DNA damage and shell malformation in Blue Mussel, *Mytilus edulis*

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**ABSTRACT.** Pollutants in waters that cause DNA damage, such as tributyltin, have been known to cause malformations in the mussel shell, which need attention to be studied. Shell malformations or deformities of blue mussels were observed in Norsminde Fjord harbour. The mean shell thickness index of blue mussels from the polluted site was greater than it from expected clean waters. DNA damages detected using the versatile comet assay which expressed as tail length and olive tail moment were evaluated in blue mussels gill cells both in situ and in vitro. The results showed that the average of DNA damages in treatments, namely the polluted site, tributyltin chloride (TBTC) (10 g/l), and hydrogen peroxide (H$_2$O$_2$) (10 M) were different from the expected unpolluted site. Although comet assay is considered as an unspecific assay, the results proposed the similarity of DNA damage character of blue mussel gill cells when exposed to tributyltin chloride and taken directly from the polluted site. The data obtained are important for assessing the environmental risks created by genotoxic agents, e.g., tributyltin, used as antifouling agents in marine paints.

1. Introduction

It is unavoidable that the development of industry around the world is proceeding successively with the extraction of nature and discharging waste into the environment, especially in aquatic habitats. Indeed, this man-made destruction of nature brings severe short and long-term effects, which endanger the sustainability of the habitat and consequently the dwellers. Some discharged products are genotoxicants or carcinogens. Numerous carcinogenic compounds have been monitored in marine sediments from polluted areas worldwide. Most of these hazardous chemicals accumulate in various trophic levels of organisms living in aquatic areas. (Bolognesi *et al.*, 1996; Lee *et al.*, 2020; Qin *et al.*, 2020; Baines *et al.*, 2021). They have significant influences in cell damage creating necrosis and neoplasia (Bebianno & Garcia da Fonseca, 2020; Medda *et al.*, 2021), DNA damage (Yaqin, 2006; Asllani *et al.*, 2019; Bolognesi *et al.*, 2019; Pellegrì *et al.*, 2020; Guimarães *et al.*, 2021; Camargo Filho *et al.*, 2022), embryonic mortality and mutation (McLaughlin *et al.*, 2020; Ganaie, 2021) and Darwinian fitness such as decrease of growth rates, reproductive output, and viability of offspring (Depledge, 2020).

Chemical-based monitoring has been used to observe and analyze pollution in the environment and thereby making a list of chemicals released in the environment. However, it only rises an idea on how many and what kind of existing pollutants are in the environment and does not depict the interaction between the existence of toxicants in an environment and the deleterious effect of organisms (Yaqin *et al.*, 2011; Métais *et al.*, 2019; Schuijgt *et al.*, 2021). Consequently, there is an urgent need to build methods that can diagnose the impact of pollutants anywhere along the hierarchy of increasing biological complexity from molecule to population. The biomarker or biological marker concept is a preferable candidate as a monitoring device to detect and assess the biological effect of pollution on organisms and the quality of the environment concomitantly (Kumari & Khare, 2018; Yaqin, 2019; Gallo & Tosti, 2020; Georgieva *et al.*, 2021).

Molecular biomarkers have been regarded as early warning systems along with the recognized correlation between the genotoxic burden of aquatic ecosystems and carcinogenic effects in humans and wildlife in an epidemic study (Black & Baumann, 1991; Baines *et al.*, 2021; Jeddi *et al.*, 2021; Liu *et al.*, 2022). Besides, the molecular biomarkers, for instance, DNA damage, serve as a good and useful early warning system of carcinogens on different organisms (Kumari & Khare, 2018; Lionetto *et al.*, 2019; Ben Ameur *et al.*, 2022). The basic reason for this is the capacity of carcinogens or genotoxicants when discharged in the environment to cause various types of DNA damage (Xu *et al.*, 2018; Pellegrì *et al.*, 2020). Likewise, DNA is a predominant important substance, which is maintained by living organisms in efficient and intricate mechanisms. Significant stresses, therefore, may disturb these mechanisms and increase observable DNA damage (Lee *et al.*, 2018; Fossi *et al.*, 2020).

DNA damage can be reflected as DNA strand breaks are considered a sensitive and non-specific predictor of a wide range of genotoxicity exposures (Mitchelmore & Chipman, 2021).
The production of DNA strand breaks correlated well with mutagenic and carcinogenic properties of environmental pollutants with diverse structures (Velali et al., 2019; Azqueta et al., 2020). DNA damage may appear via profound strand breaks as a result of the alkali labile site (Hong et al., 2020; Möller et al., 2020; May et al., 2022) and can also occur via the action of the excision repair enzyme (Park et al., 1991; Speit & Hartmann, 1995; Kim et al., 2019; Winkelbeiner et al., 2020).

Comet assay is a sensitive, simple, flexible, rapid, and inexpensive technique for detecting DNA damage as DNA strand breaks in an individual cell of almost any eukaryotic organism (Azqueta & Collins, 2013; Cvetković et al., 2018; Tatin et al., 2021). Due to its ability and practical advantages, this assay has already been used as a genotoxicity test for various genotoxicants both direct and indirect-acting (Erraud et al., 2018; González et al., 2018; Gajski et al., 2019; Cordelli et al., 2021; Rajendran et al., 2021) for various aquatic organisms from vertebrate (Żegura & Filipič, 2019; Camargo Filho et al., 2022; Cant et al., 2022; Costa, 2022; Gazo et al., 2022; Mahboob et al., 2022) to invertebrate (Erraud et al., 2018; Khan et al., 2019; Guidi et al., 2021; Consantino et al., 2022; Ivorra et al., 2022).

Mussels (Mytilus edulis and their relatives) are a very common aquatic invertebrate species that have been used as sentinel organisms in aquatic environment monitoring since Goldberg (1975) has proposed the Mussel Watch program. Mussels were thought to fulfil what was required by environmental monitoring principles because of their behaviour as a sessile organism, wide geographical distribution, availability, and resistance to general stress. By well-known antifouling compound tributyltin (TBT) mussels showed as a par excellence sentinel organism. High larval mortality of common mussels was observed at low concentrations of TBT (Beaumont & Budd, 1984; Amara et al., 2018; Bryan & Gibbs, 2020). About half of the larvae induced to 0.1 µg/l were dead by 15 days and most of the surviving larvae were dying and had grown significantly slowly (Beaumont & Budd, 1984). Reduction of Darwinian fitness as reflected in deformity of the shell was first observed in Japanese oysters (Crassostrea gigas) from the Bay of Arcachon (Alzieu, 1991). The deformity or abnormal shell thickness mechanism occurred at TBT concentration as low as 2 ng/l (Chagot et al., 1990) and Phelps and Page, (1997) used it as a TBT biomonitoring device. Page et al., (1996) and Huang and Wang (1995) also noted that there was a correlation between shell deformity and TBT burden of mussel tissue, thereby it might be a biomarker of TBT exposure in M. edulis. However, Lundebuye et al., (1997) carried out in situ studies in Danish waters and investigated that the TBT burden of mussel tissue was not correlated with Darwinian fitness, which was reflected as condition index and one of the biomarkers of exposure namely stress protein.

This triggers for using another biomarker considered as a sensitive biomarker, e.g., DNA strand breaks to detect genotoxic effects of TBT. Some studies have been carried out to recognize the cytogenetic damage induced by TBT on the early stage of mussels (Jha et al., 2000; Mičić et al., 2001; Hagger et al., 2005). Jha et al., (2000) noted that TBT is capable of inducing cytogenetic damage on M. edulis. In hemocytes of blue mussel (M. galloprovincialis) TBT was inducing apoptosis at low concentrations (Mičić et al., 2001). However, the implication of DNA damage induced by TBT on ecologically significant factors such as Darwinian fitness of mussels is still unknown. Therefore, studying DNA damages, which are indicated in DNA strand breaks and their implication on Darwinian fitness is interesting. So, this present study aimed to determine whether a highly toxicant in water, TBT, induced DNA damage on mussel gill cells and its implication on Darwinian fitness, which was reflected as shell thickness index.

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2. Materials and Methods

2.1. Samples Collection

Four hundred blue mussels 2.37 – 6.43 cm in length were collected randomly from relatively unpolluted and polluted sites, Norsminde Fjord, Denmark (Fig. 1). In the laboratory, blue mussels were kept in a well-aerated container for 2–7 days and employed for cell preparations. Blue mussels from assumed unpolluted sites were used for in vitro exposure namely H₂O₂ (hydrogen peroxide) and TBT (tributyltin chloride) and transplantation test. The mussels from the polluted as well as from unpolluted sites were used for the determination of shell thickness index and comet assay.

2.2. Shell Thickness Index

Shell thickness index or thickness index (TI) was determined on shells for 200 blue mussels collected randomly from the unpolluted and polluted site. The thickness index is a dimensionless quantity defined as 100 times shell depth (i.e., “thickness”) divided by shell length. TI was determined by measuring the greatest length and the greatest depth of the entire mussel shell with the valves closed. The depth of the shell was measured perpendicular to the plane of shell closure across both halves (Page et al., 1996).

2.3. Cell Preparation

Gill cells were selected for this study since they were easy to obtain, and in nature, they come into contact with relatively large volumes of seawater compared to the rest of the animal (average filtering rate for an adult mussel is approximately 2 l/h at 20 °C), thus conferring them with the potential for being a suitable target tissue for mutagen exposure (Dixon & Clarke, 1982; Venier & Canova, 1996; Pearson et al., 2018; Qu et al., 2019). The gills are initial sites of uptake TBT by M. edulis (Laughlin et al., 1986), and accumulation of TBT in gills and viscera is greater than in other parts of the bivalve mussels (Huang & Wang, 1995). Regarding cell types, the mussel gills provide an additional advantage as gill cells are relatively homogenous. Likewise, it can easily be made into a single-cell suspension without any need for chemical dissociation (Wilson et al., 1998).
Before the experiment, gill cells were isolated as described in Wilson et al., (1998). Four pairs of gills were dissected out and placed in 2 ml Ca⁺²-Mg⁺² free saline (CMFS) buffer (1100 mOsM; 20 mM HEPES-NaOH pH 7.3 containing 500 mM NaCl, 12.5 mM KCl, and 5mM ethylene di-aminetetraacetic acid (EDTA)), chopped for 1 minute and washed twice. The chopped pieces were transferred into 200 ml beaker glass with 10 ml buffer saline and agitated gently under the dark condition at 1°C for 1 hour. Cell suspensions were filtered through 60 µm cell strainers to remove cell clumps. The filtrates were centrifuged at 250 x g and 2500 rpm for 5 minutes to spin down cells. The supernatant was removed and cells suspensions were harvested and ready for comet assay or in vitro test. The viability of the cell solution was tested using a 0.4 % solution of the red dye EOSIN Y.

2.4 In Vitro Exposure

The mussels collected from the expected clean site were transferred into the laboratory and treated for preparing cell suspension as outlined above. Freshly prepared cell suspensions were exposed in vitro to 10 µM of H₂O₂ and 10 pg/l of TBTC. Test compounds were added to cell suspension (1 ml final concentration) in CMFS as vehicle solvent for 1 hour at 1°C in a dark room. Following incubation, 100 µl of cell suspension was used in the comet protocol outlined below, with the rest used for the viability determination using the red dye EOSIN-Y.

2.5. In Situ Exposure

Two models of in situ experiments were carried out. The first experiment was performed by collecting samples from the polluted site and used them for the comet assay. Transplantation was the second experiment where blue mussels from the expected unpolluted site were entered into a plastic net and fully immersed in the water column in the expected polluted site (harbour) for one month. Following incubation, animals were harvested and transferred to the laboratory for the next steps. This transplantation experiment was conducted to see whether there was a short-term effect of pollutants on the animals and to avoid confounding factors such as environmental changes in the polluted site.

2.6. Comet Assay

Three comet assay methods have been used in the ecotoxicology field namely, single, double and triple layers, the last method is called the ‘sandwich method’. The main difference among them is the layer of agarose used as cell suspension attachment. The double-layer method following Singh et al., (1988) was adopted with minor modification. Fully frosted microscope slide was coated with 100 µl of 0.85% normal melting-point agarose (NMP, Sigma) in Kenny’s salt solution (0.4 M NaCl, 9 mM KCl, 0.7 mM K₂HPO₄, 2 mM NaHCO₃) at 35°C and cover with 22 x 50 mm coverslip. The slides were placed in the refrigerator for 15 min to allow solidification. Cell suspensions in 2 ml were centrifuged at 3000 rpm and 250 X g for 5 min to create cell pellets. Cell pellets in 100 µl were resuspended in 125 µl of 0.65% low-melting-point agarose (LMP, Sigma) in Kenny’s salt solution. After the first agarose layer was solidified, cells suspended in LMP were transferred as 100 µl aliquots onto a coated fully frosted microscope slide. A coverslip was put onto the slide and agarose was allowed to solidify for 15 min.

After removing the coverslip, the slides were placed in lysis solution consisting of 2.5 M NaCl, 10 mM tris,0.1 mM EDTA, 1% sarcosyl, 1% Triton X-100 and 10% DMSO, pH 10 in a Coplin jar for 1 h at the refrigerator. Following a rinse two times in electrophoresis solution (10N NaOH, 200mM EDTA, pH > 13) for removing excess salts, the slides were transferred into gel electrophoresis chamber and immersed in electrophoresis solution for 15 min to allow the unwinding of DNA. Electrophoresis was carried out in the same solution for 20 min at 25 V and 300 mA. The slides were neutralized in cold neutralization solution (0.4 M TRIS) pH 7.5, 2 x 5 min.

Before the examination, the slides were stained with 50 µl ethidium bromide (in distilled water) (10 µg/ml) for 10 min with a coverslip. The slides were examined by using a fluorescence microscope (x 1,000 magnification). 50 cells/slide were visually saved randomly and the cell images were analyzed by using CASP (Comet Assay Software Project) version 1.0. The degree of DNA damage was expressed as tail length and olive tail moment. Tail length is indicated as the length of the comet tail measured from the right border of the head area to the end of the tail (in pixel). Olive tail moment considers the tail length and fractions of the DNA in comet tail, and is calculated as a percentage of DNA in the tail x (TailMeanX – HeadMeanX) ([percent DNA in tail] x [distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head in x-direction]) (Fig. 2).

Figure 2. Comet measurements (AutoCometTM. 2002).

All the steps above were performed under artificial light to minimize DNA damage induced by UV light.

2.7. Statistical Analysis

For statistical analysis, data were arcsinh transformed and a level of 0.05 was used as a significance level. One-way analysis of variance (ANOVA) was used to determine to mean differences of all treatments in comet assay and shell thickness index. The Bonferroni procedure was employed for the post hoc test.

3. Result and Discussion

3.1. Shell Thickness Index

Shell thickness index or thickness index (TI) is an index that depicts deformity or mal growth of mussel shell. The larger the value of the index is the more rounded the shell
shape. Table 1 shows the result of TI means of blue mussels from expected clean and polluted sites. Based on One-Way ANOVA there is a difference in means of TI between samples that were taken from the expected clean and polluted sites at level p < 0.01 (Table 1). It was indicated that mussels from the polluted site have a more rounded shell shape than those from expected clean sites. This reveals that pollutants, which occur in the harbour, may create a shell calcification anomaly or deformity of mussel shells (Fig. 3).

Table 1. Shell thickness index (mean ± SEM) of blue mussels, M. edulis from clean and polluted sites.

| Site       | Shell thickness index |
|------------|-----------------------|
| Clean      | 31.311 ± 0.523        |
| Polluted   | 36.205 ± 0.777**      |

** p < 0.01

Figure 3. Blue mussel shells occurred in polluted (a) and clean (b) sites.

Norsminde Fjord is a shallow inlet, which is used as a harbor for small boats, situated just south of Aarhus. Therefore, this fjord is expected as a place where TBT was widespread. From a preliminary study, it was known that many malformed blue mussels have rounded shell shapes. This study revealed that TI as TBT biomarkers is greater in blue mussels from harbour than TI in blue mussels from expected clean water. It may indicate that even TBT has been banned to use for ships with a length, of not more than 25 m since 1993 in Danish waters, the severe effects of TBT to use for ships with a length, of not more than 25 m since 1993 in Danish waters, the severe effects of TBT contamination of the local breeding waters was found to be responsible for failure to reproduce and for anomalies occurring in the shell calcification of adult oysters leading to stunted growth. The existence of the bad effects of TBT in some waters is still visible even though its use has been banned for a long time (Gallo & Tosti, 2020).

Many studies have been conducted to show the relationship between tributyltin contamination in waters with shell deformity in various bivalves, such as Japanese oyster (C. gigas) (Waldock & Thain, 1983), Portuguese oyster (C. angulata) (Phelps, 1990), and M. edulis from a marina area (Stephenson et al., 1986). Page et al., (1996) also detected that there was a correlation between TI of blue mussels with tributyltin concentration in mussels tissue. However, the mechanism by which, tributyltin creates anomalies occurring in the shell calcification is still unknown. The altered mechanism of calcification resulted in shell anomalies at TBT concentrations as low as 2 ng/l (Chagot et al., 1990). Alzieu, (1991) found that the organotin compound created oyster shell abnormalities consisting of water-like chambering with the formation of interlamellar jelly. The observation by Krampitz et al., (1983) revealed that gelatinous contents of the chambers differed from normal organic matrix protein in having lower levels of aspartic acid, glycine, and serine, and a higher level of threonine. This lead to the hypothesis, that the target of tributyltin could be the organic matrix. This hypothesis agrees with the results of Allemand et al. (1998) who demonstrated that the primary target of tributyltin in calcifying animals is protein synthesis, whose inhibition leads to the inhibition of organic matrix and consequently creates malicious calcification. Likewise, TBT used to expose the Hooded oyster Saccostrea cucullata, disrupts the calcium homeostatic process through the VDAC-3 protein present in the mantle and this is considered to be a key mechanism of TBT in disrupting shell calcification with the result of bivalve shell deformity (Khondee et al., 2016).

3.2. DNA Damage

The red dye EOSIN-Y was used to test the viability of the cells. Using this dye the viability of the cells was consistently above 90 % for all treatments.

After electrophoresis, the presence of DNA strand breaks causes a fraction of DNA to migrate from the nucleoid core toward the anode by forming a comet (Singh et al., 1988). DNA damage expressed as DNA fragments migrating to the tail region were measured by tail length and olive tail moment as comet parameters.

In this study, the expected unpolluted site was used as a control and compared by other in situ experiments, polluted area, and transplantation experiments, an in vitro experiment, TBTC (10 µg/l), and H2O2 (10 µM) exposure. The comet did not follow a normal distribution (Anderson et al. 1996), and the data were transformed using arcsinh transformation procedure to stabilize these. The transformed data were analyzed by one-way ANOVA to show if there were different means among the experiments and proceed to post hoc test using the Bonferroni procedure. The level of DNA damage in gill cells of blue mussels by various exposures was shown in Fig. 4, 5, and Table 2. The results of One-way ANOVA showed that there was a significantly different mean among the exposures (p < 0.01).

Table 2. Comet parameters, tail/length, and tail olive moment (mean ± SEM) in blue mussel gill cells from different experiments. Comparison between clean areas as controls and the treatments (transplantation, polluted area, TBTC, and H2O2).

| Experiments       | DNA damage               |
|-------------------|--------------------------|
|                   | Tail length  | Tail olive moment |
| Clean             | 28.0093 ± 0.0768        | 2.2788 ± 0.0727   |
| Transplantation   | 42.8006 ± 0.0948**      | 4.3695 ± 0.1070** |
| Polluted          | 108.2138 ± 0.0669**     | 12.9196 ± 0.0792**|
| TBTC (10 µg/l)    | 95.8958 ± 0.0703**      | 11.4798 ± 0.0854**|
| H2O2             | 109.1630 ± 0.0752**     | 20.1105 ± 0.0928**|

** p< 0.01
Gill cells of blue mussels from clean water showed undamaged cells to mainly low with a small number of cells with considerable DNA damage. Relatively small increases in the number of cells damaged and the extent of DNA damages were detected in blue mussels that were transplanted to the polluted site for one month. The level of DNA damage and the number of cells damages extended exceedingly for blue mussels from the polluted site and blue mussels exposed to TBTC (10 µg/l). The effect of TBTC leading to DNA damage in this study agrees with Gabbianelli et al., (2002) who demonstrated that TBTC led to a significant increase in the extent of DNA damage as measured by three tail parameters (tail length, tail intensity, tail moment) and the number of cell damages. The coordination number and the nature of the organic group attached to the central tin atom determine the binding ability of organotin(IV) compounds to DNA (Devi & Pachwania, 2018).

The highest level of DNA damages and the number of cell damages were revealed by exposing gill cells of blue mussels to H$_2$O$_2$ (10 µM) as a positive control (Fig. 4, 5 & Table 2). Increasing the percentage of cell damages indicated as the percentage of DNA fragments in the tail has been pointed out in this treatment. Hydrogen peroxide (H$_2$O$_2$) has been frequently used as a positive control compound in many comet assay studies investigating its application to various cell types (Singh et al., 1988; Gajski et al., 2019; Odongo et al., 2019; Zegura & Filipič, 2019; Erikel et al., 2020; Muruzabal et al., 2021). This compound is considered to cause base modifications directly, particularly following the Fenton reaction to form the highly reactive hydroxyl radical (OH) (Halliwell, 2001; Madkour, 2019; Lelieveld et al., 2021). Mitchelmore et al., (1998) showed that the addition of H$_2$O$_2$ produced a significant concentration-dependent increase in the percentage of DNA in the comet tail.

In this study, both tail length and olive tail moment were considered as appropriate parameters for quantifying DNA strand breaks. The mean of DNA strand breaks expressed by two parameters showed a similar sensitivity at level p < 0.01 (Tabel 2). However, the Bonferroni procedure showed that the tail length endpoint did not reveal the difference in means among polluted sites, 10 µg/l of TBTC and 10 µM of H$_2$O$_2$ exposure. On the other hand, in terms of olive tail moment polluted site and 10 µg/l of TBTC exposure revealed a similar degree of DNA damages, whilst the effect of H$_2$O$_2$ (10 µM) on gill cells of blue mussels shown increasing significantly in DNA damages level (Table 2). In many cases, tail moment and olive tail moment are more precise as endpoints than tail length, because these parameters consider not only the length of comet tail but also the intensity of DNA in the tail and the mean distance of DNA migration in the tail (Olive et al., 1990; Dutta & Bahadur, 2019; Cui et al., 2021; Gajski et al., 2021; Rajendran et al., 2021).

The extent of DNA damage taken place along with an increasing number of gill cell damages of transplanted blue mussels described that transplanting method could be used for assessing the impact of such geno-toxicants like tributyltin to marine animals. It was also explained that transplanting mussels facilitated measuring biomarkers like DNA damage and made this tool more flexible when used for manipulative field testing and hypothesis testing (Bolognesi et al., 2019; Gecheva et al., 2020; Perovic et al., 2020; Dos et al., 2020; Muruzabal et al., 2021).

Figure 4. The level of DNA damage of blue mussel gill cells from different exposure.

Figure 5. Distribution of DNA damage based on percentage DNA in the comet tail of blue mussels gill cells in different treatments.
Santos et al., 2022; Sussarellu et al., 2022). Despite that the level of DNA damage in blue mussel gill cells for this experiment was not more than those in gill cells of blue mussels taken from the polluted site directly, the result also described the time of transplanting exposure was appropriate for exposing mussels in the in-situ experiment. A field study by taking mussels from the polluted site directly described that DNA damage of gill cells was more severe than those from transplanting experiments and expected unpolluted sites. D’costa et al., (2018) showed a relationship between DNA single-strand breaks and environmental contaminants in the field. This result suggested that Norsminde fjord harbor contains potential genotoxicants. In addition, the results depicted that there was no difference in means between the levels of DNA damage in term olive tail moment in gill cells when taken from polluted site as when exposed to TBT (10 µg/l). On the other hand, the extent of DNA damages in gill cells when exposed to H$_2$O$_2$ (10 µM) differed from those exposed by TBT (10 µg/l) and taken from the polluted site. However, it is uncertain from this study to suggest that the main genotoxicant in Norsminde Fjord harbour which induced DNA damage on gill cells of mussels was tributyltin. This suggestion was needed to evaluate tributyltin contain and other potential genotoxicants in the water column, sediment, and mussel tissues, which was not performed in this study. Moreover, the results indicated that DNA damages on gill cells exposed by TBT (10 µg/l) have a similar character, in the light of the olive tail moment, with those of blue mussels taken from the polluted site. It might indicate that the effect of TBT on these gill cells was similar to the effect of genotoxicants contained in the harbour.

Evaluation of the genotoxic potential of tributyltin on marine invertebrates like blue mussels using comet assay could give important information on the DNA alteration induced by this compound. Tributyltin has been observed to cause programmed cell death or apoptosis in human cell lines and tissue of marine sponge, Geodia hydronium (Ratel et al., 1993). Apoptosis was also detected in M. galloprovincialis exposed to tributyltin chloride (Mićić et al., 2001). The study showed that in TBT-treated mussels, DNA fragmentation and degradation to higher-order DNA structures appeared to be the critical lesions responsible for the induction of apoptosis. Cytogenetic damages (sister chromatid exchanges and chromosomal aberration) in larvae of blue mussel induced by TBT have also been evaluated (Jha et al., 2000). In addition, a study on rainbow trout erythrocytes using comet assay showed that TBT has a profound genotoxic effect, whilst DBT (dibutyltin chloride) creates lesser DNA damage and DNA damage is completely absent for MBTC (monobutyltin chloride) (Tiano et al., 2001). Likewise, a study on the adverse effects of different organotin compounds on DNA of Sparus aurata (freshwater fish) erythrocytes using comet assay showed different genotoxicities among TBT, DBTC, and MBTC (Gabbianelli et al., 2002). TBT and DBT have pronounced effects on tail length, tail intensity, and tail moment, though TBT is more efficient in producing DNA damage. MTBC caused a fast genotoxic effect that did not change with incubation time.

The current study has evaluated the genotoxic effect of TBT, and other pollutants both in situ and in vitro that caused DNA damage on blue mussel gill cells. However, the cause of the DNA damage is not yet established, especially for TBT.

DNA damage induced by TBT may act through an indirect mechanism by perturbation of Ca$^{2+}$ homeostasis. It has been reported that intracellular Ca$^{2+}$ was increased by 10–5 M TBT exposure in Ciona intestinalis (Urochordata) ovaries (Puccia et al., 2001), which played an important role in inducing DNA cleavage in apoptotic cells. Another possibility is a perturbation of mitochondrial activity. It is possible that TBT disturbance of the mitochondrial proton gradient could divert electrons from the respiratory chain, leading to the formation of reactive oxygen species (Puccia et al., 2001). In gill cells of M. edulis (Wilson et al., 1998), Littophaga littophaga (Essawy et al., 2021), Donax incarnatus (Dias et al., 2021), and M. coruscus (Xu et al., 2018), oxidative DNA damage generated by reactive oxygen species has been attributed to the high and variable DNA damage.

4. Conclusion

The results of this study demonstrated that deformity has been detected on blue mussels living in Norsminde fjord harbor that might be caused by a mixture of genotoxicants, probably dominated by tributyltin. The evaluation of DNA damage using comet assay is a simple and effective method to assess the existence of genotoxicants in both in situ and in vitro experiments. Therefore, the results obtained are important for assessing genotoxicants that produced a severe impact on marine animals. Further studies on tributyltin contained in the environment and tissue of animals are requested to specify which genotoxicant causes an exact severe impact on the animals since this assay is an unspecified but versatile tool for detecting the presence of genotoxicants.

Acknowledgement

This study was supported by The Danish Agency for Development Assistance (DANIDA). I would like to thank Vibeke Simonsen and Janeck Scott-Fordsmand for the useful guidance and discussion on this study. Thank Hans Løkke the director of the National Environmental Research Institute (DMU), Denmark for providing a laboratory to conduct comet assay analysis. I also thank Anni Christiansen for kindly helping during laboratory work.

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How to cite this article:
Yaqin, K. 2022. DNA damage and shell malformation in Blue Mussel, Mytilus edulis. Akuatikisle: Jurnal Akuakultur, Pesisir dan Pulau-Pulau Kecil 6(1): 65–74. https://dx.doi.org/10.29239/j.akuatikisle.6.1.65-74