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Ecotoxicological Assessment of Aquatic Genotoxicity Using the Comet Assay

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Comet assay is a novel biological analysis, which is a sensitive, flexible, simple, rapid, and inexpensive method to assess aquatic genotoxicant. Since Singh and co-workers developed the method in 1988, its use has increased exponentially in various fields. This review discourses on the application of this assay in aquatic ecosystems. Various types of cells from various aquatic organisms have been tested by various genotoxicant both direct- and indirect-acting using the comet assay. The applications of this assay suggest that it is a useful assay to assess aquatic genotoxicants. However, there are some factors, which should be taken into account when using this assay as aquatic ecotoxicological assessment device such as inter-animal and cell variability.

Key words: comet assay, aquatic genotoxicant, aquatic organism, ecotoxicological assesment

Due to the increasing input of genotoxic agents into aquatic environment, growing interest in the worldwide problem in aquatic pollution has recently focused on attention on the assessment of the effect of genotoxic agents to organisms and ecosystems. Genetic approaches offer powerful tools for examining the current status of populations, inferring the history of population changes, and anticipating future population direction (Belfiore & Anderson 2001). On living organisms, which have a sensitivity to genotoxicants, DNA damage owing to genotoxicants exposure in aquatic environment can be used as a biological indicators or biomarkers (Mitchelmore & Chipman 1998b; Depledge 1998). They provide the possibility of genotoxicant assessment into aquatic environment and recently using them to assess ecosystem or organism health is a popular concept (Downs *et al*. 2001). Although, DNA damages as biomarkers do not reveal specific response of genotoxicants; it is still useful on the ecotoxicological assessment since it serves as an early warning system (Tice *et al*. 2000) that determines the bioavailability and the effect of genotoxicants. Furthermore, the non-specific nature of the assay coupled with a high sensitivity and applicability to many cell types may be considered favourable for environmental monitoring, provided that a more detailed understanding of the causes of any observed effect is pursued (Mitchelmore & Chipman 1998b). Currently, there is much interest in determining the level of DNA strand breakage (SB) as a sensitive indicator of genotoxicity (Shugart, 1990). It is a potential assessment tool since it may be produced by a wide range of agents and mechanisms including compounds, which do not produce bulky DNA adducts or other endpoints (Mitchelmore & Chipman 1998b). Production of SB correlates well with the mutagenic and carcinogenic properties of environmental pollutants with diverse structures (Sina *et al*. 1983). Hence, there is a strong need to discuss the screening assay that is capable to estimate the level of DNA strand breakage to assess aquatic genotoxicity in order to establish better understanding of the assay.

Nowadays, Indonesia faces aquatic environmental problems due to increasing of anthropogenic activities such as industrialization and urbanization which produced many pollutants that recognized induce DNA damage, such as organotin, PAH, organochlorine, heavy metals into aquatic ecosystem (Williams *et al*. 2000; Sudaryanto *et al*. 2002). Therefore, the development of risk assessment of pollution that elucidates deleterious effects of pollution in the molecular level is demanded to save public health and national food resources based on prevention approaches. This present review discourses on single cell gel electrophoresis (SCGE) or named as comet assay, which is a sensitive, rapid and simple screening assay to measure the level of DNA strand breakage, as an aquatic genotoxicity assessment device (Dixon & Wilson 2000). This assay offers an early warning system (Tice *et al*. 2000) which would not only reveal the initial levels of damage, but would also provide direction for control strategies and precautionary measures (Hansen 1997). Conceivably, it is aimed to generate an understanding of the application of comet assay and consequently, to stimulate the use of genotoxicity biomarkers as complimentary devices for a classic chemical based monitoring and risk assessment of released pollution into aquatic environment in Indonesia.

Brief History

The comet assay is one of biological analyses in ecotoxicology for detecting the effect of genotoxicants as DNA damage on living organisms. It primary detects DNA damage, expressed as single-strand breaks, and single-strand breaks associated with incompletely repair excision by measuring the migration of DNA from nuclear DNA (Mouchet *et al*. 2005). It was carried out firstly by Rydberg and Johanson (1978) by quantification of DNA damage in individual cell under mild alkali condition to allow the partial unwinding of DNA. To enhance the sensitivity for the damage detection, Östling and Johanson (1984) developed a microelectrophoretic procedure for direct visualization of DNA damage in single cells. Accordingly, Singh *et al*. (1988) developed a microgel technique involving electrophoresis under alkaline (pH > 13) condition that could permit not only the detection of the double-strand breaks, but also single-strand breaks. This version is known as SCGE, however many investigators call to this method as the "Comet assay" (Rojas *et al*. 1999) since the visualization of DNA migration under fluorescence microscope after electrophoresis similar with a comet form. Because almost all genotoxic agents induce orders of magnitude more single-strand breaks than double-strand breaks, this version of assay offered greatly increased sensitivity for identifying genotoxic agents (Tice *et al*. 2000). Subsequently, Olive *et al*. (1992) introduced another version of this assay in which DNA is electrophoresed at pH of ~ 12.3 to detect single strand breaks. Despite the fact that the Singh and Olive method are identical in practice, the Singh method appears to be at least one- or two-orders of magnitude more sensitive (Rojas *et al*. 1999).

Since the introduction of the alkaline (pH > 13) comet assay in 1988, the extensiveness of application and the number of investigators using this technique have increased almost exponentially (Tice *et al*. 2000). Comet assay offers considerable advantages over other cytogenetic methods for DNA damage detection, like chromosome aberrations, sister chromatid exchanges (SCEs) and micronucleus test (MN), because the cells studied need not to be mitotically active (Dixon & Wilson 2000; Pavlica *et al*. 2001). The assay is applied comfortably since it can be applied to any eukaryotic organism and cell type (Tice & Strauss 1995; da Silva *et al*. 2000; Hartmann *et al*. 2003). Besides, it provides information on the intercellular distribution of damage and repair as data are collected at the level of the individual cell (Tice & Strauss 1995). For conducting the assay, it is required not only small number of cells (Tice & Strauss 1995), but also the relatively short time period within few hours (da Silva *et al*. 2000; Tice *et al*. 2000; Dixon & Wilson 2000). Hence, it is suitable for environmental investigations and risk assessments. Another advantage of the technique includes it demonstrates the highly sensitivity for detecting low levels of DNA damage compare to many others of genotoxicity assay such as alkaline elution (Leroy *et al*. 1996; Tice *et al*. 2000) and micronuleus assay (Mouchet *et al*. 2005). The detection limit of this assay is 5 cGy gamma rays in human lymphocytes (Tice & Strauss 1995). In addition, Mitchelmore and Chipman (1998a) reported that the level of detection as low as one break per chromosome. Compare to others DNA damage test such as sister chromatid exchange (SCE), micronuclei test and alkaline unwanding assay, this assay show relatively cost-effective and flexible (Tice *et al*. 2000; Dixon & Wilson 2000). McNamee *et al.* (2000) have highlighted the comet assay advantages by modifying the basic comet protocol to increase productivity and efficiency without sacrificing assay reliability. This modified technique offers a rapid processing of multiple samples. Moreover, using Tebbs *et al*. (1999) modification, DNA strand breaks can be detected in extremely small tissue samples as embryonic tissues.

Comet Assay Methodology

This assay has undergone several modifications, but the basic principles are resting upon the neutral and alkaline version. The alkaline version introduced by Singh *et al*. (1988) is more commonly adopted because it is more sensitive than neutral version and can detect alkali-labile sites (ALS) (Anderson *et al*. 1998; Rojas *et al*. 1999; Tice *et al*. 2000). However, the selection of which method to use in aquatic environment depends largely on the type and the degree of information required in the study.

Briefly, after a suspension of cells is obtained, it is embedded and immobilized in low melting agarose at final concentration of 0.5-1.0% at 35-45 ^{o}C on a fully frosted microscopic slide. The numbers of agarose layers, which are used per gel are the single layer gels and ´sandwich' gels (where the cells are contained in the middle layer of three distinct layers of agarose). In slide preparation, sufficiently stable gel is a need for subsequent manipulation, as well as to ensure during comet analysis, the frosting of the slide does not contribute to fluorescent noise, which may obscure details of the comets (Fairbrain *et al*. 1995). After the agarose gel has solidified, the slides are placed, generally for at least 1 h, in a lysis solution consisting of high concentration of salts and detergents. DNA damage caused by iron released during lysis from erythrocytes which exist in blood and tissue samples can be avoided by adding 10% dimethyl sulfoxide (DMSO) (Tice *et al*. 1991).

Before electrophoresis, the slides are equilibrated in alkaline (pH > 13) electrophoresis solution to produce single-stranded DNA and to express ALS as SSB (single strand breaks). Singh *et al*. (1988) used 1 mM EDTA and 300 mM sodium hydroxide, pH > 13 as alkaline solution. This solution is still used most frequently in comet studies, because it maximizes the expression of ALS as SSB (Rojas *et al*. 1999).

After alkali unwinding, the single-stranded DNA in the gel is electrophoresed under alkaline condition to create comets. Following electrophoresis, slides are washed and stained with a fluorescent DNA binding stain for image analysis. A variety of stains have been used effectively; propidium iodide is a popular choice especially for image analysis. Singh *et al*. (1994) have proposed the use of YOYO-l (benzoxazolium-4-quinolinum oxazole yellow homodimer),

which increases the assay sensitivity by giving better visualization. To prevent additional DNA damage, all steps are performed under dimmed light.

The method of quantifiying DNA migration is as varied as the application of which the comet assay has been used. Since comets are formed upon the principle of releasing damaged DNA from the core of the nucleus by electrophoresis, several different attempts have been made to evaluate and quantify comet formation pattern (Fairbrain *et al*. 1995). The simplest method for quantifying comet data is based on determining the proportion of cells with altered migration. However, this method can be used only for the majority of control cells exhibit no or little DNA damage and fails to provide information about the extent of DNA damage among more damaged cells (Tice *et al*. 1991). Gedik *et al*. (1992) proposed an advanced and useful technique by classifying comet image into four categories of increasing damage and calculated the proportion of comets in each category.

The most common measurement used in comet studies is the length of DNA migration. This measurement assumes that the migration length to be related directly to fragments size and would be expected to be proportional to the level of SSB. The migration length could be measured using different tools; with a micrometer in the microscope eyepiece, a rule on photographic negative/positive of cell images or in the camera monitor, and by using image analyser (Tice *et al*. 2000). Furthermore, a variant of this measurement is to present the ratio of length/width or width/length with cell exhibit no damage having a ratio approximately 1 (Rojas *et al*. 1999; Tice *et al*. 2000). To enhance the sensitivity of data scoring, Olive *et al*. (1990) introduced the concepts of tail moment, which was defined as the product of percentage of DNA in the tail and the displacement between the centre of mass of the head and the centre of mass of the tail as measures for DNA migration. This measure showed the sensitivity to detecting DNA damage (Fairbrain *et al*. 1995; Kent *et al*. 1995). Subsequently, Hellman *et al*. (1995) proposed a tail inertia (the moment of inertia of the tail), which was showed more sensitive than the tail moment.

The Application of the Comet Assay in Aquatic Environment

Various types of isolated cells from various aquatic organisms were tested to various genotoxic agents both direct- (not requiring metabolic activation) and indirect-acting using the comet assay. In the literature, the most common organisms, which were used as sentinel organisms in aquatic ecotoxicology were fish and then followed by mussel, and amphibian. The employment of other aquatic organisms as sentinel organisms to detect genotoxic agent for instance plankton (Erbes *et al*. 1997), tunicate (Kamer & Rinkevich 2002), shrimp (Lee *et al*. 2000), and aquatic mammals (Betti & Nigro 1996; Taddei *et al*. 2001) are seldom used in comet assay. Some researchers (i.e. Mitchelmore & Chipman 1998b) pointed out the direct acting compound including H_2O_2, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) capable to produce DNA strand breaks in hepatocytes or blood cells isolated from either brown rainbow trout or flounder, in cells from the digestive gland and gill cells from mussel, and in cells from blood of tunicate as well. The employed various cells, for instance fish hepatoma cell line indicated also their capability to detect DNA strand breaks induced by indirect-acting agents, for example benzo[a]pyrene and 1-nitropyrene. The production of DNA strand breaks was also detected when hepatocytes of rainbow trout (*Onchorynchus mykiss*) exposed *in vitro* by organic extracts from site contaminated with PAHs/PCB (Devaux *et al*. 1998).

An intriguing study was shown by Pruski and Dixon (2002) who showed a genotoxic effect of Cd to gill cells of mussel unsuccessfully under acute and chronic exposure condition using comet assay *in vitro* and *in vivo*. However, this study revealed that pre-exposure to low concentrations of Cd was found to enhance the genotoxicity of another mutagen, H_2O_2 and the capability of Cd inhibits DNA repair at the post-treatment. On the contrary, a genotoxic effect of Cd was demonstrated in the sponge *Suberites domunculata*, where an increase in frequency of DNA strand breakage was measured in specimens from polluted environments and followed by exposure to Cd in laboratory (Mueller *et al*. 1998).

Tiano *et al*. (2000) demonstrated effect of organotins, tributyltyn-chloride (TBTCl), dibutyltin-chloride (DBTCl), and monobutyltin-chloride (MTBCl) to produce DNA strand breaks on trout-nucleated erythrocytes. This *in vitro* study presented that TBTCl displays a marked genotoxic effect, whereas the genotoxic effect was less pronounced for DBTCl and it was completely absent for MBTCl. This trend was in accordance to the general evidence that the toxicity of organotin is determined by the number and nature of organic substituents on (Sn^{4+}); in general, the toxicity decreases from tri to mono-alkyltins. Interestingly, this research employed different parameters such as a tail length, a tail intensity, and a tail moment to measure the level of DNA strand breaks. The result of this research revealed that the tail length was less sensitive to detect DNA strand breaks than others. This was in agreement with the experiment on Chinese hamster ovary (CHO-KI) cells (Kent *et al*. 1995), which demonstrated that the tail length increased linearly with dose, but reached a plateau at higher doses. On the other hand, the tail moment increased linearly with dose as well, but over a larger dose range than the tail length and had no tendency to plateau.

DNA strand breaks in kidneys cells from gold fish (*Carasius auratus*) exposed to heat shock were evaluated *in vitro* by Anitha *et al*. (2000). The heat shock temperatures used were 34, 36, and 38 ^oC. The results showed that heat shock caused the induction of micronucleus at the three temperatures studied. Heat shock also inhibited cell proliferations at 38 ^oC and caused aberrations in the metaphase chromosomes at 34 and 36 ^oC. The comet assay demonstrated single strand breaks at all three temperatures.

The application of comet assay for investigating *in vivo* effect of genotoxicant on the aquatic organism was explored. Although a wide range of the comet assay application

focussed on the aquatic macro organisms, the employment of aquatic microorganisms depicted excellent results. Green algae (*Chlamydomonas reinhardtii*) responded very sensitively to a treatment with increasing doses of 4-nitoquinoline-1-oxide, N-nitrosodimethylamine, and hydrogen peroxide (Erbes *et al*. 1997). The results of this research, and particularly after exposure to hydrogen peroxide, depicted that a treatment in light enables *Chlamidomonas reinhardtii* to cope with oxidative stress more efficiently than under dark conditions. DNA strand breaks have been detected in Cryptophyta, *Rhodomonas* sp., induced by UV radiation (Sastre *et al*. 2001). Cells of *Rhodomonas* sp. exposed to 12 h visible + UV A + UV B and visible alone (control) showed approximatetly 200% more damage than control if these were treated with T4 endonuclease V. DNA strand breaks induced by visible + UV A and UV B were observed increased along with the exposure time.

Freshwater planarian (*Dugesia schubarti*) blood cells were used to test the genotoxic potential of copper sulphate ($CuSO_4$) (Guecheva *et al*. 2001). To study the influence of copper ions on the persistence of mutagen-induced DNA damage, the blood cells were treated with methyl methanesulphonate (MMS), and then incubated in the absence or presence of $CuSO_4$. This experiment indicated that $CuSO_4$ inhibited DNA repair of MMS-induced DNA damage. The inhibition effect of copper ions on repair of MMS-induced DNA damage suggested that copper could modulate the genotoxic effects associated with a complex mixture exposure in the environment.

Sasaki *et al*. (1997) performed *in vivo* study of two species of shellfish *Patunopecten yessoensis* and *Tapes japonica* which exposed to the well-known genotoxic compounds such as benzo[a]pyrene (B[a]P). All the tested chemical compounds demonstrated their genotoxicity by damaging DNA of cells isolated from the gills. On the other hand, *in vivo* exposure of blue mussels, *Mytilus edulis*, to hydrogen peroxide, resulted in more uncertain dose dependent responses compared to the *in vitro* studies (Wilson *et al*. 1998). Interestingly, the authors showed that the application of antioxidant supplementation like vitamin E can improve the sensitivity of the assay by lowering the baseline damage in untreated animals.

By exposing grass shrimp (*Palaemonetes pugio*) embryos to four compounds (anthracene, pyrene, a-terthienyl, methylene blue) along with solar exposure, Lee and Kim (2002) demonstrated the capability of comet assay to detect both DNA damage and repair. The authors found that the DNA damage more pronoun when the embryos exposed to the chemicals and solar than those exposed to the chemicals solely. Decreasing of DNA damage when solar exposed embryos were transferred to the dark, suggested DNA repair systems were active.

Tropical green mussel (*Perna viridis*) has been used as an experimental animal to detect DNA damage induced by water-borne benzo[a]pyrene (B[a]P) in serial concentrations (Siu *et al*. 2004). The experiment used circulating hemocytes of green mussel. The results suggested that an increase in the proportion of DNA damage was occurred dose-dependently, but a significant decrease of DNA damage was observed after 12 days exposure. This indicated that recovery or DNA repair on circulating hemocytes of green mussel was occurred. Moreover, previous study using the same type and concentration of contaminant Ching *et al.* (2001) demonstrated that digestive gland of exposed green mussel showed an increase of DNA damage after prolongation of the animals exposure until 24 days. These results suggested that a chronic *in vivo* test is needed to ensure the mechanism DNA damage and repair induced by genotoxicant since both concentration and duration can influence DNA damage in *Perna viridis*. Overall, two studies offered a considerable potential of green mussels used as a sentinel organism for risk assessments of released genotoxicants in tropical region like Indonesia. Indeed, the extrapolation of laboratory results should be consorted with field studies in order to recognize which environmental factors that influence the use of molecular biomarkers such as DNA strand breaks.

Tadpoles (*Rana hexadactyla*) were used as a sentinel organism to evaluate potential genotoxicant of four sulfur dyes used in textile and tannery industries (Rajaguru *et al*. 2001). The dyes, along with their active ingredients, were Sandapel Basic Black BHLN, Negrosine, Dermapel Black FNI, and Turquoise Blue. The dyed-treated tadpoles showed significant DNA strand breaks, measured as mean DNA length-width ratio, when compared with unexposed control. Among the four tested dyes Sandapel Basic Black BHLN appeared to be highly genotoxic, Dermapel Black FNI was least genotoxic, and Negrosine and Turquoise Blue were moderately toxic to the tadpoles. DNA repairs were observed when the tadpoles placed in dechlorinated tap water after for a 24 h period exposure to the dye solution.

Regarding *in vivo* experiment in fishes, a frequent way of exposure is to place fish in spiked water. For example, Belpaeme *et al*. (1998) with some modification exposed blood, gill, liver, and kidney cells of turbots (*Psetta maximus*) to the ethyl methanesulfonate (EMS). A statistically significant increase in DNA strand breaks expressed as a tail moment was observed for all the cells following EMS exposure. Erythrocytes of brown trout (*Salmo trutta farion*) were examined to know the potential genotoxicity effect of a planar PCB77 (Belpaeme *et al*. 1996) using micronucleus test and comet assay. Ethyl methanesulfonate (EMS) at a concentration of 25 mg/l water was used as a positive control. Although EMS induced a statistically significant increase of single strand breaks in the comet assay, in neither of the two tests, mutagenic effect due to PCB exposure was observed.

In term of *in situ* study, Frenzilli *et al*. (2001) investigated spatial and temporal variability of DNA integrity as responses to environmental disturbances in a highly eutrophicated coastal lagoon. Mussels were collected four times at five different sites. Longer DNA migration distances were obtained for individuals sampled in the inner part of the lagoon. The analysis of variance also exhibited some seasonal variability with the higher level of DNA migration generally occurring in late summer in mussels from the inner part of the lagoon. This

study also found that specimens with the lower DNA integrity possess a low ability to neutralize three strong cellular oxidizing species, namely peroxyl radicals (ROO·), hydroxyl radicals (·OH) and peroxynitrite (HOONO). This indicates that the reactive oxygen species are involved in mediating DNA damage. Furthermore, the freshwater zebra mussel *Dreissena polymorpha* Pallas, was used to examine a potential genotoxin in the Sava River downstream, Zagreb, Croatia (Pavlica *et al*. 2001). This river receives effluents from chemical industries situated on the outskirts of the city along with domestic sewage. The mussels were kept in plastic mesh bags, fully immersed in the water column during the whole exposure. A statistically increase in DNA strand breaks measured a tail moment was observed after *in situ* exposure in the Sava river. By comparing *in vitro* and *in situ* study, Yaqin (2002) figured out that exposed gills of *M. edulis* by 10 mg/l of TBTCl had a similar magnitude of DNA strand breaks with those collected directly from suspected TBT-polluted marina, Norsmind fjord Denmark. Moreover, transplanted blue mussels to the marina for one month revealed a stimulation of DNA damage of blue mussel gills when compared to those which collected from reference site. However, DNA damage of the transplanted blue mussel gills was lower than those of blue mussel gills which collected from polluted area. These results demonstrated not only a quick response of DNA strand breaks to pollutants, but also the latency of TBT to promote DNA damage in sessile animals even tough it has been banned for long time. Besides, this study emphasized that three levels of ecotoxicology studies which are *in vitro*, *in situ* and transplantation are necessary for underpinning the application of biomarkers as a risk-based management tool.

Can the Comet Assay Service as an Aquatic Genotoxicity Assessment Device?

The information given above demonstrates the potential of comet assay for detecting and assessment a range of genotoxicants in aquatic environment. Interestingly, most of the studies reported in this review demonstrate that chemicals may be investigated *in vitro* and *in vivo* in numerous aquatic organisms. Furthermore, it is of some concern that environmentally complex mixtures can be tested with the comet assay protocol. Mollusks, fish, and amphibians may be employed for *in situ* evaluation of genotoxicity of water.

The valuable of the comet assay as an assessment tool is supported by the fact that this assay fulfils the requirement of ecotoxicological assessment strategy, namely, sensitivity, easy to use, flexibility, low cost and rapidity (reviewed in cited literature above; see also Dixon *et al*. 2002). It opens a challenging opportunity for developing countries like Indonesia which is threatened by many genotoxicants such as PAH (Williams *et al*. 2000), organochlorine (Munawir 2005), heavy metals and organotin (Sudaryanto *et al*. 2002) to apply comet assay for assessing hazardous risk of discharged genotoxicants wastes into aquatic environment. Some environmental regulations in Indonesia are still based on the lethal screening test which elucidates nothing about biotransformatinon and biomagnification of xenobiotic substances within aquatic organisms and less sensitive compare to the DNA damage endpoint. Those substances do threaten not only for edible aquatic organisms but also for endanger human being. Therefore, it is a time for Indonesia to go beyond curative approaches for protecting public health and its national aquatic food resources from genotoxicants catastrophes through enhances the sensitivity of the threshold level of aquatic environmental health by employing the comet assay. However, it is also clear that there are some aspects to take into account for avoiding misconduct and misinterpreting of collected data when applying this assay.

The interesting point of the comet assay application on aquatic ecotoxicological assessments is the use of various parameters by researchers for measuring DNA damage which range from the simplest method to the complicated one. The percentage of comets is the simplest method to determine DNA damage. Regarding the tail length of the comet, Cotelle and Férard (1999) recognized at least three parameters which were commonly used for examining DNA damage i.e. the displacement between the leading edge of the nucleus and the end of the tail (Singh *et al*. 1988) which can also termed as the total DNA length, the displacement between the centre of the nucleus and the end of the tail, and the displacement between the trailing edge of the nucleus and the end of the tail (Ashby *et al*. 1995). Those measurements may appear problems when data comparisons are conducted since each parameter has a different sensitivity for detecting DNA damage.

The DNA damage measurement has been strengthened by using image analyzing system which based on the fluorescence intensity (Cotelle & Férard 1999). Based on that system, Olive *et al*. (1990) proposed a suitable index of the DNA damage by considering the distance of the genetic material migration and the relative amount of DNA in the tail. Olive *et al*. (1990) termed this parameter as a tail moment which defined as the product of the percentage of DNA in the tail and the distance between the means of the head and tail distributions. It has been showed in the literatures cited here that the negative result to evaluate DNA damage induced by genotoxicity in aquatic organisms frequently used the tail length rather than the tail moment. It may be more rationale to use the tail moment instead of the tail length. Moreover, a major advantage of using the tail moment as the index of DNA damage is that both the amount of damaged DNA and the distance of migration of genetic material in the tail are represented by a single number (Hellman *et al*. 1995). However, the disadvantage of this parameter is that it depends on the centre of gravity of the tail (Cotelle & Férard 1999). The same tail moment might be resulted from the tails with different lengths, numbers of fragments, and relative amounts of DNA (Hellman *et al*. 1995). Furthermore, the use of this metric eliminates potentially useful information on the relationship between the length of migration and the percentage of migrated DNA (Tice *et al*. 2000). Therefore, Hellman *et al*. (1995) proposed other parameter, namely a tail inertia as a substitution of the tail moment.

Regarding ecotoxicological assessment of aquatic genotoxicity, which type of parameter should be used in order to determine the appropriate interpretation of the genotoxicity effects in DNA damage has to be considered. Despite preference of parameter depends upon the resources of the investigator and the experimental design, however, combining some parameters such as the tail length, the tail moment, the tail intensity, and the length-width ratio will be an appropriate method. Besides, a standardization of used comet assay parameters is necessary for possible and justified comparisons among comet assay studies, which establishes a robustness and reproducibility of the comet assay application on aquatic ecotoxicological assessments.

Inter-animal and inter-cell variability that can influence the reflecting of DNA damage induced by genotoxicity have been observed and discussed by Mitchelmore and Chipman (1998a). The coefficient of variation (CV) between different hepatocytes preparations (i.e., between different fish) was a mean of $29.5 \pm 6.4\%$ for direct-acting compounds and $50.6 \pm 2.8\%$ for those requiring metabolic activation. Nacci *et al*. (1996) also reported marked variability in comet data from haemocytes from individual oysters and in hepatocytes, blood cells and gut cells from flounder exposed to B(a)P-spiked diet and sediment. For 4-8 fish per treatment, an effect of about 75% difference from the control was required for statistical significance in hepatocytes. Furthermore, Nacci *et al*. (1996) recognized a hepatocyte heterogeneity within the same tissue sample depending on the DNA damaging agent. With direct acting genotoxicants (H_2O_2, MNNG, and MX), the CV decreased with increased concentration, whereas with B(a)P and 1-nitropyrene, the CV remained similar to the control (Mitchelmore & Chipman 1998a). Finally, in the light of ecotoxicological assessment the comet assay provides a sensitive, rapid and economic method for the observation of DNA strand breaks, which is ideally suited as a non-specific biomarker of genotoxicity in aquatic organism.

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