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Application of the comet and micronucleus assays to the detection of B[*a*]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*)

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Abstract

Green-lipped mussels (*Perna viridis*) were exposed to water-borne benzo[*a*]pyrene (B[*a*]P) at nominal concentrations of 0, 0.3, 3 and 30 $\mu g\,l^{-1}$ for up to 12 days, and both the relative levels of DNA strand breaks (assessed using an alkaline comet assay) and the proportion of micronucleus (MN) formation were monitored in mussel haemocytes at days 0, 1, 3, 6 and 12. The results of the comet assay indicated that an increase in the proportion of strand breaks occurred generally with increasing B[*a*]P concentration, but a significant decrease in the levels of DNA damage was observed after exposure for 12 days at all concentrations tested, suggesting that the patterns of changes in the levels of DNA strand breakage can be explained by the threshold dependent DNA repair theory. Moreover, the relatively slow development and recovery of the DNA damage response in mussel haemocytes in comparison with previous findings utilizing *P. viridis* hepatopancreas suggests that the response of DNA alteration upon exposure to B[*a*]P may be tissue-specific in this species. Monitoring the frequency of micronucleus development in mussel haemocytes indicated both dose– and time–response relationships within the exposure period. Furthermore, the levels of DNA strand breakage correlated well with the levels of micronucleus induction, suggesting a possible cause and effect relationship between the two damage types. We suggest that DNA strand breakage and micronucleus formation in mussel haemocytes can potentially be used as convenient biomarkers of exposure to genotoxicants in the marine environment.

Keywords: Benzo[*a*]pyrene; Haemocyte; DNA damage; Micronucleus; *Perna viridis*; Comet assay

1. Introduction

Persistent organic pollutants (POPs) are chemicals that are almost entirely anthropogenic in origin and are generally regarded as being resistant to degradation

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through photolysis, chemical transformation and/or biological action (Eduljee, 2001). POPs pose considerable risk to all living organisms because of both their persistence and toxicity. In particular, due to their environmental persistence, many organisms may be subjected to long-term exposure to low levels of POPs, eventually leading to the development of serious chronic effects in a variety of cell types and through which the whole organism may eventually be deleteriously affected. As a result of bioaccumulation in aquatic environments, the concentration of POPs may be up to several orders of magnitude higher in a living organism than in the ambient water, and this may result in a higher probability of both acute and chronic poisoning to living organisms (Bernes, 1998). In addition, many POPs are genotoxicants, meaning they are substances that can modify both the structure and integrity of DNA (Shugart, 1995), and many genotoxic POPs can also further damage DNA through the generation of intermediates of greater genotoxicity that are formed via metabolism during complex cellular processes (Newman, 1998; Mitchelmore and Chipman, 1998a,b; Shugart, 1995, 2000; Holbrook, 2001). As a result, alteration of DNA by genotoxic POPs may cause mutagenesis (changing genetic information), teratogenesis (causing developmental malformations), clastogenesis (breaking of the chromosome) and carcinogenesis (cancer or neoplasia).

The genotoxicity of various POPs can be reflected, and therefore assessed, by the level of damage to genetic material, for example by the relative increase in DNA adduct formation, DNA strand breaks and/or micronucleus (MN) formation. There is now a growing trend to use single cell gel electrophoresis (SCGE) assays (or the comet assay) for the detection of DNA damage in individual cells, a method first developed to detect double strand DNA breaks induced in X-irradiated cells (Östling and Johanson, 1984). Originally, this assay used individual cells embedded in agarose to minimise mechanical shearing artefacts, in which both the cellular and nuclear membranes are disrupted by immersing the sample in a lysis solution containing high salt concentrations and detergents. The exposed nucleoid DNA is then subjected to an electrophoretic field under neutral conditions and the cleaved DNA fragments are forcibly migrated away from the nucleoid cores by the electric field. Thus, after staining with a DNA-specific fluorescent dyestuff, the DNA appears as a “comet” under a fluorescence microscope, thereby giving the assay its name. Moreover, the distance that the DNA has migrated from the core (i.e. the comet tail length) can be used to assess the level of double stranded DNA breakage, as the comet tail length is positively correlated with the amount of DNA damage (Singh et al., 1988). In addition, the size and staining intensity of the comet tail has also been shown to provide a measure of the degree of DNA damage (Wilson et al., 1998). Although many modifications to the comet assay have subsequently been proposed, most current protocols are still largely the same as those developed by Singh et al. (1988), incorporating an alkaline unwinding procedure in the assay to allow the sensitive detection of both double- and single-strand breaks as well as to generate breaks at alkaline labile sites created through DNA adduct formation.

Thus, the major advantage of the comet assay over other techniques is the highly sensitive detection of both double- and single-strand breaks. The levels of detection have been reported to be as low as one break per chromosome (Mitchelmore and Chipman, 1998a) or as few as 200 breaks per cell (Rojas et al., 1999). Moreover, the comet assay is generally considered to be rapid, simple, relatively inexpensive and a reliable method for the detection of DNA damage and repair within virtually any eukaryotic cell population. Furthermore, it requires only small cell samples ($<10\,000$ cells) to detect these differences (Wilson et al., 1998; Rojas et al., 1999; Shugart, 2000), an important consideration when the amount of sample is limiting (Theodorakis et al., 1994). Since the comet assay measures DNA damage at the single cell level, it is possible to correlate the results of DNA strand breakage with other cytogenetic responses observed in single cells, such as the micronucleus induction assay (Wrisberg et al., 1992).

The comet assay has been used extensively in the assessment of both chemical- and radiation-induced DNA damage and repair mechanisms and has been widely utilized for predicting the genotoxic, mutagenic and carcinogenic properties of a range of substances (Duthie and Collins, 1997; Cotelle and Ferard, 1999), although these studies have mainly been restricted to human and other mammalian cell types (Collins et al., 1995; Van Goethem et al., 1997; McNamee et al., 2000). In an ecotoxicological

context, the comet assay has been applied to aquatic vertebrates including the bottlenose dolphin (*Tursiops truncatus*) (Taddei et al., 2001), the zebra fish (*Danio rerio*) (Schnurstein and Braunbeak, 2001), rainbow trout (*Oncorhynchus mykiss*) (Devaux et al., 1997), butterfish (*Pholis gunnellus*) (Bombail et al., 2001), bullhead (*Ameriurus nebulosus*) (Pandrangi et al., 1995), carp (*Cyprinus carpio*) (Pandrangi et al., 1995; Kammann et al., 2001), brown trout (*Salmo trutta*) (Belpaeme et al., 1996), and flounder (*Pleuronectes americanus*) (Nacci et al., 1996). Comparably fewer studies have been conducted with aquatic invertebrates and these have been restricted to bivalve species such as the marine mussel (*Mytilus edulis*) (Wilson et al., 1998; Accomando et al., 1999), the zebra mussel (*Dreissena polymorpha*) (Pavlica et al., 2001), Mediterranean mussel (*Mytilus galloprovincialis*) (Frenzilli et al., 2001) and the oyster (*Crassostrea americanus*) (Nacci et al., 1996).

As for the micronucleus assay, it can be used to detect cytogenetic damage. MN arises when complete chromosomes or chromosomal fragments fail to incorporate into the daughter nuclei during the anaphase of cell division and remain in the cytoplasm throughout the life cycle of the cell.

The MN assay has proven most suitable for assessing genotoxic effects of environmental contaminants in effluents and polluted water bodies (Mersch and Beauvais, 1997). It has been applied to both laboratory and field studies in amphibians, different species of fish (*Umbra limi*, *Ictalurus nebulosus*, *Umbra pygmaea*, *Genyonemus lineatus* and *Heteropneustes fossils*) (Gauthier, 1996 and references therein), and different species of bivalves (*Anodonta cignea*, *Crassostrea gigas*, *Crassostrea virginica*, *M. galloprovincialis*, *M. edulis*, *Mya arenaria* and *D. polymorpha*) (Mersch and Beauvais, 1997 and references therein).

In Hong Kong, *Perna viridis* (Linnaeus, 1785) (Bivalvia: Mytilacea) is widely distributed from oceanic waters to estuarine systems and is a dominant inter-tidal species on polluted rocky shores. Apart from their abundance and wide distribution within local water bodies, their sedentary and filter-feeding characteristics make them an ideal pollution indicator. Specifically, these animals tend to bioaccumulate pollutants in their tissues. Their body burdens of trace metals and trace organics have often been used to reflect levels of contamination in surrounding waters (Cheung et al., 2001). To use DNA damage and MN induction as biomonitoring tools, comprehensive studies on the time-dependent effects of exposure to various concentrations of genotoxicants are necessary. Furthermore, there is a lack of detailed study on the relationship between the levels of DNA strand breaks and micronucleus induction. According to Heddle et al. (1983), MN can be caused by chromosomal fragmentation. Although the mechanism of chromosomal fragmentation has seldom been illustrated, it would most probably be preceded by DNA strand breakage. A positive relationship between DNA stand breaks and MN would lend support to the above hypothesis.

The present study aims to investigate the dose- and time-dependent responses of DNA strand breaks and micronucleus formation in haemocytes of *P. viridis*, and to examine the correlation between the level of DNA strand breaks and micronucleus frequency in the mussel haemocytes. To this end, we exposed the green-lipped mussel (*P. viridis*) to benzo[*a*]pyrene (B[*a*]P) over 12 days and assessed the dose- and time-dependent responses of two cytogenetic endpoints in the mussel haemocytes. The levels of DNA strand breaks were monitored using the alkaline comet assay and the extent of MN formation was measured using the MN assay.

2. Materials and methods

2.1. Dosing experiment

Green-lipped mussels (*P. viridis*) with shell length of 7–10 cm, were collected from Kat O, a relatively clean site in Hong Kong. After collection, the mussels were cleaned and acclimated in 300 l fibreglass tanks containing aerated, sand and charcoal filtered, recirculating seawater at a density of around 2 l of seawater per mussel for 7 days. The salinity and temperature of the seawater were maintained at 35 ± 2‰ and $20 \pm 1\,^{\circ}C$, respectively, and the mussels were fed daily with *Dunaliella tertiolecta*. The seawater was renewed on a daily basis and any dead mussels were removed and discarded immediately. Acclimated mussels, with their bysus removed, were divided into five duplicated groups: an untreated group (seawater only), a solvent control and three groups exposed to different levels of B[*a*]P, and were transferred to 60 l glass

aquaria filled with filtered natural seawater to provide a volume of 2 l seawater per mussel. Appropriate volumes of B[*a*]P dissolved in acetone were added to the seawater to yield nominal final concentrations of 0.3, 3 and 30 $\mu g\,l^{-1}$; in each case, the final acetone concentration was 0.006% (v/v). The toxicant-laden seawater was renewed, and mussels were fed daily with *D. tertiolecta* (3×10^6 cells l^{-1} seawater) with feeding preceding water exchange by 1 h; all other conditions were maintained as for acclimation. Four individuals were sampled randomly from each replicate on days 0 (untreated group only), 1, 3, 6 and 12. The haemolymph from individual mussels was collected from the sinus near the posterior adductor muscle using a hypodermic syringe under dim yellow light. The sample was transferred to a microcentrifuge tube and held on ice to prevent endogenous damage occurring during sample preparation and to inhibit DNA repair in the unfixed cells. The samples were subsequently analysed by comet and MN assays.

2.2. Comet assay

The alkaline comet assay was adapted from the method described by Singh et al. (1988) with slight modifications. All steps described were performed under dim yellow light to prevent DNA damage from ultraviolet irradiation (Singh et al., 1988). The chilled haemolymph (15 μl) with a density of $\sim 1 \times 10^5$ cells ml^{-1} was combined with 500 μl of 1% low melting point agarose (LMAgarose) in Ca^{2+}- and Mg^{2+}-free PBS at 42 °C and 50 μl of the mixture was immediately pipetted onto each of the two sample areas of a CometSlide™ (Trevigen, Gaithersburg, MD, USA). The agarose was allowed to solidify at 4 °C in the dark for no longer than 10 min and the slides were then immersed into a cold, freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) for at least 1 h at 4 °C in a Coplin jar. Following lysis, the slides were drained to remove any residual salts from the solution, which might otherwise affect DNA electrophoretic migration and introduce variability in the results (Singh et al., 1988; Fairbairn et al., 1995) and the slides were then aligned in 2 rows in a horizontal electrophoresis tray and covered with an alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 45 min at room temperature to allow the DNA to denature. Electrophoresis was performed in the same buffer at 1 V cm^{-1} and 300 mA for 30 min and the slides were then drained, fixed in absolute ethanol for 5 min and allowed to air-dry for storage. Prior to the analysis of comets, 50 μl of 1% SYBR Green staining solution (Molecular Probe, Eugene, OR, USA) was added to each agarose spot and the stained slides were kept in a humidified dark-box. Since laser-scanning microscopy allows for improved analysis of the comet images (Meyers et al., 1993), the slides were analysed with an Axiovert 100 M confocal microscope (Zeiss, Germany) at 200X magnification. A total of 100 cells (50 cells from each spot) were scored for each sample and the captured images were analysed using VisComet (1.5) image analysis software (Impuls, Germany). The parameters assessed included tail length (measured from the middle of the head to the end of the tail), tail DNA content (tail% DNA) and Olive Tail Moment (tail length × tail DNA content), which can be more accurately defined by the formula:

$$M_{\text{Tail Olive}} = (|\text{CG}_{\text{Tail}} - \text{CG}_{\text{Head}}|) \times \%\text{DNA}_{\text{Tail}}$$

where $M_{\text{Tail Olive}}$ is the Olive Tail Moment, CG_{Tail} the centre of gravity of the tail, CG_{Head} the centre of gravity of the head and $\%\text{DNA}_{\text{Tail}}$ is the percent of migrated DNA in the tail compared to the head.

2.3. MN assay

The haemolymph was dropped onto a microscopic slide and allowed to air-dry completely at room temperature. The cells were then fixed with absolute methanol for 1 min, allowed to air-dry and were subsequently stained with 5% Giemsa in phosphate buffered saline, pH 6.6 for 10 min. A total of 2000 intact cells were scored for each sample using a light microscope under oil immersion at 1000X magnification and the results were recorded as micronucleus frequency (MN frequency); the micronuclei were scored using the criteria described by Tates et al. (1980).

2.4. Statistical analyses

For the comet assay, the data obtained from assessment of the tail DNA content and the tail length

were used without transformation and the data obtained from assessment of the Olive Tail Moment were log-transformed. Normality and the homogeneity of variance were verified by the Kolmogorov–Smirnov test and the Barlett test, respectively. Two-way analysis of variance (ANOVA) was used to examine the separate effects of pollutant concentration and exposure on the level of DNA strand breakage; the α level used was 0.05. Where significant effects were detected, pairwise Tukey tests were used to identify differences between specific treatment groups (Zar, 1984). Since the sample size and the MN frequencies were low for the data resulting from the MN formation assay, an arcsine $\sqrt{P}$ angular transformation of MN frequency was used to stabilize the variance and approximate normality (Burgeot et al., 1995, 1996; Scarpato et al., 1990a,b). Subsequently, two-way ANOVA and Tukey pairwise comparisons were applied, as above. The correlation between the comet parameter, 'Olive Tail Moment', and the MN frequency was determined by Spearman rank correlation analysis.

3. Results

3.1. Comet assay

The results of DNA strand breakage, expressed as the mean tail DNA content, the mean tail length and the mean Olive Tail Moment, are presented in Fig. 1. All three measured comet parameters in both the untreated group and the solvent control remained stable and consistent throughout the 12-day exposure period and, regardless of the parameter tested no significant differences ($P > 0.05$) were observed between the untreated group and the solvent control. In contrast, each of the measured comet parameters were observed to increase in a dose-dependent manner at each time interval, with the exception of the mean tail length at days 1 and 6, after exposure to B[*a*]P. Furthermore, comparisons of the data among different treatment groups showed that there were significant differences ($P < 0.05$) among the 0.3, 3 and the 30 $\mu g\,l^{-1}$ B[*a*]P-treatment groups, regardless of the duration of exposure. Similarly, comparisons of the treatment groups within given time intervals indicated that there were significant differences ($P < 0.05$) between the 0.3 and 30 $\mu g\,l^{-1}$ B[*a*]P-treatment groups on days

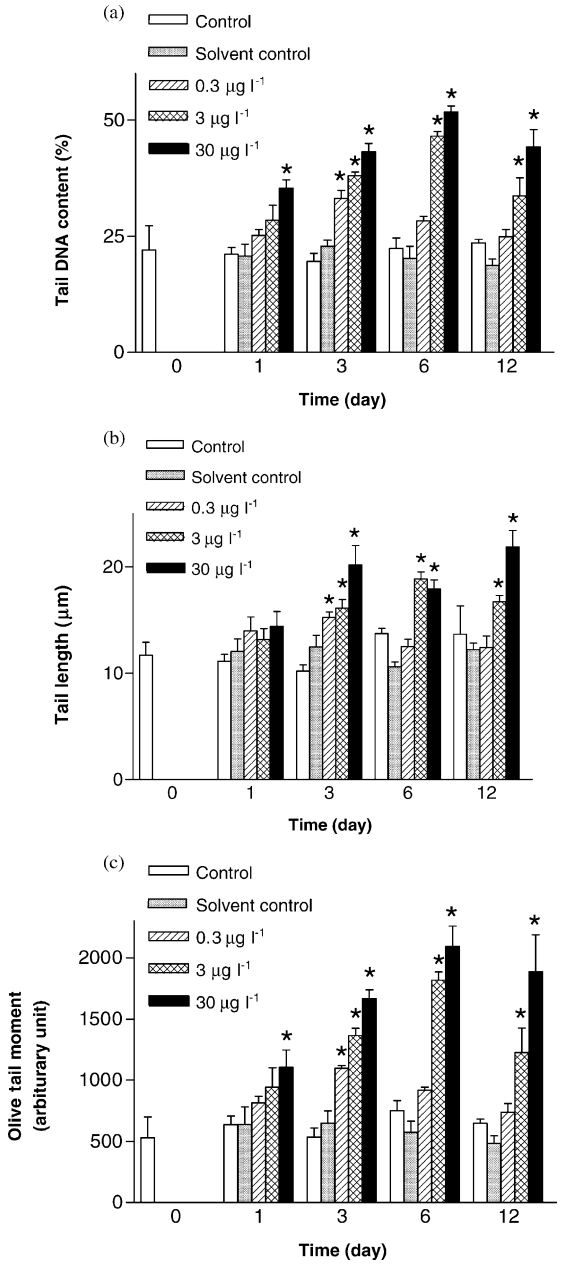

Fig. 1. Comparison of the (a) tail DNA content, (b) tail length and (c) Olive Tail Moment of haemocyte comets from *Perna viridis* from controls and various treatment groups subjected to various duration of exposure to various concentrations of B[*a*]P. All comet parameters were reported as mean ± S.E.M. Significant differences in comparison with the experimental control are indicated by $^{*}P < 0.05$.

3 and 6 and among the three treatment groups on day 12.

Amongst the 0.3 $\mu g\,l^{-1}$ group, all measured comet parameters showed an increasing trend from days 0 to 3, yet they were only significantly greater than the control values ($P < 0.05$) on day 3 and each parameter had essentially returned to background levels by day 6. For the 3 $\mu g\,l^{-1}$ exposure group, all comet parameters were elevated from days 0 to 6, but decreased from days 6 to 12 and the three parameters were significantly greater ($P < 0.05$) than that of the control values on days 3, 6 and 12. Interestingly, when the comet parameters for days 6 and 12 were compared, it was found that both the mean tail DNA content and mean Olive tail moment at day 12 were significantly lower ($P < 0.05$) than the same parameters at day 6. Finally, amongst the 30 $\mu g\,l^{-1}$ exposure group, all comet parameters varied in a similar manner as that observed in the 3 $\mu g\,l^{-1}$ group. Both the mean tail DNA content and the mean Olive tail moments were significantly greater than their control values ($P < 0.05$) at all time intervals, while the mean tail lengths were significantly greater than that of the control values ($P < 0.05$) on days 3, 6 and 12. No significant differences were observed when the comet parameters at day 6 were compared to those of day 12.

3.2. MN formation frequency

The results of the MN assay are displayed in Fig. 2. The MN formation frequencies of both the untreated group and the solvent control group remained low (ranging from 0.00 to 0.50‰) during the entire 12 days of exposure, and were statistically similar ($P > 0.05$). Similar to the results observed in the comet assay, the MN frequencies of the B[*a*]P-treated mussels appeared to increase in a dose-dependent manner at all time intervals. Moreover, as was seen in the comet assay results, comparison of the MN frequencies among different treatment groups showed that there were significant differences ($P < 0.05$) among the three B[*a*]P-treatment groups, regardless of the duration of exposure. Comparisons of the MN frequencies among the various treatment groups within particular time intervals indicated that there were significant differences ($P < 0.05$) between the 0.3 and the 30 $\mu g\,l^{-1}$ B[*a*]P exposure groups on days 3, 6 and 12.

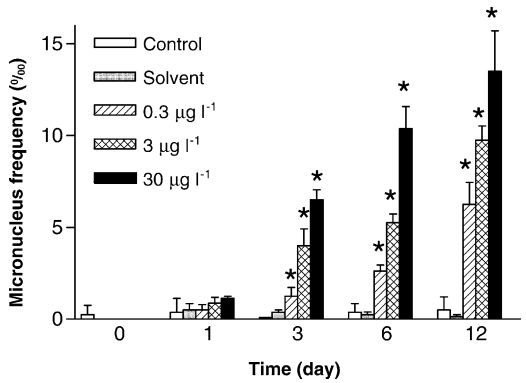

Fig. 2. Comparison of the MN frequencies (mean ± S.E.M.) in haemocytes of *Perna viridis* in control and various treatment groups subjected to various duration of exposure to various concentrations of B[*a*]P. Significant differences in comparison with the experimental control are indicated by $^{*}P < 0.05$.

The MN frequencies of all B[*a*]P-treatment groups increased continuously until the termination of the experiment. Interestingly, the greatest MN frequencies were recorded on day 12 (6.25 ± 1.2, 9.75 ± 0.78 and 13.63 ± 2.13‰ for 0.3, 3 and 30 $\mu g\,l^{-1}$ B[*a*]P, respectively); comparisons of the data within different time groups indicated that the frequencies of MN formation at day 12 were significantly higher than those at days 0, 1 and 3 regardless of the exposure concentration tested.

3.3. Correlation between comet assay and MN formation frequency

The comet assay results revealed a very high correlation among the tail DNA content, the tail length and the Olive Tail Moment and since significant positive relationships were found between the three parameters (tail length against tail DNA content: $r_s = 0.725$, $P < 0.01$; tail length against Olive tail moment: $r_s = 0.832$, $P < 0.01$; Olive tail moment against tail DNA content: $r_s = 0.932$, $P < 0.01$), only the data for the Olive tail moment was used to determine the correlation between the level of DNA strand breaks and micronucleus frequencies. A significant and positive relationship was found between MN frequencies and levels of DNA strand breaks as indicated by Olive Tail Moment ($r_s = 0.741$, $P < 0.01$).

4. Discussion

Haemocytes are the cells of the open vascular system of the mussel (Mersch et al., 1996) and have been used routinely for monitoring cytogenetic damage (Pavlica et al., 2001). The preferential usage of haemocytes in this type of research results in part from three favourable characteristics. Firstly, haemocytes provide a relatively non-invasive source of material for bio-monitoring (Fossi et al., 1994; Mitchelmore and Chipman, 1998b; Taddei et al., 2001) and, secondly, these cell types are suitable for the comet assay and the MN assay because they can be rapidly and easily sampled and cell dissociation is not required (Belpaeme et al., 1998). These advantages have the benefit of shortening the time for slide preparation and facilitating sample processing. Moreover, since cell dissociation is not required, the degree of artificial cellular damage from mechanical and/or proteolytic cell dissociation is minimized; in particular, low background levels of DNA damage simplify the identification of micronucleated cells. Finally, haemocytes also play an important physiological role in immune defence, phagocytosis, transport, excretion and detoxification of xenobiotics (Cheng, 1975); their multifunctional role has been suggested to render them more sensitive than other cells towards external factors such as the genotoxic xenobiotics (Venier et al., 1997).

The background level of DNA damage in the control and the solvent control was low. No significant differences were found between the control and the solvent control, suggesting that the solvent at the concentration used caused no effect on DNA strand breakage. It is noted that the levels of DNA strand breakage recorded in the present study were relatively high (e.g. the tail DNA content of the control group was almost 25%). This may be attributed to endogenous strand breaks or artificial strand breaks generated during sample processing. According to Mitchelmore et al. (1998), the control response of DNA damage in invertebrate cell types (e.g. digestive gland cells of *M. edulis*) using the comet assay should be relatively higher than that of the vertebrate cell types (hepatocytes and blood cells of *S. trutta*). Mitchelmore et al. (1998) argued that the relatively higher control response is not the results of the endogenous or artificial strand breaks mentioned above but is a general feature of the invertebrate, which is most likely due to the way their DNA is packaged. This phenomenon of relatively high control levels has also been demonstrated by other invertebrate studies using different DNA strand break detection techniques (Nacci et al., 1992; Everaarts, 1995; Steinert, 1996).

Using the comet assay we have demonstrated a dose–response relationship between the level of DNA strand breaks and environmentally relevant concentrations of B[*a*]P. All comet parameters have shown that there are significant differences among the three B[*a*]P-treatment groups (0.3, 3 and 30 $\mu g\,l^{-1}$) when they were compared independently of the duration of exposure. Moreover, comparisons of the treatment groups within particular time intervals have also shown clear dose-dependent responses since there are significant differences between the lowest (0.3 $\mu g\,l^{-1}$) and the highest (30 $\mu g\,l^{-1}$) B[*a*]P-treatment groups on days 3 and 6 and significant differences among all three treatment groups on day 12. In addition, the DNA damaging effect in the animal occurs earlier and lasts longer if a higher dose of B[*a*]P is applied. This can be observed from a significant increase in both the parameters of tail DNA content and Olive tail moment measured on day 3 only for the low dose, on days 3, 6 and 12 for the medium dose and at all time intervals for the highest dose. Furthermore, our results suggest that strand-breaking effects are time-dependent in *P. viridis* haemocytes. The tail DNA content and Olive tail moment values increased over time with their highest values recorded on day 3 for the 0.3 $\mu g\,l^{-1}$ group and day 6 for the 3 and 30 $\mu g\,l^{-1}$ groups. These recorded values gradually decreased to the background level for the 0.3 $\mu g\,l^{-1}$ group and to levels that were still significantly higher than the background on day 12 for the 3 and 30 $\mu g\,l^{-1}$ groups. These observed time-dependent variations in the levels of DNA strand breaks can be explained by the DNA repair theory suggested by Ching et al. (2001) and suggest that a DNA repair system may be activated after the mussel tissue has accumulated sufficient toxicant above a threshold level (Siu et al., 2003). Below this level, the DNA repair activity may be facilitated by only a basal level of DNA repair enzymes.

Unfortunately, despite its inherent advantages, the comet assay lacks standardization (Belpaeme et al., 1998). A large number of different protocols have currently been employed in different laboratories and

variations in the composition and pH of reagents (e.g., lysis solution and electrophoresis buffer) and the electrophoretic conditions (e.g. voltage, amperage, unwinding time and running time) may affect the sensitivity of the assay by affecting the shape and size of the resultant images (Rojas et al., 1999). Moreover, to date there is no uniform application of the parameters used as the measure of DNA migration (Bauer et al., 1998). For example, these parameters have been reported in many different ways, including comet moment (Kent et al., 1995), tail length (Ashby et al., 1995; Nacci et al., 1996), tail DNA content (tail% DNA) (Mitchelmore et al., 1998) and tail inertia (Vaghef and Hellman, 1995). Surprisingly, even where the same parameter is applied, the definition can be different amongst different laboratories; for example, Olive et al. (1990) defined tail length as the displacement between the mean distribution of the head and the mean position of the tail, whilst Ashby et al. (1995) alternatively recommended that the tail length should commence at the trailing edge of the cell. As a result of these differences, inter-laboratory comparison of the results obtained using different species and tissue with the comet assay is difficult.

Taking advantage of image analysis systems allowing capture and storage of comets for multiple analyses, the tail DNA content, the tail length and the Olive tail moment were simultaneously measured as endpoints of DNA damage in the present study, enabling at least intra-laboratory comparisons to be made. In our study, the tail DNA content and the Olive Tail Moment appear to give corresponding results and display similar trends. Indeed, consistent results between these two parameters have also been reported in other studies with hepatocytes and gill cells of the zebra fish (*D. rerio*) (Schnurstein and Braunbeak, 2001) and KB epithelial cells (Chauvel-Lebret et al., 2001). In contrast, the results of the tail length measurements varied from the other two parameters; unlike the tail DNA content and the Olive tail moment, the tail length did not demonstrate a significant increase in the level of DNA strand breaks in the 30 $\mu g\,l^{-1}$ B[*a*]P-treatment group on day 1 and also displayed inconsistent decreases in DNA damage on day 6 in the 3 $\mu g\,l^{-1}$ and 30 $\mu g\,l^{-1}$ treatment groups. Our study further demonstrates the inconsistency between these parameters of comet measurement within a given data set and emphasizes the need for standardization of the assay protocol, as has previously been recommended (Schnurstein and Braunbeak, 2001). Similarly, previous studies have also suggested that tail DNA content and tail moment are the most satisfactory endpoints to express the recorded DNA damage (Devaux, 1998; Hartmann and Speit, 1997; Helbig and Speit, 1997; Mitchelmore and Chipman, 1998a,b), whilst the measurement of tail length has been criticized for not adequately representing the extent of DNA damage, because the DNA migration is easily affected by differences in the gel (Collins, 1992; Fairbairn et al., 1995). It has also been noted that comets can regularly be observed with the same tail length but with different fluorescence intensities (Schnurstein and Braunbeak, 2001).

Surprisingly, the results of our study differ significantly from the results of a similar study carried out in the same laboratory in which it was reported that the same nominal levels of B[*a*]P, exposure time and regime caused a non-linear dose-dependent increase in the number of DNA strand breaks in the hepatopancreas (digestive gland) of *P. viridis* (Ching et al., 2001). In that study, it was observed that levels of DNA strand breaks increased significantly after one day of exposure at 0.3 and 3 $\mu g\,l^{-1}$ B[*a*]P. Furthermore, Ching et al. (2001) suggested an immediate activation of the DNA repair system in the hepatopancreas in the 30 $\mu g\,l^{-1}$ exposure group, since no DNA strand breaks were observed during the 12-day exposure period. The differences between the present and previous findings suggest that DNA strand breakage is a tissue specific response. Indeed, Monteith and Vanstone (1995) reported different responses involving DNA strand breakage are possible when different cell types are used. Similarly, Mitchelmore and Chipman (1998a) have previously demonstrated that in vitro exposure of the digestive gland cells of the brown trout (*S. trutta*) to B[*a*]P (0–200 μM) results in significant dose-dependent increases in DNA strand breaks, whilst the haemocytes of this organism show only a slight and statistically insignificant response when using DNA strand breaks as a marker. Indirectly, these results also suggests that the digestive gland cells may have higher metabolic activity for B[*a*]P than haemocytes and may lend weight to the notion of tissue specific differences in the mussel.

It has previously been suggested that the rapid increase in DNA damage (and concurrent chromosomal

damage) following exposure of mussels to B[*a*]P may be due to metabolically activated intermediates such as quinone and reactive by-products, for example ROS (Venier et al., 1997). B[*a*]P metabolism has been previously demonstrated in the digestive gland of mussels, *Mytilus* spp. (Michel et al., 1992, 1994; Lemaire et al., 1993; Martinez and Livingstone, 1995), and the same metabolic mechanisms are believed to occur in the haemocytes, since circulating cells are believed to metabolise xenobiotics (Kennedy et al., 1991). However, there may be significant differences in xenobiotic metabolism in circulating cells between vertebrates and invertebrates. For example, in an in vitro study of brown trout (*S. trutta*) blood cells, B[*a*]P (0–200 μM) caused only a slight and statistically insignificant increase in the proportion of DNA strand breaks, indirectly implying that circulating cells have low metabolic activity for genotoxicants (Mitchelmore and Chipman, 1998a). Moreover, the same study reported that significant increases in the proportion of DNA strand breaks in brown trout blood cells can occur after a single IP injection with 50 mg kg^{-1} of B[*a*]P for 24 h and it was suggested that exposure of the circulating cells to reactive metabolites produced from hepatic metabolism was the primary cause of cellular DNA damage in circulating cells. There is evidence that the circulating cells of an invertebrate may be more sensitive to DNA damage than the vertebrate cell; significant DNA damage has been reported in the haemocytes of the oyster (*C. virginica*), but not in the blood cells of flounder (*P. americanus*) following in vivo B[*a*]P exposure (Nacci et al., 1996). Thus, further investigation is required to characterize the relative B[*a*]P-metabolizing abilities of the circulating cells in invertebrates and vertebrates. However, irrespective of the metabolic basis for such differences between tissues, it is clear that the dose- and time-dependent DNA strand breakage monitored in the current study indicates that the response in the haemocytes of *P. viridis* will provide a more suitable genotoxicant monitoring tool than the cells of the hepatopancreas.

In the second part of the current study, the clastogenic properties of B[*a*]P were assessed by the MN assay. According to Mersch and Beauvais (1997), a minimum sample size of 500 cells from each of 4 individuals should provide representative information about the induction of MN; the results presented here should be reliable, because a sample size of 2000 cells from each of 4 individuals was taken. In addition to the advantages of haemocytes in assessing genotoxicity previously mentioned, it has also been suggested that MN can be more accurately identified in the haemocytes than in the more commonly used gill cells, because haemocytes are non-granular and have relatively lower spontaneous MN levels (Mersch et al., 1996). Indeed, in the present study the spontaneous MN frequencies ranged from 0.00 to 0.50‰ and these values are considerably lower than those of gill cells of *P. viridis*, which ranged from 0.50 to 1.1‰ (Siu et al., unpublished data). Comparable tissue-specific differences in spontaneous MN rate between gill cells and haemocytes have also been reported in other studies with different bivalve species (see Mersch et al., 1996 and references therein). Interestingly, the spontaneous MN formation frequencies of haemocytes recorded here are also lower than those values obtained in other studies using haemocytes from other bivalve species. For example, the spontaneous MN rate averaged 1.2‰ in the zebra mussel (*D. polymorpha*), ranged from 0.9 to 1.4‰ in the blue mussel (*M. edulis*) and was approximately 1‰ in the oyster *C. gigas* (Wrisberg and Van der Gaag, 1992; Wrisberg et al., 1992; Burgeot et al., 1995); the lower spontaneous frequency of MN formation in the current study is likely due either to inherently lower spontaneous rates of formation in *P. viridis* or to the improved experimental procedures in the assay, or both.

The present study has indicated that B[*a*]P-treatment of *P. viridis* caused significant MN induction in the haemocytes. It has already been suggested that DNA double strand breaks are the chief contributor to MN induction (Van Goethem et al., 1997), however it should be noted that MN induction in haemocytes may not be due to DNA strand breaks generated by direct B[*a*]P exposure in the open vascular system, since normal haemocytes do not divide in the haemolymph (Cheng, 1981). Since formation of MN requires mitotic division, micronucleated haemocytes should be formed elsewhere before they enter the open vascular system, although it is still not clear from where the haemocytes are derived. Smolowitz and Reinisch (1993) have suggested that the connective tissue could be the haemocyte-producing organ, since several types of haemocytes are commonly found there, implying that the chromosome damage

resulting in the MN formation may be occurring in the connective tissue (Dopp et al., 1996).

Although the mitotic rate of mussel haemocytes has seldom been documented, the cells should divide rapidly under chemical stress and Venier et al. (1997) showed that haemocytes are quickly recruited during defence and immune reactions. In the present study the earliest significant MN responses were observed on the third day of exposure, implying that haemocytes should divide within 72 h. Indeed, other studies have suggested that the mitotic rate of haemocytes could be as rapid as 48 h; for example Wrisberg et al. (1992) showed that a 48 h period is sufficient for observing significant MN induction following ethyl methanesulfonate (EMS) treatment of haemocytes of marine mussels (*M. edulis*). Moreover, significant MN induction was reported in haemocytes of Mediterranean mussels (*M. galloprovincialis*) and oysters (*C. gigas*) exposed to B[*a*]P for 48 h (Burgeot et al., 1995; Venier et al., 1997). However, this rapid mitotic activity does not appear to lead to a high MN response in the haemocyte. The highest mean MN frequencies recorded in the current study were only 13.63‰. Moreover, the low MN induction capacity seems to be a general feature of mussel cell types, since comparably low values have also been found in the gill cells of *P. viridis* exposed to a genotoxicant mixture (Siu et al., unpublished data).

The results of both the comet assay-based and MN frequency-based study presented here have demonstrated clear dose- and time-dependent responses to genotoxicant (B[*a*]P) exposure in the *P. viridis* haemocyte and that these responses in this cell type can be sensitive indicators (biomarkers) of a genotoxicant within an environmentally realistic range. Overall, the assays provide a set of convenient, highly sensitive, non-invasive monitoring tools of environmental exposure to genotoxicants and both the comet assay parameter ‘Olive Tail Moment’ and the frequency of MN formation correlate extremely well.

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