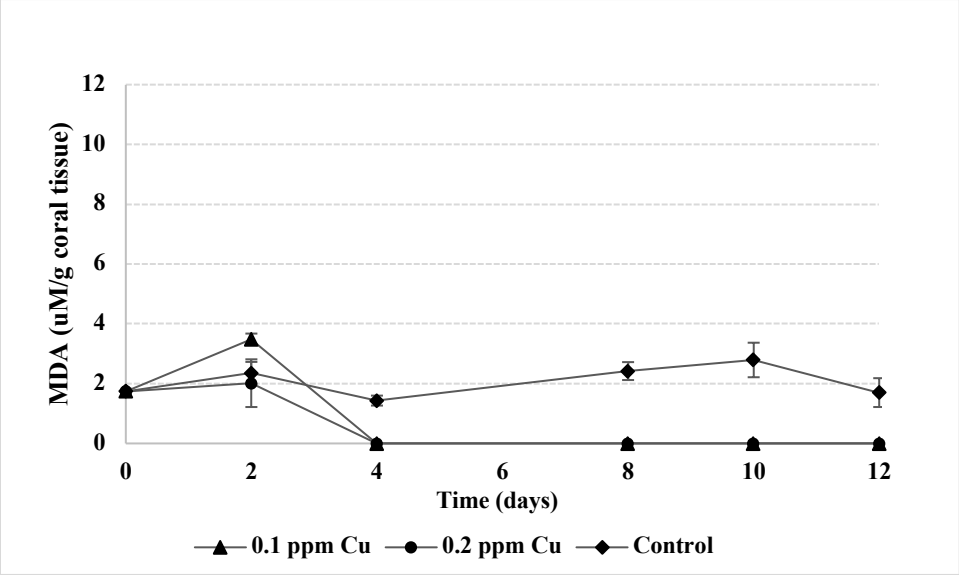


Fig. 7. MDA levels in *S. pistillata* incubated with 0.1 and 0.2ppm copper.

13. Discussion

In this study, *S. pistillata* were used as proxies for environmental pollution using DNA damage and LPO as stress indicators (regardless of the type of pollutant) and demonstrate the magnitude of response towards pollutants as prognostic tools. The sites selected (MSS, IC and PB) are distributed along the Jordanian GoA, to determine levels of sublethal responses of corals towards environmental stresses at those sites.

Heavy metals pollution is central environmental issue due to their toxicity, which influence growth and longevity of organisms, biochemical persistency and tendency to bioaccumulate in biological systems (Jia *et al.,* 2011; Al-Rousan *et al.,* 2012). Exposure to heavy metals generates ROS, which can damage DNA and cells and lead to LPO through oxidative stress (Richier *et al.,* 2005; Lesser, 2011).

The DNA damage in coral cells was measured by comet assay. After one year of planting corals *in situ*, the DNA damage score was highest in samples planted at PB site, followed by samples at IC site, while samples at MSS had the lowest DNA damage score. Those results suggest that PB and IC sites have sublethal levels of pollutants that can damage DNA in corals. Also, in PB and IC sites, both the 1yr planted samples and the natural colonies collected from the sites had no significant differences among them, however they were statistically higher than the 1wk planted samples in both sites. Those results suggest that corals introduced to the sites became biologically similar to natural coral in the site after long time of deployment.

The 1wk planted samples at MSS had the highest DNA damage, followed by samples at PB and IC sites, respectively. This suggests that there might be some environmental disturbances presented at the MSS site during this period, which caused DNA damage. Although the MSS site is protected area with restricted access, its proximity to Containers and Passengers Ports, where oil spills and antifouling paints may reach the MSS site from time to time, and have the ability to cause stress on corals. The DNA damage found in natural colonies at the MSS site was significantly lower than that in the natural colonies at the IC and PB sites, while no significant differences were found between the IC and PB samples. These findings

suggest that the MSS site can be considered as control site, despite the environmental disturbances described above. It is possible that corals at MSS site had DNA damage due to natural factors prevailing at the site. For example, it was found that anemone cells exhibit high control or background levels of DNA strand breaks (Mitchelmore & Hyatt, 2004). DNA damage in corals may result from thermal stress, exposure to UV radiation and/or from oxidative stress due to the symbiotic algae in corals exposed to sunlight (Baruch *et al.,* 2005; Nesa *et al.,* 2012). It was observed in corals that photoreactivation (a light-stimulated response) is the main repair pathway for UV-induced DNA damage in which photolyase proteins repair damaged DNA (Reef *et al.,* 2009). It is possible that the efficiency of repair systems differs among the different coral species as well as among individuals of the same species.

The *ex situ* incubations of corals with Cu and Pb produced significantly higher DNA damage than control samples, which showed some DNA damage that can be reasoned to background DNA damage or to incubation stress (Kteifan *et al.,* 2017). Heavy metals may damage DNA, cause bleaching and final death of corals (Jia *et al.,* 2011; Schwarz *et al.,* 2013). It was also suggested that cnidarians with symbiotic algae experience high levels of oxidative stress, which may result in DNA damage (Lesser, 2011).

The results suggest that IC and PB sites have pollutants that can produce stress at cellular levels of corals. It also suggests that these corals have efficient repair systems since they could survive such stress factors, though their growth, survival, and fecundity might be affected due to energy diversion during repair process when combatting adverse environmental conditions (Banaszak, 2003). Other factors that might affect coral survival include aging, which impair coral ability to counter oxidative stress, while larvae produced under stressed conditions are less sensitive to stress (Pacific & Davies, 1991; Farina *et al.,* 2008).

Lipid peroxidation through its product (MDA), was used as biomarker for subcellular structural integrity and gives indication that ROS exceeds the capacity of antioxidant defense system, which might lead to fatty acid degradation and destabilize membranes (Rikans & Hornbrook, 1998; Wahsha *et al.,* 2012). Heavy metals generate ROS and increase MDA production in corals (Kteifan *et al.,* 2017). MDA production was a direct response towards stress, which indicates health status of corals during the study.

Except for samples at IC site, there were no significant diffenrences of LPO results for field samples, which suggests higher stress levels at IC site. Also, when corals were incubated with Pb and Cu, the MDA concetrations increased before the corals died. The MDA level increased gradually for 10 days incubation with 0.1ppm Pb, which decreased afterwards, while the level was more than doubled when upon incubation with 0.2ppm Pb concentration and died by the end of the incubation period. The corals could not survive after two days of incubation with Cu, as it was also suggested earlier (Jia *et al.,* 2011). Copper is considered the most toxic metal as it affects fertilization, larval settlement, cause tissue necrosis and impair photosynthesis in corals (Reichelt-Brushett & Harrison, 1999; Schwarz *et al.,* 2013).

14. Conclusions

The bioindicators used in this study were useful tools for detecting sub-lethal effects of pollutants on corals. The comet and lipid peroxidation assays are useful tools for monitoring stressed marine environmental areas, and certainly have potential for use as early warning

applications for environmental disturbances. The results obtained suggest that the use of DNA damage as biomarker is suitable for the long-term monitoring of coral health, while LPO is only suitable for short-term purposes. The results also revealed that corals in study sites are facing environmental disturbances resulting in high levels of LPO and DNA damage, which were also shown by *ex situ* testing of Cu and Pb added to incubation tanks. The results found here are promising, though further research is still needed to verify their application with other coral reef organisms.

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