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Genotoxicity biomarkers in aquatic bioindicators

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Abstract Pollution of the aquatic environment is an ever-growing problem, as waters are the ultimate sink for the large number of xenobiotics from multiple sources. DNA damaging agents have a significant ecological relevance since they are implicated in many pathological processes and exert effects beyond that of individual being active through following generations. A large number of methods have been applied to evaluate genotoxic damage in different aquatic species. Comet assay, as method for detecting DNA alterations, and micronucleus test, as an index of chromosomal damage are the most widely applied and validated methods in field studies. These methods were applied in different vertebrate and invertebrate aquatic species, but only mollusk and fish species have been employed in routine biomonitoring programs. Mussels, due to their widely geographical distribution and the suitability for caging represent the bioindicator of choice in field studies. *Mytilus* species is the most used marine mussel. The use of fish is limited to specific geographic areas. The present review mainly focuses on the application of comet assay and micronucleus test in mussels. A number of biomonitoring studies in mussels, using comet assay or micronucleus test, revealed exposure to different classes of genotoxic compounds with a good discrimination power. The different evidence from the two assays, reflects different biological mechanisms for the two genetic endpoints, DNA damage and chromosomal damage, suggesting their combined application in the field. Different endogenous and exogenous factors have been shown to modulate the genotoxic responses in mussels, acting as confounding factors in environmental monitoring. The use of standardized protocol for caging, sampling and genotoxity evaluation is critical in biomonitoring studies. The use of a multimarker approach coupling genotoxicity biomarkers with physiological and biochemical factors allows to have a complete picture of the environmental pollution [*Current Zoology* 60 (2): 273–284, 2014].

Keywords Aquatic animals, Genotoxicity, Bioindicator, Biomarker, Micronucleus test, Comet assay, Bivalve, Mussel

1 Introduction

Over the last decades, the aquatic environments faced increasing contamination from multiple sources. Industrial development, intense urbanization, agricultural practices have introduced large quantities of different biologically active substances, including organic and inorganic chemicals. Many thousands of persistent pollutants are present in the environment at very low levels, but they can accumulate in tissues of aquatic organisms at concentrations several orders of magnitude higher than that of the environment. In addition the contamination of water by emerging still unregulated contaminants, such as drugs, diagnosis products, steroids and hormones, personal care products, is a matter of growing concern (Boleda et al., 2009; Santos et al., 2010).

Organisms in aquatic environments are usually exposed to a complex mixture of chemicals including parent compounds and their transformation products causing multiple damages at the organisms, population and ecosystem level, in organ function, reproductive stages and biological diversity (Ginebreda et al., 2014; Vorosmarty et al., 2010). A wide variety of environmental contaminants, directly or indirectly affecting DNA, have a significant toxicological relevance since they are implicated in many pathological processes, such as carcinogenesis and reproductive effects, exerting a damage beyond that of individual and being active through following generations (Deavaux et al., 2011; Lewis and Galloway, 2009). Exposure to these chemicals can lead to abnormal physiological responses and cause adverse effects on the development, growth, behavior, and reproduction (Bistodeau et al., 2006; Giesy et al., 2000; Ginebreda et al., 2014; Eganhouse and Sherblom, 2001; Lee and Peart, 2000). Epizootic neoplasms have been found in a variety of ectothermic species, such as shellfish, echinoderms, and fish (Mix, 1986; Malins et al., 1988; Bolognesi, 1990) in association with the exposure to specific classes of DNA damaging pollutants (Meyers et al., 1987, 2003, 2008).

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Due to its biological significance the genotoxic effects were considered the endpoints of major importance in assessing pollution-related toxicity. Large-scale biomonitoring programs in marine environments have demonstrated the associations between genotoxicity and chronic health effects at the population level (Mix, 1986; Hose, 1994).

2 Genotoxicity Biomarkers in Aquatic Organisms

DNA alterations induced by chemical and physical pollutants include single and double strand breakages, induced directly or indirectly by an interaction with oxygen radicals, DNA-DNA crosslinks, and DNA-protein crosslinks.

Different methods have been established to evaluate DNA alterations. The 32P-postlabelling assay, is a highly sensitive procedure mainly applied to the detection of bulky aromatic adducts deriving from complex mixture of environmental pollutants. This technique was applied in a number of aquatic species (Malins et al., 1990; Stein et al., 1992; Venier and Canova, 1996; Harvey et al., 1999; Dolcetti et al., 2002), but, due to the complexity of the experimental protocol, is not commonly used in large environmental biomonitoring programs.

Alkaline elution is based on the evidence that the rate at which DNA single strand fragments pass through a membrane filter under alkaline conditions is related to the length of the DNA strand itself (Kohn et al., 1976). This assay measures the extent of single and double strand breaks, DNA-DNA and DNA-protein crosslinkings and it was successfully applied in fish and invertebrates exposed to chemical compounds in aquatic environment (Batel et al., 1994; Bolognesi et al., 1996,1999, 2004, 2006).

The DNA alkaline unwinding assay was also used to detect DNA damage caused by complex environmental contamination in aquatic test organisms (Batel et al., 1999; Sarkar et al., 2008, Oliveira et al., 2010). In this assay, whole cells or crude DNA extracts are subjected to alkaline assay conditions to allow controlled "unwinding" of double-stranded DNA into single-stranded DNA, beginning at each strand break. The method for quantifying strand breaks uses fluorescent dyes, binding with an high affinity to the intact double stranded.

More recently Comet assay was developed for detecting DNA damage at the individual cell level. Its use has spread to a variety of areas, including environmental monitoring and genetic ecotoxicology. This technique is now considered one of the most promising genotoxicity biomarkers to detect a broad spectrum of DNA lesions with a very high sensitivity in aquatic species (Jha, 2008; Frenzilli et al., 2009; Frenzilli and Lyons 2013).

Chromosomal damage expressed after cell replication represents an accumulated effect associated with long term exposure to the genotoxic chemicals. The low amount of DNA per cell, the small size and high number of chromosomes and the low mitotic activity in lower vertebrates impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges. The micronucleus assay due to its potentiality to be applied in any proliferating cell population regardless to the karyotype was successfully applied in aquatic organisms (Hayashi et al., 1998; Bolognesi and Hayashi 2011).

The present review focuses on the application of comet assay and micronucleus test, the most validated genotoxicity biomarkers in biomonitoring studies in the field.

2.1 Comet assay

The comet assay or single-cell gel electrophoresis (SCGE) is a rapid and sensitive technique that detects DNA strand breaks, measuring the migration of DNA from immobilized individual cell nuclei (Fairbairn et al., 1995). In this method, cells are embedded in agarose gel on microscope slides, first they are lysed and then electrophoresed. Under the force of the electric field, cells with damaged DNA show a migration of DNA fragments from the nucleus. The length of the migration indicates the amount of DNA breakage, and it could be compared as a direct measure of the damaged DNA level. The DNA damage could be estimated by both manual microscopic and computerized image scoring analyses. This assay has a number of advantages with respect to other assays: mainly it needs a small number of cells and detects damage at single cell level.

The comet assay is widely used in human biomonitoring studies and was also applied on a wide variety of aquatic species like fish, clamps, crustaceans, worms and snails. A number of studies are available in the literature on aquatic invertebrates, such as crustaceans amphipods *Gammarus fossarum* (Lacaze et al., 2010, 2011) on which the comet assay was established on germ cells and haemocytes or the grass shrimps *Palaemonetes pugio*, on hepatopancreas (Kuzmick et al., 2006) or embryos (Kima and Lee, 2004). For what concerns the other marine or freshwater aquatic species, like snails (Ali et al., 2012; Vincent-Hubert et al., 2012),

worms (Guecheva et al., 2001; Cong et al., 2011; Osterauer et al., 2011) or echinoderms (Taban et al., 2004; Canty et al., 2009), the comet assay is seldom applied, without having a specific target tissue or bioindicator species, and still few studies have reported successful and reproducible results. A number of studies exist using more complex organisms, as amphibian (Feng et al., 2004) or even marine mammals (Diaz et al., 2009). However the most promising studies have been carried out in fish and bivalves species for which a validated protocol is currently available (Frenzilli and Lyons, 2013). In fish the test was applied in liver, gill, kidney but the large majority of available studies were carried out on peripheral erythrocytes. In marine bivalves haemolymph, gill cells and digestive gland cells were considered for the DNA strand-breaks detection with comet assay (Lee and Steinert, 2003). Haemolymph is the major constituent of mussel's soft tissue: a large part of it is composed by haemocytes, which, as circulating cells of an open vascular system, are continually exposed to waterborne contaminants (Soares- da-Silva et al., 2002). The heaemolymph is easy to collect without killing the animal, and provides a single-cell suspension ready to use for comet assay. Digestive gland is involved in transformation of xenobiotics and represents a direct target for DNA damaging agents. Gill cells are component of the filter feeding apparatus and the respiratory organ and they represent the first barrier against potential pollutants. For these reasons gills are considered as a possible target tissue in many studies using comet assay. However the main limitation of using solid tissues for comet assay is the need of cell dissociation with the potential risk to induce DNA damage. The observed variation in basal DNA damage in different tissues and organisms using different experimental procedures points out that standardized protocols have to be established for each indicator species and target tissue. Among the aquatic invertebrates, the freshwater zebra mussel *Dreissena polymorpha* and the marine mussels (*Mytilus sp.*) have been successfully used to evaluate the potential effects of anthropogenic compounds in laboratory studies. Large interindividual and intercell variability have been observed in different studies confirming the importance to optimize the experimental design selecting proper controls and reference area when the studies are conducted in the field (Dixon et al., 2002; Lee and Steinert, 2003). A time and dose dependent increase in DNA damage was observed up to 12 days with significant variations among the different classes of chemical and physical agents, followed by a reduction in DNA damage at the end of the exposure, which was thought to be related to DNA repair (Frenzilli et al., 2009). The fluctuations of DNA strand breaks were analysed in mussels *Mytilus galloprovincialis*, seasonally sampled over a three year period from a reference site along the Adriatic coast, showing changes in DNA damage extent in haemocytes, with marked differences between various sampling years (Pisanelli et al., 2009). Basal level of DNA strand breaks could be influenced also by factors unrelated with chemical exposure and a better knowledge of the natural variability of wild animals would be really important to discriminate the effects of human activity from the natural ones.

2.2 Micronucleus test

The micronucleus assay has become one of the most widely used methods for measuring structural and numerical chromosomal changes in different systems *in vitro and in vivo*. Micronuclei (MN) are cytoplasmic masses of chromatin which are not integrated in the daughter nuclei during mitosis and that remain in the cytoplasm after cell division. Micronuclei result from acentric chromosome fragments, or whole chromosomes lagging behind during metaphase/anaphase transition induced by clastogens or by spindle dysfunctions respectively. MN are formed in the process of cell division and their expression can occur at different times after the DNA damage event, depending on the cell cycle kinetics and the mechanism of induction.

The MN assay, originally developed with mammalian species is today widely applied in fish and other aquatic organisms, including sea urchin, mussels, oysters, crabs and worms, in wild and transplanted animals. The large majority of studies with the MN assay have been applied in different species of fish and bivalves (Bolognesi and Hayashi, 2011).

Micronuclei in fish can be visualized in different cell types such as gill, kidney, hepatic cells and fins (Bolognesi and Hayashi, 2011) although the use of peripheral erythrocytes is more widespread because it avoids the complex procedures of cell preparation and the killing of animals.

Hemocytes and gill cells are the targets tissues most frequently considered for the evaluation of micronuclei frequency in bivalves species. The hemolymph provides easily collectable single-cell suspension, but its main limitation for the application of micronucleus test is the complexity of cell types. Different subpopulations, including granular and agranular cells were described in the hemolymph with different origin and functions not completely known (Bolognesi and Hayashi, 2011; Bo-

lognesi and Fenech, 2012)

Gills as active proliferating tissue represents an ideal target for the evaluation of the micronuclei frequency showing a more sensitive response to genotoxic agents compared with hemocytes. The cell suspension from gills is heterogeneous in composition including larger epithelial cell types with or without cilia, and with large cytoplasm and well-spread nuclear chromatin, as well as some smaller cells with a higher nucleus/cytoplasm area ratio. A fraction of the cell population is composed by hemocytes moved into the tissue from the circulatory system. The available papers in the literature report micronuclei frequency in different cell populations with a large variability. However the evaluation of only the agranular cells in both the target tissues is strongly suggested for the MN scoring, in order to avoid the confounding factors represented by the cytoplasmic granules (Bolognesi and Fenech , 2012).

Dose-related induction of micronuclei by different pollutants has been reported in mussels exposed under laboratory conditions with up to ten fold increases over the control values after continuous exposure to genotoxic compounds without any significant mortality effect. Time-dependent increase in MN frequency was observed. The persistence of MN frequencies in gill cells following a short-term treatment with genotoxic agents was shown to be more than 1 month (Bolognesi and Hayashi, 2011).

3 Field Studies

Molluscs and fish have both been employed as sentinel organisms in routine biomonitoring programs. The use of fish in monitoring programs is believed to be of importance because of the key position of these organisms in the trophic chain and their high commercial value. However, due to large variability in sensitivity to genotoxic damage, the availability of a sufficient number of individuals and to logistic problems, cost of sampling, caging, and transportation, their use is limited to certain areas. Molluscs act a crucial role in ecosystem function (Dixon et al., 2002) and are now taken as the bioindicators of choice in the environmental studies, on the basis of their wide geographic distribution, their straightforward availability in the field and through aquaculture, and their suitability for caging experiments. Bivalves such as mussels are used for monitoring aquatic contaminants in freshwater and marine environment due sessile lifestyle, easy sampling, tolerance to a considerable range of salinity, high accumulation of a wide range of chemicals and resistance to stress (Viarengo et al., 2007). The *Mytilus* sp. which are abundant along extensive areas of the Mediterranean Sea, are commonly used to assess the water quality. The most used marine mussels in biomonitoring studies are *Mytilus edulis* and *Mytilus galloprovincialis*.

Our analysis of field studies was focused on the application of comet assay and micronucleus test in marine mussels. The collected experimental evidence on the sensitivity of the MN assay allows it to be recommended as a test of genotoxicity in the battery of biomarkers in the Mussel Watch programs. The MN assay was proposed as the only core genotoxicity biomarker in international marine pollution programs (Mediterranean Pollution (MED POL) United Nations Environment Programme (UNEP) Mediterranean Biomonitoring Program; Raphael Monaco Genoa Pollution Program (RAMOGEPOL); Oslo and Paris (OSPAR) Conventions and Helsinki Commission (HELCOM) convention). The comet assay has been shown to be the most promising test for the evaluation of different parameters of DNA damage.

3.1 Comet assay

Table 1 summarized the results on DNA damage evaluated by comet assay in a number of studies on mussel (*Mytilus* spp) collected from contaminated sites in different geographical areas. The large majority of studies were carried out in native animals or in both native and transplanted ones. Hemocytes and/or gill cells were the target organs evaluated in all the studies with two exceptions, considering the digestive gland (Shaw et al., 2004) or the germ cells (Steinert et al., 1998).

The molecular responses to ionizing radiations was evaluated in the native mussel *Mytilus edulis* collected at the Ravenglass Estuary (RE), U.K. a site impacted by ionizing radiation discharges. Increased DNA damage, detected using comet analysis, and elevated RAD51 mRNAexpression, a gene involved in the repair of DNA double strand breaks specifically induced upon exposure to ionizing radiation, was observed at the impacted site compared with the reference area (Alamri et al., 2012).

A number of studies are available reporting increased DNA strand breaks in mussels collected in sites contaminated with heavy metals, PAH or PCB, compared with reference area. The extent of DNA increase, expressed as frequency ratio ranges from 1.3 and 15.5

Table 1 Assessment of DNA damage by the comet assay on mussel (*Mytilus* spp) collected from contaminated sites

Animal	Target Tissue	Geographic Location	Contaminant	Study type	Parameter	Response (frequency ratio)[a]	Ref.
M. edulis	Haemocytes	Ravenglass Estuary (RE), U.K.	alpha, beta and gamma-emitting radionuclides	Native	% tail DNA	+ (4.8)	Alamri et al., 2012
M. edulis	Digestive gland	New Brighton, Blackpool, Whitstable, River Swale Port Quin, UK	PAHs	Native	mean % tail DNA	-	Shaw et al., 2004
M. edulis	Haemocytes	Tamar estuary, UK	Heavy metals Cr, Mn, Zn, Ni, Se, Pb	Native	Tail length Tail moment	+/-	Dallas et al., 2013
M. edulis	Haemocytes, Gill cells	Køge Bay, Copenhagen DK	wastewater from many Industries and municipalities	Native	Tail moment	+ (2–6.7) +(1.8–14.5)	Rank et al., 2005
M. edulis	Gill cells	Chemical dumping sites DK	Zn, Ni, Pb, Hg, As organophosphate pesticides	Native	Tail Moment	+(1.9–3.9)	Rank et al., 2007
				Transplanted		+ (1.8–7.5)	
M. edulis	Gill cells	Small harbour, DK	Heavy metals, PCBs, PAHs, Butyltin compounds	Native	Tail Moment	+ (2.1)	Rank, 2009
M. edulis	Haemocytes, Gill cells	Reykjavik harbour, IS	PAHs	Transplanted	% tail DNA	+ (1.4) + (1.7)	Halldorsson et al., 2004
M. edulis	Haemocytes, Sperm	San Diego Bay,California, U.S.	PAH,s Heavy metals	Transplanted	Tail length	+ (1.3–3.0) + (1.7–8.2)	Steinert et al., 1998
M. galloprovincialis	Haemocytes	Kaštela and Trogir Bays HR	Monitoring genotoxic pollution in coastal waters	Native and Transplanted	% tail DNA	+ (3.7–5.2)	Klobucar et al., 2008
M. galloprovincialis	Haemocytes Summer Spring	Ria Formosa Lagoon.P	Heavy metals, PAHs	Native	% tail DNA	+ (9.3) + (8.0)	Almeida et al., 2013
M. galloprovincialis	Gill cells	Galicia coast, SP	Oil spill (Prestige)	Native	Tail length	+ (1.7)	Laffon et al., 2006
M. galloprovincilis	Gills	Estuary of river Cecina IT	Industrial and urban wastewater, heavy metals	Native and Transplanted	% tail DNA	+ (1.3–1.5)	Nigro et al., 2006
M. galloprovincialis	Gills	Genova, IT	PAH, heavy metals	Transplanted	% tail DNA	+ (1.9)	Regoli et al., 2004, Frenzilli et al., 2004

Frequency ratio= DNA fragmentation in polluted site/DNA fragmentation in reference area.

with large variability among the different areas. The application of different experimental protocols and score systems, and the use of different comet parameters prevent any comparison among the studies.

Three studies were carried out in Denmark in different polluted areas. A first study shows significant increase of DNA damage, expressed as tail moment, in wild blue mussels *Mytilus edulis* sampled from coastal waters at Køge Bay, DK, an area receiving wastewater from many industries and municipalities compared with levels of DNA damage in mussels from the non-polluted coastal areas. Higher levels of DNA damage in gill cells compared with haemocytes are reported with a positive correlation between tail moment and concentrations of chromium, nickel and cadmium detected in waste water (Rank et al., 2005). A further study in gill cells native mussels collected in a Denmark coastal area impacted by a disused chemical dump site shows increased DNA damage with significant seasonal fluctuations in the impacted sites compared with the reference area. Hg, Ni, Pb and As are the most relevant pollutants measured in water samples from the studied sites. The paper highlights the importance to control the biotic and abiotic effects on the biomarker response in native animals (Rank et al., 2007). A more recent study investigated the genotoxic impact induced by the exposure to heavy metals (Cd, Cu, Pb and Zn), butyltin compounds, PCB and PAH in mussels collected at two sites in a highly contaminated harbour. The results show a significant increase of DNA damage clearly reflecting the levels of measured chemicals, with the highest effect in mussels from the highly contaminated old harbour and the lowest effects at the reference site (Rank et al., 2009).

A study carried out in the UK assessed the relationship between genotoxic responses and heavy metal concentrations in mussels exposed to industrial waste in the Tamar valley in SW England. *Mytilus edulis*, were sampled from five locations along the Tamar estuary and one reference location on the south Devon coast. A link between DNA damage and concentration of Cr and Mn in soft tissues was observed in mussels in the most polluted site. (Dallas et al., 2013).

Transplanted mussels of *Mytilus edulis* was use in a study in San Diego Bay to evaluate the biological effects caused by environmental contaminants, including bioaccumulation, organism growth and the determination of DNA single-strand breaks in hemocytes and sperm cells. The chemical analysis of the sediments, showed that the Hg, Cu, and Zn, were the most notable contaminants associated with the DNA damage extent (Steinert et al., 1998).

A multimarker approach using native and transplanted mussels was applied in monitoring the Estuary of river Cecina IT. A significant alteration of all the biomarkers investigated was observed in association with an increase of tissue metal levels. Similar levels of DNA damage were detected in gill cells from native and transplanted mussels collected in the same areas (Nigro et al., 2006).

DNA damage was detected by Comet assay in gill cells of mussels directly exposed to the Prestige oil spill and after 7 day recovery in the laboratory. Significant increase of DNA single strand breaks was detected in contaminated animals with respect to reference mussels, as a result of the high levels of total polycyclic aromatic hydrocarbons (TPAH) in seawater. Comet tail length was only slightly reduced during the recovery stage, as a result of DNA repair processes in exposed mussels. (Laffon et al., 2006)

A biomonitoring study was carried out in Kaštela Bay and the neighbouring Trogir Bay, Croatia, using the micronucleus test and Comet assay in haemocytes from native and caged mussels *Mytilus galloprovincialis*. The results show increased DNA damage along a pollution gradient with higher levels in caged mussels than in native ones. (Klobucar et al., 2008).

A number of studies were carried out to evaluate the role of oxyradical metabolism in inducing genotoxic damage associated with different pollution gradients.

Several biomarkers, including DNA integrity, damage, oxidized bases, oxyradical metabolism and the impairment of lysosomal membrane stability, were applied in haemocytes of mussels *Mytilus galloprovincialis* caged for four weeks in Genova harbour IT. Organisms were collected at different time intervals to better characterize the sensitivity, temporal variations and interactions of analysed responses. All the indices showed significant increases in mussels transplanted to Genova harbour, as a result of potential chemical contamination due to the presence of PAHs and trace metals. The levels of oxidised DNA bases significantly increased in harbour deployed mussels only at the end of translocation period when the efficiency to neutralise peroxyl radicals was shown to be compromised (Regoli et al., 2004; Frenzilli et al., 2004).

DNA damage was evaluated by comet assay in the haemolymph of *Mytilus galloprovincialis* from the Ria Formosa lagoon (south Coast of Portugal). Coupled with genotoxic effect, lipid peroxidation was analyzed to verify if the conditions that induce DNA damage can

be related with injury to cell membranes. DNA damage was low, reflecting the low levels of genotoxic contaminants in the lagoon with any correlation with the lipid peroxidation. Seasonal differences were observed, revealing higher environmental stress in summer, due to the increase of anthropogenic pressure in the area. The study concludes that DNA damage, evaluated by Comet assay is a sensitive biomarker that could allow to detect also low level of exposure (Almeida et al., 2013)

The relationship of DNA damage with other biomarkers, such as lipid peroxidation, cytochrome P450 and immunopositive proteins was also analysed in *Mytilus edulis* digestive gland at different seasons and at different sites around the UK coast. (Shaw et al., 2004). The study failed in detecting the difference in pollution gradient: higher levels of DNA damage, expressed as mean % tail DNA were observed at all time points in animals collected at Port Quin, the reference station, compared with the other sites. The Comet assay results indicated a seasonal influence on DNA strand breaks with lowest values, like CYP1A-immunopositive protein, occurring in December.

A biomonitoring study using transplanted mussels in different polluted sites at the Reykjavik harbor, Iceland shows higher DNA damage in hemocytes of mussels deployed in intertidal zone compared to subtidal animals pointing out the intra-site variability of biomarkers and the need to standardize the deployment and sampling protocols (Halldrsson et al., 2004).

3.2 Micronucleus assay

Table 2 summarized the results on micronuclei frequency in a number of biomonitoring studies carried out on mussel (*Mytilus* spp.) in different geographical areas. Application of the MN assay in the field has revealed the effects of exposure to different classes of pollutants (e.g., polycyclic aromatic hydrocarbons, heavy metals, organochlorinated compounds), showing good discrimination power and allowing the identification of genotoxicity events along a pollution gradient and facilitating recovery effects after accidental pollution events.

A statistically significant negative association was found between the MN frequency and the distance of sampling site from the effluent in transplanted mussels deployed at regular distances from effluents with unspecific pollutants, such as the urban wastes in different ecological and geographic locations (northern Spain and South Argentina). (Izquierdo et al., 2003). Significant MN increases were found in haemocytes and gill cells of native mussels from polluted sites in the Venice lagoon, associated with increased DNA adducts and with high levels of PAH, PCB, heavy metals and organochlorinated compounds (Dolcetti and Venier, 2002; Venier and Zampieron, 2005). A general stress condition was also detected in mussels from urban sites located in the canals of the Venice historic centre where an increased MN frequency (up to 6 fold with respect the control level) was significantly correlated with DDD, DDT and PCBs (Pampanin et al., 2005).

A year-round biomonitoring study on *Mytilus galloprovincialis* was carried out in selected sites along the Gulf of Oristano (Sardinia, Italy) including a commercial port and lagoon areas characterized by intensive agricultural and mining activities. The extent of increase of MN frequency shows seasonal fluctuations attributed either to changes in pollution input level, composition or seasonal physiological changes. The study indicates that the use of a battery of biomarkers of DNA and chromosomal damage together with biochemical markers could provide a comprehensive indication of the impact of chemical pollutants in coastal marine ecosystems (Magni et al., 2005).

The genotoxic pollution along the Spanish Mediterranean coast was studied through the determination of the frequency of micronuclei (MN) and other nuclear abnormalities (NA) in gill cells of native mussels *Mytilus galloprovincialis* collected from 17 sites. The highest MN and NA levels were found in mussels from metal polluted sites, where genotoxicity seemed to be mainly related to the oxidative stress. A lower association between MN levels and organic contaminants was observed (Fernandez et al., 2011).

The MN test was applied in caged mussels in assessing the toxic impact associated with remobilized chemicals during dredging and disposal operations in a harbor area (Piombino, Tyrrenian coast, IT). Significant increased MN frequency was detected in haemocytes of mussels during and three months after the dredging activities in association with an increased level of PB and PAH in tissues (Bocchetti et al., 2008). The MN test applied in a multi-biomarker approach with caged mussels showed high sensitivity in revealing the impact of an offshore gas platform in the central Adriatic Sea and it could be used to implement the biomonitoring plan to assess the ecotoxicological effects of the extraction activities (Gorbi et al., 2008; Gomiero et al., 2011). A recovery effect was shown by a study carried out with caged mussels in two stations in front of the Haven oilship sinking area (Italy) revealing a significant decrease of the MN frequency (from 10 to 2 fold compared to the

Table 2 Micronuclei frequency on mussel (*Mytilus* spp.) collected from contaminated sites

Animal	Target Tissue	Geographic Location	Contaminant	Study type	Response (frequency ratio)	Ref.
M. edulis	Gill cells	Gijon, SP	Urban waste	Native	+ (4.0)	Izquierdo et al., 2003
M. edulis	Gill cells	Puerto Madryn (Patagonia) AR	Urban waste	Native	+ (2.6)	Izquierdo et al., 2003
M. edulis	Gill cells	North sea	PAHs, TBT and other organic pollutants	Native	+ (6.6)	Barsiene et al., 2004
M. edulis	Gill cells	Baltic sea	PCBs, PAHs, chlorinated pesticides	Native	+(4.9)	Schiedek et al., 2006
M. edulis	Gill cells	Baltic sea	PCBs, PAHs, chlorinated pesticides	Native	+ (6.0,)	Barsiene et al., 2012
M. edulis	Gill cells	Palanga(Oil terminal area) LT	Oil spill	Native	+ (18.1)	Barsiene et al., 2006
M. galloprovincialis		Venice lagoon, IT	Industrial discharges (PAHs)	Native		Dolcetti and Venier, 2002
	Haemocytes, Gill cells				+ (3.2-5.1) + (1.4-1.9)	
M. galloprovincialis		Venice lagoon, IT	Industrial discharges (PAHs, PCBs, HCBs)	Native		Venier and Zampieron, 2005
	Haemocytes, gill cells				+ (2.0) + (5.3)	
M. galloprovincialis	Haemocytes, gill cells	Canal of Venice, IT	Urban waste	Native	+ (6.0)	Pampanin et al., 2005
M. galloprovincialis	Gill cells Spring	Ligurian coast, IT	PAH, Heavy metals		+	Bolognesi et al., 2004
				Native	+ (9.89)	
	Autumn			Transplanted	+ (13.7)	
				Native	+ (4.2)	
				Transplanted	+ (3.1)	
M. galloprovincialis	Gill cells	West Ligurian coast, IT	Oil spill	Transplanted	+ (1.9)	Bolognesi et al., 2006
M. galloprovincialis	Gill cells	Cecina estuary, IT	Industrial activity and urban waste	Native	+ (4.2)	Nigro et al., 2006
				Transplanted	+ (1.7)	
M. galloprovincialis	Haemocytes	Adriatic sea, IT	Offshore platform	Transplanted	+ (8.0)	Gorbi et al., 2008
M. galloprovincialis	Haemocytes	Adriatic sea, IT	Offshore platform	Native	+ (1.8-2.35)	Gomiero et al., 2011
M. galloprovincialis	Haemocytes	Piombino harbour, IT	PAHs, heavy metals	Transplanted	+ (7.4)	Bocchetti et al., 2008
M. galloprovincialis	Gill cells	Oristano Gulf, IT	Industrial and agriculture activity (heavy metals)	Native	+ (1.9)	Magni et al., 2005
M. galloprovincialis		Strymonikos gulf, GR	Urban and industrial waste (PAHs, PCBs, organochlorinated compounds)	Native		Dalianis et., 2003
	Gill cells Spring				+ (2.1)	
	Autumn				+ (2.3)	
	Haemocytes Spring				+ (5.6)	
	Autumn				+ (2.2)	
M. galloprovincialis	Gill cells	Patras gulf	Urban, industrial and agricultural wastewater (heavy metals)	Caged		Kalpaxis et al., 2004
	Spring				+ (4.4)	
	Summer				+ (3.0)	
	Autumn				+ (5.0)	
M. galloprovincialis	Gill cells	Spanish Mediterranean coast	Heavy metals (Hg, Cd, Cu, Zn, and As), PCBs, DDTs, PAHs	Native	+ (3.8-6.1)	Fernandez et al., 2011

[a] Frequency ratio= MNx1000 cells in polluted site/MNx 1000 cells in reference area

control level) ten year after the oil spill (Bolognesi et al., 2006).

A number of studies were carried out with the aim to compare the MN frequency in native and transplanted mussels collected in areas with different pollution gradients in different seasons. Native and transplanted mussels were sampled two times in a year in different stations along the west Ligurian cost in ltaly . MN frequency in gill cells of animals showed a progressive increase associated with increased DNA damage and bioaccumulation of PAH and heavy metals. Native mussels accumulated significant concentrations of chemicals and showed a higher induction of MN compared with transplanted mussels. Seasonal effects were also observed with highest values in spring time (Bolognesi et al., 2004). This result was confirmed by another study carried out to evaluate the chemical impact of the estuary environment (Cecina estuary, IT) where the MN frequency in transplanted mussels doubled after 30 days of deployment, while in native mussels it was more than four times those of control specimens (Nigro et al., 2006). Two studies applied the MN test in biomonitoring polluted areas along the Greece coast with wild or caged mussels. The first study carried out with native mussels reports results difficult to be interpreted with some inconsistency with the pollution gradient. Higher sensitivity and lower inter-individual variability of gill cells compared to haemocytes was observed in response to genotoxic damage (Dalianis et al., 2003). A second study shows an increased MN frequency in gill cells of caged mussels along a pollution gradient, characterized by the presence of heavy metals. Seasonal effects were observed only in some stations with lower values in summer (Kalpaxis et al., 2004).

A number of studies carried out in Baltic and North sea using native mussels show increased MN frequencies in gill cells along a pollution gradient with significant inter-location and seasonal differences. MN baseline levels were ten times lower in mussels from these areas compared with those detected from the areas in the Mediterranean Sea. Water temperature and salinity are the main factors responsible for these differences. Water temperature was shown to have a direct effect on the mitotic rate and consequently on the formation of MN (Barsiene et al., 2004, 2006, 2012; Schiedek et al., 2006).

4 Future Perspectives

Overall the results of the biomonitoring studies carried out in different geographical locations and using different experimental conditions show the efficacy of the comet assay and micronucleus test as biomarkers of genotoxicity in the *Mytilus* species, the most widely used bioindicator in field studies. Increased genotoxic effects were detected in association with the extent of pollution with different sensitivity for diverse chemical classes and mixture of pollutants. Different evidence coming from the two genotoxicity biomarkers, reflects different biological mechanisms for the two genetic endpoints, DNA damage and chromosomal damage, suggesting their combined application in field biomonitoring. The evaluation of DNA damage allowed the detection of a recent exposure, while the micronucleus test, reveals the accumulated damage during the lifespan of the cell providing an index of a time integrated exposure to the complex mixture of contaminants.

The importance of factors other than chemical pollution on the genotoxic responses of mussels is a common finding of the large majority of the field studies. Inter-individual variability observed by the large majority of studies with both the biomarkers indicates the importance to use standardized experimental protocols and to test a large number of individuals for each station. Intra-site variability of biomarkers outlines the need to standardize the deployment and sampling protocols (Halldrsson et al., 2004). Seasonal effects were also observed in many studies to affect the extent of DNA fragmentation (Nigro et al., 2006; Rank et al 2007; Shaw et al., 2004) and modulate the MN expression (Barsiene et al., 2004, 2006, 2012, Bolognesi et al., 2004; Kalpaxis et al., 2004; Magni et al 2006; Nigro et al., 2006; Schiedek et al., 2006). Endogenous factors including physiological changes related also to the reproduction cycle in bivalves, affect the metabolism and the bioaccumulation of pollutants and modify the biomarker responses.

Climatic changes affecting salinity, temperature and primary production influence the bioavalaibility of pollutants and induce also biochemical and enzymatic changes. Water temperature was shown to have a direct effect on the mitotic rate and consequently on the formation of MN (Barsiene et al., 2004, 2006, 2012, Schiedek et al., 2006).

Higher variability and sensitivity were detected in heterogeneous wild populations of mussels compared with the caged animals in different field studies (Bolognesi et al., 2004; Dalianis et al., 2003 Nigro et al., 2006). Other factors, including induced cytotoxicity and adaptation to polluted environments, which depend on the genetic make-up of the individual and populations could explain this apparent variability. The use of caged

animals from a mussel farm or from an unpolluted site, following the Mussel Watch approach, is strongly recommended in field studies. The parallel assessment of native mussels, in areas where they are naturally present, could be considered to improve the characterization of the studied sites. Repeated samplings, at least two in a year, need to be planned to account for the effects of seasonal variations.

A multimarker approach, including genotoxicity biomarkers and different biochemical physiological parameters allows to have a comprehensive picture of the pollution-related effects and to evaluate the ecosystem status. A number of studies on the application of micronucleus test reported also the results related to the frequencies of nuclear abnormalities such as nucleoplasmic bridges, nuclear buds, binucleated or eight shaped cells, showing pollution related increases of these parameters. The validation of a standardized Mussel Cytome protocol including the identification of different cell types and evaluation of the different cytotoxic and nuclear alterations, following the same procedure applied for the cytokinesis-block micronucleus cytome assay in human lymphocytes (Bolognesi and Fenech, 2012) could help to evaluate genotoxic and cytotoxic effects induced by the cumulative exposure to the mixture of different kinds of contaminants in the studied areas.

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