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Evaluation of single-cell gel electrophoresis data: Combination of variance analysis with sum of ranking differences

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ABSTRACT

Specimens of the mussel *Mytilus galloprovincialis* were collected from five sites in the Boka Kotorska Bay (Adriatic Sea, Montenegro) during the period summer 2011–autumn 2012. Three types of tissue, haemolymph, digestive gland were used for assessment of DNA damage. Images of randomly selected cells were analyzed with a fluorescence microscope and image analysis by the Comet Assay IV Image-analysis system. Three parameters, *viz.* tail length, tail intensity and Olive tail moment were analyzed on 4200 nuclei per cell type. We observed variations in the level of DNA damage in mussels collected at different sites, as well as seasonal variations in response. Sum of ranking differences (SRD) was implemented to compare use of different types of cell and different measure of comet tail per nucleus. Numerical scales were transferred into ranks, range scaling between 0 and 1; standardization and normalization were carried out.

SRD selected the best (and worst) combinations: tail moment is the best for all data treatment and for all organs; second best is tail length, and intensity ranks third (except for digestive gland). The differences were significant at the 5% level. Whereas gills and haemolymph cells do not differ significantly, cells of the digestive gland are much more suitable to estimate genotoxicity. Variance analysis decomposed the effect of different factors on the SRD values. This unique combination has provided not only the relative importance of factors, but also an overall evaluation: the best evaluation method, the best data pre-treatment, etc., were chosen even for partially contradictory data.

The rank transformation is superior to any other way of scaling, which is proven by ordering the SRD values by SRD again, and by cross validation.

1. Introduction

The mussels of the *Mytilus* sp. are commonly used as sentinel organisms for the screening of pollution and potential environmental harm [1–3]. As members of cosmopolitan species, they have been employed in numerous environmental studies from all parts of the world [4]. Several characteristics such as filter feeding, sessile life form and ability to accumulate pollutants in addition to their wide distribution, makes them favored organisms for estimating environmental pollution levels [5,6]. With a range of physiological, histological and molecular responses, including abnormal morphology, alterations of anti-oxidative status, induction of DNA strand-breaks, etc., they have been shown to be applicable for *in situ* and *ex situ* assessment of the effects of pollutants present in environment [7–9]. Most importantly, they are widely employed for assessing genotoxicity [10–13].

The comet assay or single-cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detection of DNA damage at the level of individual cells [14]. The assay is based on the ability of negatively charged loops/fragments of DNA to migrate through an agarose gel in response to an electric field. The extent of DNA migration away from the nucleus depends directly on the DNA damage present in the cells. The modification of the assay, such as alkali conditions or combination with certain enzymes (e.g. endonucleases), enables detection of DNA single-strand breaks

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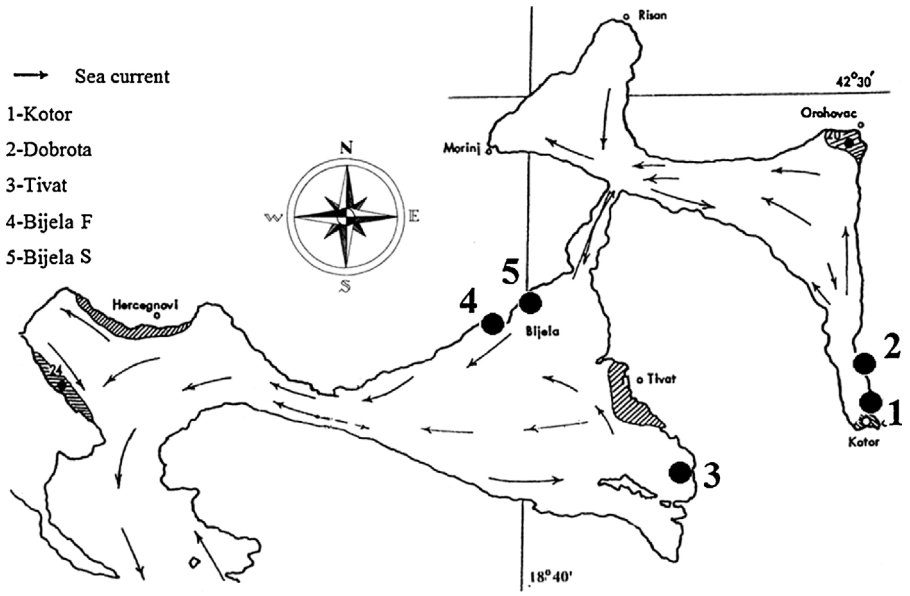

Fig. 1. Sampling sites at the Boka Kotorska Bay.

(strand breaks and incomplete excision-repair sites), alkali labile sites and cross-linking [15,16].

Since 1998, when the comet assay was first performed on *Mytilus* sp., there is a steady and continuous interest each year in applying the comet assay on this mussel species. However, there are issues related to inter-laboratory differences in the comet-assay procedure. The factors that are the most variable are the preparation of cell suspensions, the conditions of denaturation and electrophoresis, and the determination of the shape, size and amount of DNA within comets. To make the assay more robust, several approaches have been developed to quantify the extent of damage more reliably, reproducibly and meaningfully. Such quantification includes both visual examination (i.e. photographic, oculometer or non-specific image-analysis systems) and the usage of commercially available (or public domain-specific) software packages for image analysis. Such specific software packages also facilitate statistical analyses, plotting, and documentation of the data [16]. In addition, an automated system has an advantage over manual scoring, not only for easier management, but also because it diminishes the effects of observer subjectivity.

The availability of several parameters for the selection of comets (Olive tail moment, tail length, and tail intensity) had led to controversy among researchers on the question which is the most suitable for assessment of DNA damage. Similarly, which tissue (of the mussels) is best suitable for a comet assay is an important aspect to consider.

The objective of this study was to find out which estimated parameters are the most reliable for the *in situ* assessment of genotoxicity, by sampling from sites with different anthropogenic impacts in the Boka Kotorska Bay in the southern Adriatic Sea (Montenegro). The Mediterranean mussel (*Mytilus galloprovincialis*) was selected as bio-indicator organism and the data were obtained from comet assays performed on haemolymph, gills and digestive glands. The ranked measurement parameters were tail length, tail intensity and Olive tail moment. Moreover, we wanted to find out which type of scaling is most appropriate for such type of data.

2. Materials and methods

2.1. The specimen collection

The study was carried out on 84 specimens of *M. galloprovincialis* from the southern Adriatic Sea. The specimens had a shell length of 35–50 mm and were collected in July and December 2011 and May, July and October 2012 from five sites with different levels of pollution in the Boka Kotorska Bay, Montenegro (Fig. 1).

The Kotor site is under the impact of waste-waters originating from the town of Kotor and from intense marine traffic. The Dobrota site is located approximately 2 km from the Kotor site, down the current. The Tivat site is located nearby the airport and also under the impact of wastewaters originating from the town of Tivat. The Bijela S site is under the impact of wastes originating from the shipyard Bijela. At the Bijela F site, mussels were collected from the mussel farm located approximately 1 km from the shipyard, up the current. None of the sites can be considered as a true unpolluted reference site. The Dobrota site has the lowest level of pollution.

2.2. Haemolymph collection and preparation of cell suspensions from gill and digestive gland

Mussels were transferred to the laboratory in cooling boxes and subjected to the comet assay. For each sampled group of mussels (for each site) the osmolarity of Hank's balanced saline solution (HBSS) was adjusted to correspond to the level of salinity measured at the sampling site. Haemolymph collected from the adductor muscle of 3–5 mussel specimens was mixed with an equal volume of osmotically adjusted HBSS into 1.5-mL micro-tubes, centrifuged for 10 min at 2000 rpm (500 g) and the pellets were re-suspended in 60 μL of residual supernatant.

Single-cell suspensions of gills and digestive gland tissue were prepared by the method of Coughlan *et al.* [17]. Tissue was excised and chopped-up separately in 0.2 mL of osmotically adjusted HBSS by use of two fresh scalpel blades in a scissor-like movement on a petri dish, washed off gently into a 15-mL centrifuge tube with a further 2.8 mL osmotically adjusted HBSS and 0.03 mL of trypsin (0.5%). The suspensions were gently rocked for 10 min at room temperature, then 10 mL of adjusted HBSS was added and the suspension was passed through a sieve to remove any large remaining fragments. After centrifugation (2000 rpm (500 g) for 5 min), the supernatant was discarded and the pellets were carefully suspended in 1 mL of osmotically adjusted HBSS. The suspension was then centrifuged for 10 min at 2000 rpm (500 g) and the cell suspension was made in 60 μL of residual supernatant.

2.3. Comet assay

The alkaline comet assay procedure was performed under yellow light, basically as described by Singh *et al.* [14]. Microscope slides were coated with 1% normal melting-point agarose (NMP) and air dried for 24 h. To form a second, supportive layer, 80 μL of 1% NMP was gently placed on top of the 1% NMP layer and spread over the slide by means of a coverslip. The slide was placed on ice for 5 min to allow complete solidification of the agarose. After the coverslips were removed, 30 μL of cell-pellet suspension was gently mixed with 70 μL of 1% low melting-point agarose (37 °C) and pipetted on the supportive layer of 1% NMP and covered with a coverslip. After 5 min on ice the coverslips were removed and the slides were immersed in freshly made cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h. To allow DNA-unwinding, slides were placed in an electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. Electrophoresis was performed by setting the power supply at 0.5 V/cm and adjusting the current to 300 mA for 20 min. After electrophoresis, the slides were placed into freshly made neutralizing buffer (0.4 M Tris,