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Bivalve haemocyte based inexpensive biomarkers in aquatic environment monitoring: scopes and challenges

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ABSTRACT

The aquatic environment of this planet is constantly being challenged by pollutants from known and unknown sources which render threat to its biodiversity and bioresource. These natural waterbodies are densely populated with molluscs of diverse species, including bivalves. Bivalves are recognised as sentinel species in major global environment biomonitoring programme owing to their ability to accumulate aquatic pollutants from surrounding environment. Development and deployment of inexpensive biomarkers based on the circulating haemocytes of these molluscs is a popular and reliable biomonitoring approach, widely adopted by the western world.Data on haemocyte density profile,haemocyte functions, like, lysosomal stability, phagocytic efficiency, generation of reactive oxygen/nitrogen intermediates and haemocyte nuclear abnormalities are documentedto assess the species-specific reactions to pollutants.Such data is consulted to formulate effective environment biomonitoring and bioremediation management strategies against potential and actual xenobiotic insults. The present review work focuses on environmental monitoring against aquatic pollution with the help of haemocyte profile of bivalve as biomarker. It further reviews the scope of deployment of the concept for effective and sustainable bioremediation.

Introduction

Pollution is a major threat to the aquatic habitats causing the massive degradation of the biodiversity and bioresource(Naiman and Turner, 2000; Jackson et al., 2001; Malmqvist and Rundle, 2002; Rachel, 2002). The aquatic ecosystem throughout the world is experiencing dynamic stress mediated by diverse pollutants of natural and anthropogenic origin. It has attracted global attention which has escalated the efforts to monitor the environmental conditions through the development and deployment of selective biological and ecological measurements, or indicators. Biological indicators are thus considered as useful means to obtain effective information about the condition of an ecosystem. Bivalves are common aquatic invertebrate organisms widely considered as bioindicators in environment monitoring (Sanders, 1993; Chakraborty et al., 2012; Burgos-Aceves and Faggio, 2017). As filter feeders, these species are known to be ideal indicators of chemical contamination (Fournier et al., 2001; Guidia et al., 2010). Filter-feeding bivalves can accumulate chemical contaminants in tissues,

several times higher than those in the waterbody (Chakraborty et al., 2010; Bolognesi and Fenech, 2012). Chemical analysis of bivalve tissues can thus be used to describe contamination profiles for different sites (Al-Subiai et al., 2011; Politakis et al., 2018). This exposure monitoring approach was used in northern Europe and the United States under the International Mussel Watch Program (National Academy of Sciences, 1980), and continues as the National Mussel Watch Program conducted by the National Oceanographic and Atmospheric Administration. The measures of bivalve mollusc defence activities, such as haemocyte density, lysosomal stability, phagocytic activity and production of cytotoxic molecules have potential to be established as indicators and appear to be responsive to xenobiotic insults in the aquatic environment (Oliver and Fisher, 1999; Nicholson, 2001; Sauve et al., 2002;Guidia et al., 2010;Patetsini et al., 2013). It has been widely accepted that the measurement of physiological and biochemical responses of individual bivalves may be used as indicators of the health of the larger population and community (Bayne et al., 1980; Guidia et al., 2010). Such measurements are termed 'biomarkers' (McCarthy and Shugart, 1990; Huggett et al., 1992; Patetsini et al., 2013) and the overall impact of a specific pollutant on multiple biomarkers have been designated as the integrated biomarker responses (IBRs) (Beliaeff and Burgeot, 2002; Cao et al., 2018).

Bivalve Haemocyte

Haemocytes are the cells of the circulating haemolymph present in the haemocoel of the molluscs. In bivalves, the internal defence system is based on the structural and functional integrity of haemocytes which display phagocytic and microbicidal activities (Cooper and Knowler, 1992; Burgos-Aceves and Faggio, 2017). They act as the major immune effector cells (Cheng, 1977; Adema et al., 1991b) and mediate non-self phagocytosis that provides natural immunity in the bivalves (Lopez et al., 1997a,b). They also remain associated with a variety of physiological and pathological functions including nutrient transport, digestion, wound and shell repair, internal defence as well as excretion (Cheng, 1981; Bayne, 1983; Fisher, 1986;Glinski and Jarosz, 1997). In molluscs, bivalves in particular, haemocytes represent the major component of their immune system although their types (Figure 1)andspecific functions are not fully understood (Table 1). However, substantial information is with haemocytes of bivalves (Cheng, 1984; Adema et al., 1991 a,b; Jing and Wenbin, 2003). Cheng (1975) has demonstrated that apparently different types of haemocyte (hyalinocyte or granulocyte) may actually represent the same type of cell during different functional or maturational stages. However, it is not clear if the variety of haemocytes described in the literature represents distinct cell lineages, or due to the differences in the maturation and/or physiology of the haemocytes, or variations in the techniques being applied (Cheng, 1975). Bivalve haemocytes are believed to be responsible for the transport of contaminants from the organ of entry (e.g. gill, mantle, digestive gland) to the kidneys or other tissues where detoxification

or accumulation may occur (Pirie et al., 1984). Alterations of the immunosurveillance have been reported for bivalve molluscs exposed to metals (Cheng and Sullivan 1984; Pipe et al.,1999) and xenobiotics (Fries and Tripp, 1980; Beckmann et al., 1992; Cimaet al.,1998). It has been established that the efficiency of haemocytes may be affected by environmental contaminants (Anderson et al., 1988; Canesi et al., 2003). Xenobiotics may alter functional profiles in molluscan haemocytes, such as phagocytosis (Fries and Tripp, 1980; Anderson, 1988; Cimaet al., 1998), lysosomal enzyme activity and lysosomal membrane stability (Lowe et al.,1995; Grundy et al.,1996; Nicholson, 2001; Sauve et al., 2002; Guidia et al., 2010; Patetsini et al., 2013). Consequently, toxic effects on haemocytes potentially affect the survival of these animals. Haemocytes act as the major immune effector cells in invertebrates including molluscs (Cheng, 1977; Adema et al.,1991b). Characterisation of haemocyte function is an important step towards understanding their immune capacity and its potential failure during toxic exposure and disease development.

Haemocyte Diversity and Count

Morphological analyses and functional consequence of molluscan blood cells under toxic exposure is poorly understood. Leydig (1850) studied circulatory haemocytes of mollusc and provided the basic information in understanding the function of haemocyte in healthy state, disease condition and toxic exposure. An ideal analysis of haemocyte population dynamics involves the simultaneous approach to a series of parameters throughout the animal's life, namely, total haemocyte count (THC) and differential haemocyte count (DHC) (Shapiro, 1979; Arnold and Hinks, 1976; Liu and Zhao, 2018). Marked variations in the density of haemocytes may be related to irregular haemocyte release from haemocytopoietic organs into the open circulation (Crossley, 1975). Detoxification and phagocytosis have been attributed primarily to granular haemocytes and the proportion of this cell type is reported to be elevated in polluted environment (Pirie et al. 1984; Liu and Zhao,

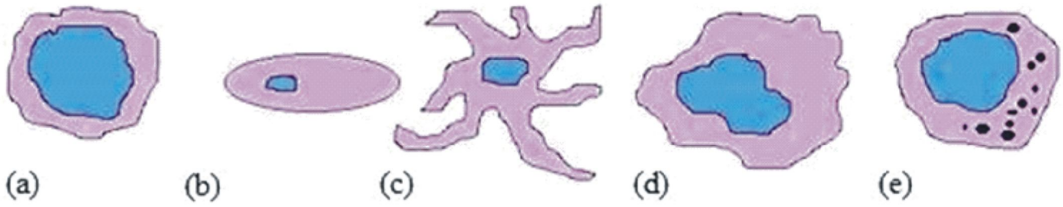

Figure 1. Illustration onthe variety of circulating haemocytes in the bivalve molluscs **(a)** blast like cell or pro-haemocyte **(b)** hyalinocyte **(c)** spreading haemocyte or asterocyte **(d)** agranulocyte **(e)** granulocyte [Source: Illustration is author's own creation]

Haemocyte types	Characteristics	Reference
Blast like cell	i. Small cells, non-spreading in nature ii. Often designated as the pro-haemocytes and molluscan undifferentiated/stem cells	Caraballal et al., 1997; Hine, 1999;Cima et al., 2000;Martin et al., 2007; Chang et al., 2005; Chakraborty et al., 2008
Agranulocytes	i. Cells are large and have ovoid to round nuclei ii. Cytoplasm with scarce secretory granules	Auffret 1988; Chang et al., 2005;Martin et al., 2007; Chakraborty et al., 2008;
Hyalinocytes	i. These cells are ovoid in shape ii. Pale hyaline cytoplasm with small and distinct nucleus iii. Cytoplasm with scattered secretory granules iv. High phagocytic ability and reactive oxygen intermediate (ROI) and nitric oxide (NO) generation	Hine, 1999; Caraballal et al., 1997; Chang et al., 2005; Martin et al., 2007; Lambert et al., 2007; Liu and Zhao, 2018
Granulocytes	i. Cells have variable in size and shape - spherical or oval ii. Small nucleus with granular cytoplasm iii. High phagocytic ability ROI generation	Cheng, 1981; Caraballal et al., 1997; Hine, 1999; Martin et al., 2007; Liu and Zhao, 2018
Asterocytes or spreading haemocytes	i. Cells are spreading and variable in their morphology ii. Projects pseudopodia/filopodia iii. Cytoplasm contains few granules	Hine, 1999; Chang et al., 2005; Mahilini and Rajendran, 2008**;** Chakraborty et al., 2009

Table 1. The structural and functional attributes of the molluscan haemocyte as bio-indicators under the influence of diverse environmental challenges.

2018). An understanding of the types of haemocytes in molluscs is essential in studying basic cell responses to environmental changes (Fisher et al., 1989; Pamparinin et al., 2002), handling of the animals (Ballarin et al., 2003; Malham et al., 2003); and infections (Canesi et al., 2002; Cochennec-Laureau et al., 2003). In freshwater ecosystem, bivalves are dominant filter-feeders that exert control over ecosystem structure and function (Strayeret al., 1999). Considerable data obtained from controlled exposures have demonstrated that oyster defence activities do respond to anthropogenic chemicals such as heavy metals. Cheng (1988 a, b) reported lower percentage of hyalinocytes in oysters exposed to 1 ppm copper sulphate and significantly higher percentage of hyalinocytes in oysters exposed to 1 ppm of cadmium chloride. Coles et al. (1995) reported a significant increase in circulating haemocyte numbers in mussels *Mytilusedulis*resulting from exposure to 400 ppb of cadmium for 7 days. Haemocytes of freshwater zebra mussels (*Dreissenapolymorpha*) exposed to lead and zinc contained enlarged and/or more numerous lysosomes compared with controls (Giamberini and Pihan, 2005). Exposure to 40 ppb of cadmium suppressed the release of degradative enzymes from the haemocytes during phagocytosis. Mussels (*Mytilusedulis*) exposed to copper also had increased granular blood cells by factors of three to four fold over unexposed controls (Pickwell and Steinert, 1984). Shift in haemocyte count of bivalves on interaction to pollutants and environmental cue are often found to be species-specific (Chakraborty et al.,

2008; Matozzo et al., 2010) while differential sensitivity of the haemocytes of clam *Chameleagallina*and the mussel *Mytilusgalloprovincialis*to acidification and temperature alterations are in report (Matozzo

of lysosomal membranes may cause undesired release of hydrolases into the cytosol, resulting consequent damage of self-cells (Lowe et al.,1995). Lysosomal hydrolytic phosphatase enzymes remain compartmentalised within

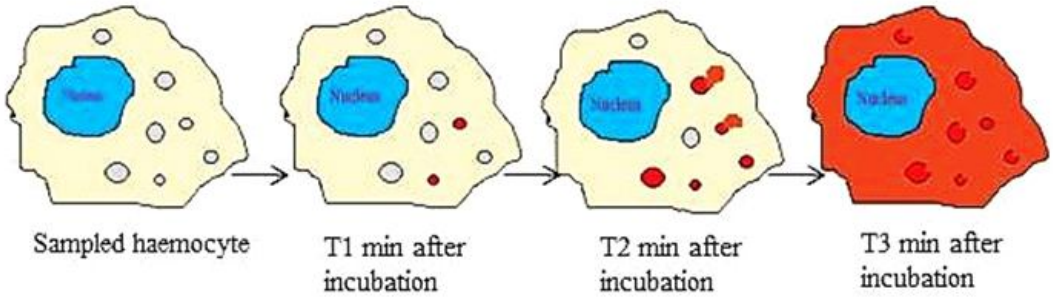

Figure 2. Schematic representation of the method of analysing the lysosomal stability of haemocyte following the principle of linkage of neutral red cationic probe from the lysosomes (T-time interval) [Source: Illustration is author's own creation]

et al., 2012). Total and differential haemocyte count of the freshwater mussel *Anodonta woodiana* has been examined to analyse the water quality of river and waterbodies in Taiwan (Wijayanti *et al.*, 2018).

Lysosomal Stability of Haemocyte

Lysosomes play an important role in the immune responses of bivalve molluscs. On phagocyte stimulation, lysosomal hydrolases are released out of cells to degrade foreign materials (Mohandas et al.,1985) or into phagosomes, thus participating in the degradation of internalized foreign particles (Cheng, 1981). It is known that the haemocytes of bivalve molluscs may accumulate high levels of metals, mainly in lysosomes (Moore, 1990; Bordin *et al.*,1996). Alteration of the integrity

electron-dense specific granules of haemocytes and granular cells (Pipe, 1990). Cytochemical studies have demonstrated the occurrence of several lysosomal enzymes associated with the cytoplasmic granules in haemocytes of several bivalve molluscan species (Moore, 1985; Gelder and Moore, 1986). Degranulation is associated with the release of lysosomal enzymes in the serum during phagocytosis which is vital for maintenance of tissue homeostasis (Cheng and Dougherty, 1989).

The neutral red retention assay (NRR) is a useful technique applied for monitoring the alterations in the permeability of the lysosomal membrane caused by environmental pollutants (Figure 2.). It has been recognised as a sensitive indicator to estimate and assess the period of

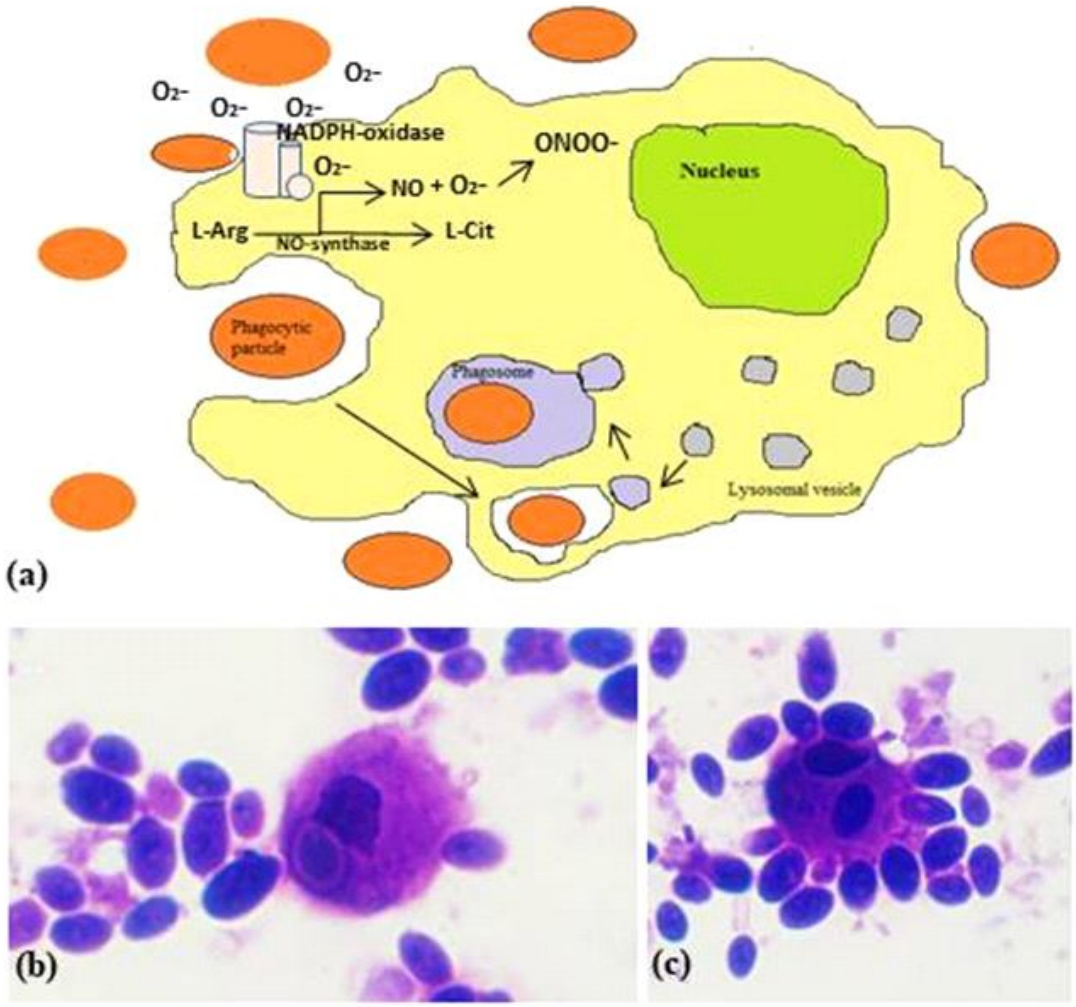

Figure 3. The event of phagocytosis of foreign particles by haemocytes is coupled with the generation of reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI) **(a)** illustration representing the process of phagocytosis by a haemocyte mediated by its lysosomes supplemented with the generation of ROI (O_2^-) and RNI (NO, $ONOO^-$) with the assistance of the membrane bound NADPH oxidase system and nitric oxide synthase enzyme **(b, c)**the phenomena of phagocytosis by the granulocytes of a freshwater bivalve *Lamellidensmarginalis*when experimentally challenged with yeast particles. [Source: Illustration (a) is author's own creation; photographic plates (b, c) are author's own unpublished work]

contaminant exposure (Fernley et al., 2000; Nicholson, 2001; Guidia et al., 2010; Matozzo et al., 2012). Reports suggest that the NRR assay is least affected by natural factors, like temperature and salinity, but is mainly influenced by pollutants (Ringwood et al.,

1998). This assay has been applied to several studies to examine the effects of diverse toxins (Fernley et al., 2000; Wedderburn *et al*., 2000) and heavy metals (Svendsen and Weeks, 1995). A reduction in NRR time under exposure to xenobiotics was observed in similar kind of studies conducted on molluscs reared in the laboratory (Lowe *et al*., 1995; Chakraborty and Ray, 2009) as well as specimens from natural habitat (Fernley *et al*., 2000). A reduction in lysosome membrane stability has been reported in mussels and oysters exposed to heavy metals (Regoli,1992; Ringwood et al.1998), pesticides (Patetsini *et al*., 2013) and expired drugs (Politakis *et al*., 2018).

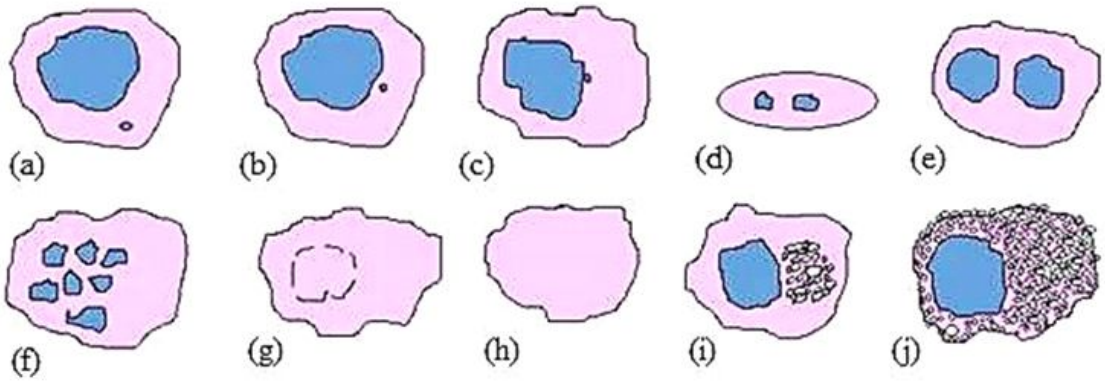

Figure 4. Illustration depicting the different types of nuclear anomalies and cellular damages as observed in the haemocytes of molluscs **(a, b)** micronuclei formation **(c)** nuclear bud formation **(d, e)**binucleation **(f)** nuclear fragmentation **(g, h)** karyolysis or nuclear dissolution **(i, j)** necrotic agranular haemocyte. [Source: Illustration is author's own creation]

Phagocytosis and Generation of ROI/RNI

In cell-mediated immune responses, non-self phagocytosis by circulating haemocytes (Figure 3) is one of the main defence reactions against pathogens and foreign materials (Cheng, 1981). Phagocytes are supposed to be ancient immune defence cells which are widely distributed throughout the body of multicellular animals (Bayne *et al*., 1979). The importance of phagocytosis in immune defence of molluscs and its sensitivity to environmental xenobiotics exposures have made it a major biomarker in ecotoxicological studies. Toxin and pollutant mediated modulation of haemocyte number and function in relation to non-self recognition, phagocytosis, respiratory burst activity etc. are in report by various workers (Adema *et al*.,1991a; Oliver and Fisher, 1999; Parisi *et al*., 2008; Chakraborty *et al*., 2009).

In invertebrates like bivalve molluscs, haemocytes mediated phagocytosis and respiratory burst activities form the major line of defence against invading pathogens andxenobiotics(Cooper and Knowler, 1992; Sami *et al*., 1992; Parisi *et al*., 2008). Nitric oxide (NO) has been described as a key component of the vertebrate immune system (Colasanti *et al*., 2002) and it has also been from

invertebrates and plants where it provides effective protection against bacterial infections (Tafalla *et al*., 2003; Zeidler *et al.*, 2004). In invertebrates including bivalve molluscs,it can kill pathogens byitself or by generating reactive nitrogen intermediate (RNI) combining with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (Figure 3a), a strong bactericidal agent (Arumugam *et al.*, 2000; Bogdan, 2001; Donaghy *et al*., 2015). Chakraborty *et al*. (2009) reported the toxicity of arsenic in the haemocytes of *Lamellidensmarginalis*in relation to phagocytosis and NOgeneration while Ciacci *et al*. (2012) have reported differential NO generation under experimental exposure of the marine bivalve *Mytilusgalloprovincialis* to nanoparticles of metallic oxides. In many bivalve species, phagocytic cells can be activated by foreign particles, or organisms and their antigens resulting in release of oxidative chemicals% this response is often referred to as an 'oxidative burst'. An oxidative burst leads to production of reactive oxygen intermediate (ROI), catalyzedby the membrane-associated enzyme NADPH oxidase. The initial metabolite O_2^- is dismutated to hydrogen peroxide (H_2O_2), which may then be converted to other toxic ROI, such as hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Buggé *et al*., 2007). ROI act as killing agents, either alone or in combination with lysosomal enzymes and are important in the elimination of viruses, bacteria, yeast, fungi, and protozoa (Chu, 2000). Production of ROI in a number of molluscan species like *Crassostrea virginica, Crassostrea gigas, Ostreaedulis, Mytilusedulis, Mytilus galloprovincialis, Pecten maximus* and *Mercenaria mercenaria,* has been studied where luminol-dependent chemiluminescence was measured in a liquid scintillation counter or the optical density of the reduction of nitroblue tetrazolium (NBT) to measure production of ROI (Bachere *et al*., 1991; Pipe, 1992; Buggé *et al.*, 2007).

Nuclear Anomalies as Genotoxicity Marker

End point of cytogenetic damage is detected by a micronucleus test, an important and authentic assay (UNEP/RAMOGE, 1999). Appearance of micronucleated apoptotic cell in mussels (Figure 4) from polluted areas has been attributed to exposure of the animals to the hazardous environmental contaminants (Steinert *et al*., 1996; Baršiene et al., 2006). The micronuclei assay is simple and relatively rapid, and is suitable for routine screening and monitoring purposes (Heddle *et al*., 1983; Bolognesi and Fenech, 2012). Micronuclei are produced from the chromosome fragments whose occurrence may be due to the defect in cytokinesis or centromere damage (Heddle *et al*., 1991). Toxic chemicals can cause genotoxic impacts on organisms by modifying the structure of DNA, consequently resulting in irreversible damage to the integrity of chromosome (Hus, 1982). These responses can be considered as biomarkers of adverse effects on the scale of cellular changes and thus can be applied as biological endpoints in genotoxicity assays (Shugart *et al*., 1992). The assay have been utilised as a biomarker of genotoxicity in marine monitoring programme (Kalpaxis *et al*., 2004; Baršiene *et al*., 2006; Schiedek *et al*.,

2006; Rocha *et al*., 2014). However, enumeration of viable, apoptotic and necrotic cells must have to be done carefully following structural conformity of the haemocytes (Figure 4).

Comet assay is another popular non-specific biomarker of genetic damage which has been shown to be applicable in the detection of DNA single strand breaks/alkali labile sites (Lee and Steinert, 2003; Cheung *et al*., 2006).It was Rydberg and Johanson (1978) who were the first to directly quantify DNA damage in individual cells on a microgel. Comet assay is advantageous over other cytogenetic methods for DNA damage detection because it requires a small number of cells for the study and the studied cells need not be mitotically active (Pavlica *et al*., 2001). The assay has been effectively used in bivalve haemocyte based biomarker study against diverse pollutants and stressors of marine and freshwater environment (Pavlica *et al*., 2001; Lee and Steinert, 2003; Cheung *et al*., 2006;Parolini *et al*., 2009; Kumar *et al*., 2014; Martins and Costa, 2015).

Conclusion

An effective biomarker based biomonitoring has the potential to provide the vital information on the temporal and spatial effect of pollutants on an ecosystem. Such information can help the environmentalist and concerned agencies to device bioremediation strategy to prevent unwanted environmental perturbation by pollutants (Galloway, 2006).Bivalve molluscs play pivotal role in aquatic ecosystems and thus they are particularly susceptible to environmental stressors (Gagnè *et al*., 2006). In this context, the biomarking approach based on the bivalve mollusc'shaemocyte structure and function provides a great opportunity for tropical countries like India for sustainable biomonitoring of its rich aquatic bioresource and biodiversity. Assays carried out on bivalve haemocytes are easy for preparation and does not necessitate expensive equipment for observation and documentation (Parolini *et al*., 2009). Simple equipment like microscope, haemocytometer, stage micrometer, spectrophotometer and some dyes/probes are good enough for designing haemocyte based cellular assays in field stations although sophistication demands expenses. The haemocytes of the bivalves remain in direct contact with contaminants and their multifunctional roles make them more sensitive than other cell lines (e.g., gills and digestive glands) to internal and environmental factors (Venier *et al*., 1997; Parolini *et al*., 2009). It is thus considered as a useful cellular tool to assess the ecotoxicity of potential environmental stressors (Parolini *et al*., 2009). However, reports suggest that though popular in Europe and North America, effective use of molluscan biomarker based aquatic environment biomonitoring has not attracted due attention in the Indian subcontinent (Verlecar *et al*., 2006). Generation of database on available molluscan species in a geographic region and the nature of interaction with potential pollutants and stressorsare the prerequisites to implement such biomonitoring strategy. Bivalve mollusc based data on the shift in haemocyte density, lysosomal stability, phagocytosis,

generation of ROI/RNI, genotoxic assay are inexpensive to document and it provides direct information on the health of an aquatic environment. The emerging concept of evaluation of multiple biomarkers (star plots) is gaining rapid acceptance (Beliaeff and Burgeot, 2002; Cao *et al*., 2018) as it has the potential to generate robust and realistic data on the wholesome reaction of a species against a stressor.

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