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REVIEW ARTICLE

Bivalves as Biomonitors of Genotoxicity: A Review

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ABSTRACT

Bivalves, particularly mussels, are already known to be valuable bio-monitors of aquatic ecosystem health. Their ecological and biological characteristics make them ideal sentinels of genotoxic pollutants in aquatic ecosystems. Direct chemical analyses of polluted samples are reported to be limited by sensitivity and cannot predict the toxicity of complex waste mixtures. However, studies exploring the use of genetic assays with other relevant organisms for monitoring the effects of these pollutants are limited. Therefore, biomonitoring can quantify specific pollutants in contaminated sites and their chronic effects on the aquatic ecosystem. This review essay provides a synthesis of the current state of knowledge on the use of bivalves as bio-monitors using genotoxic endpoints, such as DNA damage and micronuclei formation, and explores relationships between such biomarkers and exposure to pollutants such as heavy metals, pesticides, and industrial effluents. While some limitations exist, mussels hold significant potential for ecotoxicology research, especially when combined with analytical techniques like biochemical, histological, and physiological biomarkers. Additionally, it's crucial to consider the impact of natural environmental factors on the studied parameters when analysing the results.

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INTRODUCTION

In recent decades, the pollution of freshwater bodies has increased significantly. Not only has the amount of contaminants increased, but so has their diversity. Various chemicals (pesticides, PCBs, PAHs, HMs, petroleum products, etc.) adversely affect organisms and ecosystems even at very low concentrations (Klimova *et al.*, 2020).

At the same time, the generally accepted physicochemical methods for qualitative and quantitative analysis of water pollution do not reflect the impact on biota. Therefore, new approaches to assessing aquatic organisms' response to exposure to pollutants and methods for evaluating the state of the aquatic environment based on it are needed (Klimova *et al.*, 2020). In general, mussels are either floating or sediment feeders or may even use both feeding methods. They usually feed on microalgae, bacteria, and detritus via filter-feeding. During filter-feeding, they inhale water from the posterior ventral side through an inhalation siphon, and water passes through the gills and is expelled through an exhalation siphon. Through this process, they filter large amounts of water, with water filtration capacities of typical wild mussel beds calculated to be 7–12 m3m-3 h-1 (Krishnakumar *et al.*, 2018). A single adult mussel pumps approximately 50 ml of seawater per minute when actively feeding (Lüskow & Riisgård, 2018). Because mussels filter large amounts of water, their tissues absorb some of the impurities and food particles present in the water) to accumulate trace metals (El-Din *et al.*, 2018). Historically, mussels have been considered valuable aquatic organisms for environmental monitoring and used as biomonitors for chemical contamination of coastal waters (Kimbrough, 2008). Mussels also resist various contaminants, allowing them to thrive in highly polluted environments. These characteristics make it a candidate species group for biomonitoring programs worldwide. Mussels have been reported to accumulate trace metals in their tissues at 100- to 100,000-fold higher levels than concentrations observed in the seawater where they live (Benson *et al.*, 2017). Therefore, some chemical contaminants, including trace metals, are present in undetectable amounts in seawater and can be detected in mussel tissue. Various species of mussels, mussels, and oysters are widely distributed across the continent, and many of these species have been used to monitor levels of pollutants in the environment (Farrington *et al.*, 2016).

This review aims to analyse and generate information on mussels' use as molecular pollution biomarkers.

METAL BIOACCUMULATION IN BIVALVES

Metal pollution is a significant environmental concern that affects aquatic ecosystems worldwide (Huang *et al.*, 2020).

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Metals such as cadmium, lead, mercury, copper, and zinc are essential components of many industrial processes, but they can also have devastating effects on the environment and public health (Huang *et al.,* 2020). These metals can accumulate in aquatic organisms, including bivalves, and cause harm to humans who consume contaminated seafood due to their persistent nature (Rajaganapathy *et al.,* 2019).

Bivalves, such as mussels and oysters, are ideal organisms for monitoring environmental metal levels. They are sedentary, allowing researchers to monitor metal levels in a specific location (Santos *et al.,* 2020). They are also bioaccumulators, meaning they can accumulate metals from the surrounding water and sediment, allowing researchers to analyse environmental metal levels (Zhang *et al.,* 2020).

The process of using mussels as bioindicators involves several steps. Firstly, mussels are collected from the environment and transported to the laboratory. Secondly, the mussels are analysed for heavy metals using techniques such as atomic absorption spectroscopy or inductively coupled plasma mass spectrometry (Zhang *et al.,* 2020). Finally, the data are interpreted to determine the levels of metal pollution in the environment.

Several studies have demonstrated the effectiveness of using bivalves to monitor environmental metal levels. For example, a study by Li *et al.* (2020) used mussels to monitor metal levels in the coastal waters of China. The study found that mussels accumulated high levels of copper and zinc, indicating high pollution levels in the area. Another study by Kumar *et al.* (2020) used oysters to monitor metal levels in the coastal waters of India. The study found that oysters accumulated high levels of lead and mercury, indicating high pollution levels in the area.

In addition to monitoring metal levels, bivalves can also be used to assess the effects of metal pollution on aquatic ecosystems. For example, a study by Wang *et al.* (2020) used mussels to assess the impact of copper pollution on aquatic ecosystems. The study found that copper pollution caused significant changes in the community structure of aquatic organisms.

Bivalves accumulate both essential and non-essential metals in their soft tissues above the background levels in seawater or sediments, and this process is called bioaccumulation. Bioaccumulation is a good integrative indicator of the chemical exposures of organisms such as bivalves in polluted waters (Geng *et al.*, 2019). For mussels, the highest metal concentrations are reported in digestive glands and/or gill tissue, followed by mantle and muscle tissue (Krishnakumar *et al.*, 2018).

BIOMARKERS OF EXPOSURE

Chemical analysis of mussel tissue samples measures the contaminants present but does not always reveal potential biological effects on the mussels. Therefore, biomarkers have been developed to assess the health status of aquatic organisms, especially mussels. Biomarkers are early warning signs of health in mussels exposed to toxic contaminants. This is because poisonous effects or reactions become visible at the molecular or cellular level before becoming noticeable at higher biological levels (Krishnakumar *et al.*, 2018). The biomarker concept describes measurable indicators such as blood cholesterol profiles relevant to clinical endpoints such as atherosclerosis and myocardial infarction (El-Din *et al.*, 2018). Over the past decade, several biomarkers sensitive to pollutant exposure and effects have been developed as tools for environmental monitoring and risk assessment (Khan, Ho & Burgess, 2020). Biomarkers based on physiological, cellular/tissue, and molecular responses in mussels have been developed to study contaminants' effects on field and laboratory-exposed mussels, especially mussels. Research on developing and applying precise biomarker-based monitoring tools for environmental contaminants has intensified in several developed countries, using several bivalve-based biomarkers to detect environmental changes in coastal and estuarine water environments (Beyer *et al.*, 2017).

BIOMARKERS OF GENOTOXICITY

Various chemical contaminants enter the aquatic environment that can directly or indirectly damage an organism's DNA. These genotoxic chemicals can cause some changes in mussels' molecular and cellular levels (Jha, 2004; Bolognesi and Cirillo, 2014). Two well-known tests, the micronucleus test and the comet test, are commonly used to assess the genotoxic effects of environmental contaminants on mussels (Jha, 2004; Bolognesi and Cirillo, 2014). The micronucleus assay detects structural and numerical chromosomal alterations, and the comet assay (single-cell gel electrophoresis) detects DNA strand breaks in mussels (Bolognesi and Cirillo, 2014). *Salmonella typhimurium* Assay, another well-known assay, the Microbial Ames Test, is a simple, rapid, and robust bacterial assay for different strains and applications of *Salmonella typhimurium*/*E. coli* determines the mutagenic potential of environmental samples, pharmaceuticals, dyes, cosmetics, etc. (Vijay *et al.*, 2018).

Comet Assay

Single-cell gel (SCG) or comet assay is more helpful for assessing DNA damage. The comet assay is a technique for quantitative DNA damage and repair in eukaryotic and some prokaryotic cells. This technique is more advantageous because it detects minor DNA damage, requires very few cells, is cheaper than other techniques, is easier to perform, and yields better results. However, the mechanisms of genotoxic effects and the chemical components responsible for harm have not been identified (Rajinder, 2018).

Ostling and Johanson (1984) first developed a microcell gel electrophoresis method for detecting DNA damage at the single-cell level. Subsequently, Singh *et al.* (1988) introduced a microcell gel technique involving electrophoresis under alkaline (pH>13) conditions to detect DNA damage in single cells. The alkaline comet assay can detect a wide variety of DNA damage, such as DNA single-strand breaks, double-strand breaks, oxidatively induced base damages, alkali-labile sites, and sites undergoing DNA repair (Meng *et al.*, 2020).

The suitability of an in vitro version of the comet assay using primary hepatocytes and zebrafish (*Danio rerio*) gill cells was established. As part of an 18-month biomonitoring study, the genotoxic potential of water samples taken from various locations along the great rivers Rhine and Elbe in Germany was identified using primary cells and was evaluated for its sensitivity and practicality. A significant difference was found in the number of genotoxic surface water samples (Schnurstein and Braunbeck, 2001).

Genetic damage, expressed as single-strand DNA breaks, in cells isolated from the gills, hemolymph, and digestive glands of the clam *Tapes semidecussatus* was tested using the comet assay. During the three-week study after the mussels were exposed to sediment, significant differences in DNA damage for each tissue type were noted for mussels exposed to two sediment samples from different sources (Osman, 2014).

In common carp (*Cyprinus carpio*), the genotoxicity of water and sediments collected from the Noyyal River was investigated using the Alkali-His comet-His assay. After exposure to contaminated water samples, DNA damages were observed in fish red blood cells, kidneys, and liver cells. The damage degree is directly related to the exposure time. The highest DNA damage was obtained in samples collected downstream from urban centers. The results showed that the Noyyal River system was contaminated with genotoxic agents, and the comet assay was sensitive enough to detect genotoxicity (Kaur *et al.*, 2018).

An evaluation was conducted to determine whether pesticide runoff was associated with river genotoxicity in native fish, using DNA strand breaks selected as genotoxicity biomarkers for the study. The study results showed that DNA strand breaks were significantly higher in fish exposed to the San Joaquin River compared to nearby reference sites (Whitehead *et al.*, 2004).

The genotoxic potential of surface water treated with disinfectants for drinking water treatment was demonstrated by the comet assay applied to circulating erythrocytes of *Cyprinus carpio*. Genotoxic damage was seen in fish exposed to water disinfected with sodium hypochlorite and chlorine dioxide. Comet assay showed DNA damage directly induced in circulating erythrocytes when genotoxic lesions of head renal stem cells were expressed in circulating erythrocytes. Untreated surface water quality is the most important parameter for long-term DNA damage in circulating erythrocytes (Buschini *et al.*, 2004).

Siu *et al.* (2004) exposed green-lipped mussels (Perna viridis) to aqueous benzo[a]pyrene (B[a]P) for 12 days and monitored the relative levels of DNA strand breaks in mussel blood cells. The study showed increased B[a]P concentration increased strand scission.

The susceptibility of the widespread freshwater mussel *Corbicula fluminea* to the DNA-damaging alkylating agent methyl methanesulfonate (MMS) was investigated using the comet assay. Results indicated that *C. fluminea* is an optimal biomarker for measuring genotoxic contaminants in aquatic environments (Rigonato *et al.*, 2005).

The genotoxic potential of two widely used herbicides. 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2-chloro-2,6-diethyl-N-(methoxymethyl) acetanilide (butachlor) in erythrocytes of freshwater catfish *Clarias batrachus* were investigated. The comet assay results showed a significant increase in comet tail length. This demonstrates DNA damage at all concentrations of both herbicides compared to controls. The average comet tail length was determined as concentration versus time, as 2,4-D (9.59 mm) and butachlor (9.28 mm) recorded maximum tail lengths at maximum concentrations and longer durations. These results demonstrate that the comet assay applied to fish erythrocytes is a useful tool for determining the potential genotoxicity of water contaminants and may be suitable as part of surveillance programmes confirmed (Ateeq *et al.*, 2005).

DNA integrity in erythrocytes was assessed using an alkaline comet assay to study the effects of water pollution on the Balkan loach (*Cobitis elongata*) of the Sava and Kupa rivers. Results indicated genotoxicity in the aquatic environment of the Sava River and showed significantly less DNA damage in fish caught in the Kupa River (Kopjar *et al.*, 2008).

The genotoxicity of lead to the African catfish *Clarias gariepinus* was investigated using the comet assay. DNA strand breaks were observed when fish were exposed to different concentrations of lead nitrate. A strong correlation has been found between lead concentration, exposure time, and DNA strand breaks (Osman *et al.*, 2008).

After in vivo exposure of Prochilodus lineatus to various concentrations of cypermethrin as a chemical mutagen, the alkaline comet assay was applied to erythrocytes to assess DNA damage. Results showed significantly higher levels of DNA damage at all cypermethrin concentrations than controls. This technique has been proposed as a standard for one of the most common native fish species to aid in biomonitoring of genotoxicity in local polluted waters (Simoniello *et al.*, 2009).

The comet assay was used to detect multi-source genotoxicity in the peripheral blood of a native fish species (*Hyphessobrycon luetkenii*). Water samples were collected seasonally at three sampling points, and fish were evaluated under laboratory conditions. The results indicate that the Synos River, including the water bodies near the river, was contaminated with substances that are genotoxic to fish (Scalon *et al*., 2010).

Rainbow trout (RTL-W1) hepatocytes were exposed in vitro to acetone extracts of sediments collected from 10 selected points along the upper Danube and analyzed using the comet assay to determine whether sediment samples correlated with the genotoxic potential of the extracts in fish. This in vitro bioassay shows a strong correlation, demonstrating similar vitro genotoxic potential. Overall results on the ecological state of the Danube indicate a moderate to severe genotoxic potential, with widely varying localizations (Boettcher *et al*., 2010).

Genotoxicity was evaluated using the comet assay on hemocytes and gill cells of gasoline-exposed *Corbicula fulminea*. A significant DNA damage was seen in the blood and gill cells (Fedato *et al*., 2010).

The genotoxic potential of the Nile was monitored in detail using the comet assay of blood from fish collected both downstream and upstream. Significantly higher damages were observed in peripheral blood erythrocytes from Nile tilapia and African catfish collected from heavily contaminated areas. DNA damage in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) erythrocytes strongly correlated with downstream contaminant levels.

From all the above studies, the comet assay has proven to be a useful tool for studying the genotoxic effects of in vitro and in vivo exposures to chemicals on aquatic invertebrates and fish (Osman *et al*., 2014).

Micronuclei and Nuclear Lesions Tests

The micronucleus test was originally proposed by Heddle (1973) and Schmid (1975) as an alternative, simpler, and superior approach to assessing chromosomal damage in vivo. Due to its convenience and ease of application, the micronucleus (MN) test is one of the most widely used methods, especially in genotoxicological studies of aquatic organisms. Micronuclei arise from chromosomal fragments or whole chromosomes left behind during cell division due to centromere defects, damage to the centromere region, or defects in cytokinesis. These remaining fragments are incorporated into secondary nuclei called micronuclei (MNs). Micronuclei formation can occur in any of the dividing cells of any species. The number of micronuclei indicates chromosomal breaks and dysfunction of the spindle apparatus (Luzhna *et al*., 2013).

Micronucleus tests have shown several advantages over cytogenetic studies, such as sister chromatid exchanges and chromosomal aberrations. These are time time-consuming and ineffective in many aquatic species due to the relatively large number of very small chromosomes. The micronucleus test is a simple and sensitive assay for assessing genotoxic properties in the aquatic environment 'in situ, in vivo, and in vitro' as biomonitoring programs (Osman, 2014).

The brown trout *Salmo trutta*, the European eel *Anguilla anguilla*, and the European minnow *Phoxinus phoxinus* were evaluated as in vivo indicators of contamination using the renal erythrocyte micronucleus test. Field studies of wild freshwater ecosystems with varying contamination levels showed that micronuclei were induced in brown trout living in polluted sites (Rodriguez-Cea *et al*., 2003). Micronucleus testing is considered one of the most efficient approaches to assessing contamination (Fenech *et al*., 2003).

Evaluation of the genotoxic effects of *Oreochromis niloticus* in effluents from petroleum refineries and chromium processing plants was investigated using micronucleus assays. Results indicated that both effluents had genotoxic potential. The genetic damage caused by petroleum refinery effluents was significantly more significant than chromium processing effluents. Results further indicated that nuclear abnormalities other than micronuclei can be used as indicators of genotoxic damage (Çavas and Ergene Gozukara *et al.* 2005).

The genotoxicity of crude oil processed from the Stratford B platform in the North Sea was assessed by micronucleus assay in the gills of green mussels (*Mytilus edulis*). The increase in micronuclei gradually increased with increasing exposure time (Barsiene *et al*., 2006).

The genotoxic, cytotoxic, and immunotoxic potential of treated wastewater (TWE) discharged from the Vilnius sewage treatment plant was evaluated. The results showed a significant increase in micronuclei in exposed samples (Barsiene *et al*., 2006).

The abundance of micronuclei (MN) in gill cells of peripheral blood of mussels (*Mytilus edulis*), flounder (*Platychthys flesus*), and wrasse (*Symphodus melops*) collected in the North Sea region of Gothenburg was analyzed. A 10-fold higher frequency of micronuclei was detected in flounder collected in the contaminated area of Jordhammarvik, and an 8-fold higher micronucleus level in Nya Alvsborg fish in Gothenburg harbor was detected. For mussels, specimens living in the ringneck zone showed the highest response (Barsiene *et al*., 2006).

The frequency of micronuclei in blood cells of a native mussel, *Mytilus galloprovincialis*, collected along the eastern Adriatic coast of Croatia, was investigated. The maximum amount of MN was observed in summer, and the results indicated that seasonal variation was observed only in polluted sites. This was probably caused by seasonal pollution and the interaction of pollutants with mussels'

high metabolic and filtration rates, resulting in higher cytogenetic damage (Pavlica *et al.*, 2008).

Micronuclei (MN), nuclear blasts (NB), and fragmented erythrocytes in mature peripheral blood and immature erythrocytes of head kidneys of flounder (*Platichthys flesus*), dab (*Limanda limanda*), and cod (*Gadus morhua*) were studied in the Baltic and North Seas. The highest environmental genotoxicity is in areas close to oil and gas platforms in the North Sea and associated with large-scale transportation, subject to pollution from major European rivers (Elbe, Vistula, Oder).

The comet assay using blood, liver, and gill cells, the appearance of micronuclei (MN), and other erythrocyte nuclear abnormalities (ENAs) were used to assess the genotoxic potential of lead in vitro. Renal, segmental, and lobulated nuclei showed significant increases, indicating that the ENA is a better biomarker for lead exposure than MN after short-term exposure. The comet assay results performed in vitro on lead-exposed erythrocytes confirmed its genotoxic effect and showed that DNA damage increased with increasing exposure time.

The biomonitoring potential of micronucleus assays for water contaminants was evaluated. Interactions between such micronuclei formation and certain common environmental contaminants are then considered. A biomonitoring study detected six nuclear lesions (NL) along with micronuclei (MN) in the blood of Nile tilapia *Oreochromis niloticus* and African catfish *Clarias gariepinus* across the Nile. A higher incidence of MN and NL was found in fish blood collected from heavily contaminated areas. A simple progressive increase in MN and NL frequencies along the channel found in the Nile indicates that wild MN numbers are related to expected contamination levels when a contamination gradient is present along the channel. This result confirmed the utility of erythrocyte MN and NL as powerful surveillance tools for detecting genotoxic agents in freshwater environments (Obiakor *et al.*, 2012).

The genotoxic effects of toxic metals in cultivated and wild Nile cichlids, *Oreochromis niloticus*, and gray mullet, *Mugil cephalus*, collected from contaminated and uncontaminated reference sites, were evaluated. Heavy metal concentrations (Cu^{2+}, Zn^{2+}, Pb^{2+}, Fe^{2+}, Mn^{2+}) were recorded in water and sediment samples. Genotoxicity tests such as the micronuclei test (MN), DNA fragmentation test, and other nuclear abnormalities (NA) were analysed. A significant decrease in CF levels associated with significant increases in MN and NA frequencies was observed in fish from the contaminated area. In addition, mixed smearing and laddering of DNA fragments in gill and liver samples of both fish species collected in contaminated areas indicate a complex contamination status (Omar *et al.*, 2012). Similar results were reported by Yazici and Sisiman (2014) when studying European chub (*Leuciscus cephalus*) and Transcaucasian barbell (*Capoeta capoeta*) collected from contaminated sites in the Karas River.

A study from affected rivers in north-eastern Brazil investigated nuclear anomalies (NA) characterized by heavy metals and organic wastewater accumulation in four fish species. Two carnivores (*Serrasalmus brandtii* and *Hoplias malabaricus*) and two omnivores (*Oreochromis niloticus* and *Geophagus brasiliensis*) were collected in the Contas River basin over two seasons. Nuclear abnormalities (bulbs, double nuclei, lobes, micronuclei, notches, vacuoles) were found in all fish samples, species commonly found in local fish markets (Jesus *et al.*, 2016).

LIMITATIONS

The use of bivalves as bio-monitors for genotoxicity has several benefits. They can accumulate contaminants, are sedentary, and are relatively easy to collect and maintain in a lab setting. These characteristics make bivalves an essential resource for evaluating the genotoxic effects of pollutants in aquatic environments and guiding water quality management strategies. However, there are some drawbacks to using bivalves as bio-monitors. The comet assay detects DNA damage but does not identify the specific type of damage or the causative agent. This can make it difficult to determine the genotoxicity's source and develop effective mitigation strategies. Similarly, the Micronuclei test faces challenges in differentiating between micronuclei and other nuclear abnormalities, such as nuclear buds and nuclear fragments. These limitations emphasize the importance of carefully interpreting results and taking into account additional biomarkers and testing methods. Additionally, further research is recommended to develop new biomarkers and testing approaches that can address these challenges.

CONCLUSION

Research findings suggest that mussels can be incredibly useful in ecotoxicology investigations. They are ideal as model organisms because they can quickly accumulate toxins. This makes it possible to promptly identify any negative impacts of exposure and utilize these biological models as an early warning system for water pollution. Nevertheless, it is important to take into account the influence of natural environmental factors on the parameters being studied when interpreting the results.

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