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Microalga Euglena as a bioindicator for testing genotoxic potentials of organic pollutants in Taihu Lake, China

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Abstract The microalga Euglena was selected as a bioindicator for determining genotoxicity potencies of organic pollutants in Meiliang Bay of Taihu Lake, Jiangsu, China among seasons in 2008. Several methods, including the comet assay to determine breaks in DNA and quantification of antioxidant enzymes were applied to characterize genotoxic effects of organic extracts of water from Taihu Lake on the flagellated, microalga Euglena gracilis. Contents of photosynthetic pigments, including Chl a, Chl b and carotenoid pigments were inversely proportion to concentrations of organic extracts to which E. gracilis was exposed. Organic extracts of Taihu Lake water also affected activities of superoxide dismutase (SOD) and peroxidase (POD) of E. gracilis. There were no statistically significant differences in SOD activities among seasons except in June but significant differences in POD activities were observed among all seasons. The metrics of DNA fragmentation in the alkaline unwinding assay (Comet assay), olive tail moment (OTM) and tail moment (TM), used as measurement endpoints during the genotoxicity assay were both greater when E. gracilis was exposed to organic of water collected from Taihu Lake among four seasons. It is indicated that the comet assay was useful for determining effects of constituents of organic extracts of water on E. gracilis and this assay was effective as an early warning to organic pollutants.

Keywords Comet assay · Drinking water · Euglena gracilis · DNA damage · Bioassay · Microalgae

Introduction

With the rapid increase in population, as well as development of industry and agriculture in the region surrounding Taihu Lake, quality of water in the lake has been decreasing (Turgeon et al. 2004; Lu et al. 2011, 2013). Several studies of pollution of Taihu Lake and its catchments have been conducted (Haruhiko et al. 2005; Zhu et al. 2006; Fu et al. 2009; Qin et al. 2010). Although relatively small concentrations of several organic pollutants have been reported to occur in water from Taihu Lake (Katsoyiannis and Samara 2005; Zhu et al. 2005; Gao et al. 2011), the risks of these chemicals on organisms of the lake or potential effects on people through drinking water had not been assessed. There is little information on organic pollutants in Meiliang Bay, Taihu Lake and no information on genotoxicity or potential of the mixture of organic pollutants to cause damage to DNA. Quantification of individual chemicals is a first step in monitoring quality of water, but comparing these concentrations to individual water quality criteria does not take into consideration chronic exposure to small concentrations of mixtures (Pellacani et al. 2006). Furthermore, the absence of

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methods or standards can preclude identification and quantification of all the residues present. Even if all the constituents could be quantified, there are insufficient water quality standards and criteria or threshold toxicity reference values to which to compare concentrations of contaminants such that the potential adverse effects can be assessed. Finally, the effects of interactions of the constituents cannot be easily assessed based on knowledge of concentrations of individual constituents.

Alternatively, biological assays can be used to effectively assess potential effects of mixtures on particular endpoints in wildlife or humans (Ohe et al. 2004) without the need to quantify all the individual constituents. Also, bioassay-directed fraction and identification can be used to determine the potential for effect, to identify sources and institute remedial actions, can be applied to identify causative agents (De Coen et al. 2000; Snyder et al. 2000; Hecker and Giesy 2011). Different unicellular and multicellular organisms, including bacteria, protozoa, algae, invertebrate and fishes have been used for this purpose. Different physiological responses such as growth rate, biomass, chlorophyll, fluorescence, and movement and other behaviors are measurement endpoints that have been monitored as criteria for the toxicity of given water sample (Kohler and Arndt 1992).

While some phyto-toxicological research has been conducted on the effects of organic pollutants on terrestrial macrophytes, the use of algae as test species has advantages (Djomo et al. 2004). The relatively rapid rates of growth of algae and their small size make them amenable to bioassays. Euglena gracilis is a unicellular photosynthetic freshwater flagellate which occurs in many aquatic ecosystems. Due, in part, to its sensitivity, to a range of toxicants, it has been shown to be a reliable organism for use in bioassays to determine to various toxicants, including metals, herbicides and other organic compounds (Danilov and Ekelund 2000, 2001; Einicker-Lamas et al. 2002). These findings suggest that E. gracilis can be used as one of a battery of suitable bioassays. Motility, cell growth, cell shaper orientation and photosynthetic parameters of E. gracilis can be used as biomarkers in evaluating the toxicity of toxic substances (Ahmed and Hadar 2010).

Induction of reactive oxygen species (ROS)-scavenging enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) are some of the mechanisms for detoxifying ROS formed during responses to chemical stressors (Mitter 2002). Changes in activities of these enzymes can be used as sensitive, functional biomarkers of exposure and adaptive responses to pollutants. But algae have not been extensively used as indicator species for studying genotoxicity caused by environmental pollutants. A few studies of genotoxicity based on the comet assay have been conducted with the flagellated, green algae E. gracilis (Watanabe and Suzuki 2002; Aoyama et al. 2003; Li et al. 2009), Chlamidomonas reinhardtii (Erbes et al. 1997; Sviežené et al. 2004) or marine diatoms (Desai et al. 2006).

In the present study, the freshwater green microalga E. gracilis was used as the test organism to determine the effect of organic compounds extracted from waters of Taihu Lake water in each of four seasons. The measurement endpoints considered were growth (cell number), photosynthetic pigment content, activities of two antioxidant enzymes (SOD and POD), and genotoxicity as measured by fragmentation during alkaline unwinding of DNA.

### Materials and methods

#### Preparation of samples

Twenty liter water samples were collected at 0.5 m beneath the surface in Meiliang Bay, Taihu Lake (31°28’20”N, 120°13’29”E). Water was collected four times during 2008, at the end of March, June, September, and December, respectively. Two gram ascorbic acid was added into each sample and mixed, then 1–2 drops of concentrated hydrochloric acid was added to keep pH < 2. All samples were refrigerated at 4 °C and kept in the dark until extraction treatment. After storing for about 24 h, the water samples were filtered through gauze and filter paper to remove suspended materials or sediments. Filtered water was then passed through a column with non-polar neutral resin XAD-2 to adsorb organic compounds. The flow rate through the column was of 30–40 ml min⁻¹. The column was eluted with methanol, acetone and dichloromethane successively. The eluate was dried under a gentle stream of nitrogen at 40 °C. Semi-volatile organic compounds (SVOCs) in the water samples were measured by GC/MS (GCQ Finnigan MAT, USA). Standard SVOCs and a deuterated internal standard (IS) mixture were both obtained from Supelco (Bellefont, PA, USA). Equilibrim time was 0.3 min and gasification temperature was 280 °C. Transfer line was kept at 220 °C and source temperature was 220 °C. Mass spectra were obtained from m/z = 50–650 base on full scan with retention time of 4 min.

#### Treatment of micro-alga

The micro-alga E. gracilis was obtained from Mid-Sweden University, Sundsvall, Sweden. Experiments were conducted in 250 ml flasks, which had been autoclaved at 121 °C for 20 min. Inoccula were added to medium with or without organics extracts. Concentrations of organic
extract included equivalents of organic residues that were 1.0×, 5.0×, and 10× fold the concentration in the source water. DMSO was used as a solvent control. The initial cell density for each experiment was 0.5 × 10^5 cells ml^{-1}. The cultures were maintained in 250 ml Erlenmeyer flasks containing a culture medium (Checucci et al. 1976) at 23 ± 1 °C under a 12 h light/12 h dark cycle provided by cool white fluorescence lights at 85–90 μmol photon (m² s)^{-1} irradiance for a period of 6 d.

Quantification of pigments

Pigments were extracted with 80 % acetone and quantified in accordance with the methods of Jeffrey and Humphrey (1975) for chlorophyll content and Strickland and Parsons (1972) for carotenoid pigment content. Concentrations were expressed as mg pigment per 10^6 cells.

Anti-oxidative enzymes assay

Activities of enzymes were determined in *E. gracilis* that had been cultured for 6 d and centrifuged at 3,000×g for 10 min and re-suspended in 1/15 mmol l^{-1} of pre-cooled sodium phosphate buffer (pH 7.0). After sonication for 5 min in an ice bath, the cell debris was removed by centrifugation at 12,000×g for 20 min at 4 °C. SOD (EC 1.15.1.1) activity was determined by use of the ferric cytochrome c method with xanthine/xanthine oxidase as the source of super-oxide radicals, and a unit of activity was defined as that described in McCord and Fridovich (1969), which was expressed as U per 10^6 cells. POD (EC 1.11.1.7) activity was determined by use of the methods described by Li (2000).

Comet assay

The comet assay with *E. gracilis* was performed following methods described by Singh et al. (1988), Aoyama et al. (2003) and Li et al. (2009) with some modifications. The slides were examined with a fluorescent microscope (BX41, Olympus, Japan). Three slides for each treatment were prepared and at least 50 cells were analyzed from each slide. Images were analyzed according to the method of Collins et al. (1995) using the comet assay software project. The effect of dose on Tail Moment and Olive Tail Moment were analyzed by one-way ANOVA using Origin 8.0 statistical software with level set at \( P < 0.05 \) and \( P < 0.001 \).

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences between control and treated samples were analyzed by one-way ANOVA using Origin Pro 8.0 statistical software. Tukey’s multiple comparisons tests with a Type I error, \( P < 0.05 \) were applied in assessing significance in the effect of each treatment. Each experiment was conducted in triplicate.

### Results

Concentrations of SVOCs

Total concentrations of semi-volatile organic chemicals (SVOCs) in Meiliang Bay, Taihu Lake were 17.459, 11.140, 11.147 and 5.675 μg l^{-1} in March, June, September and December, respectively (Table 1). The 25 SVOCs identified and quantified during this study could be divided into four classes, including polycyclic aromatic hydrocarbons (PAHs), phthalates (PAEs), benzenes series (BTEX) and others. Concentrations of SVOCs were significantly greater in March than in December. The greatest content was PAEs, followed by a higher detection content of PAHs and BTEX. The total concentration of BTEX observed in June was more than the other two seasons.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>March</th>
<th>June</th>
<th>September</th>
<th>December</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAHs</td>
<td>0.558</td>
<td>0.300</td>
<td>0.735</td>
<td>0.399</td>
</tr>
<tr>
<td>PAEs</td>
<td>16.067</td>
<td>7.840</td>
<td>9.608</td>
<td>4.428</td>
</tr>
<tr>
<td>BTEXs</td>
<td>0.540</td>
<td>1.200</td>
<td>0.686</td>
<td>0.606</td>
</tr>
<tr>
<td>Others</td>
<td>0.294</td>
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<td>0.045</td>
<td>0.162</td>
</tr>
<tr>
<td>All</td>
<td>17.459</td>
<td>11.140</td>
<td>11.147</td>
<td>5.675</td>
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Pigments bioassay

Concentrations of the photosynthetic pigments, Chl *a*, Chl *b* and carotenoid pigments in *E. gracilis* varied among seasons and treatments (Fig. 1). Concentrations of Chl *a* and Chl *b* ranged from 1.0 × 10^{-2} to 5.0 × 10^{-2} and 4.0 × 10^{-3} to 1.2 × 10^{-2} mg per 10^6 cells, respectively. The concentration of Chl *a* exposed to the 10× extracts were significantly less by 24, 11 and 30 % than that of control cells in March, June and September, respectively, except in December which showed no statistically significant differences between the control and any of the treatments with organic extract (Fig. 1a).

Chl *b* content of *E. gracilis* was affected differently by exposure to organic extracts among seasons. In March, concentrations of Chl *b* were significantly less (29 and 37 %, respectively) in cells exposed to 5× or 10× concentrations of organic extract relative to that of controls (Fig. 1b). In June, Chl *b* content in cells exposed to 1× and

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Table 1: Content of semi-volatile organic compounds in Meiliang Bay of Taihu Lake in all seasons (μg l^{-1})

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An organic extract was greater (33 and 4%) than that of the control. In September, only the concentration of Chl b in cells exposed to the 5 × extract was significantly less (8%) than that of the control. In December, Chl b content in cells exposed to 10 × organic extract was greater (33%) than that of the control.

Concentrations of carotenoids in E. gracilis were not affected significantly by exposure to organic extracts in March or fall, but were affected in June and December. In June and December, concentrations of carotenoids were greater than that of the control (18 and 13%, respectively) in cells exposed to the 1 × organic extract (Fig. 1c).

Antioxidative enzymes assay

There were no statistically significant effects of the organic extracts on SOD activity (Fig. 2a) except in June, when SOD activity was 17, 21% (P < 0.01) and 130% (P < 0.001) greater than controls in cells treated with 1 ×, 5 ×, or 10 × concentrations of organic extracts, respectively.

In March, exposure of E. gracilis to 1 × or 5 × resulted in 89 and 27%, greater POD activity, than that of the control, respectively. In June, POD activity of E. gracilis was 25% greater when exposed to 10 × extract. In September, all three concentrations of organic extract caused
statistically significant differences ($P < 0.001$), between POD activity of cells exposed to $19$, $59$, and $10^9$ concentrated organic extract which were $46$, $47$, and $53$ % less than that of the controls, respectively. In December, the trend was similar with that of June which was $146$ % greater when exposed to $10^9$ extract.

Comet assay

Organic extracts of water from Meiliang Bay caused concentration-dependent damage to DNA of *E. gracilis*. The magnitude of damage was directly proportional to exposure concentration and statistically significant, regardless of whether the endpoint monitored was Olive tail moment (OTM) or tail moment (TM). The response increased gradually with increasing concentration of organic extract among all seasons except for the $1\times$ exposure in the March (Fig. 3). When OTM was used as the metric (Fig. 3a), in March exposure to $5\times$ or $10\times$ concentrated organic extract resulted in $87$ and $174$ % greater DNA damage, respectively, relative to that of the control. In June, September and December, based on OTM, there were statistically significant ($P < 0.05$, $P < 0.001$) differences among treatments and the control. Exposure to $1\times$, $5\times$ or $10\times$ resulted in $119$, $116$ and $206$ % in June, $293$, $563$ and $1,127$ % in September, $77.36$, $88.22$ and $326.20$ % in December more DNA damage than in the controls.

Based on TM (Fig. 3b), the amount of DNA damage in March was $122$ and $238$ % ($P < 0.001$) greater than that of the control cells when exposed to $5\times$ or $10\times$ water equivalents, respectively. In June, September and December, there were statistically significant differences ($P < 0.05$, $P < 0.001$) in TM for all exposure concentrations. TM of cells exposed to $1\times$, $5\times$, or $10\times$ organic extract were greater than the controls by $154$, $192$ and $350$ % in June, $500$, $917$ and $2,336$ % in September, $101$, $46$ and $228$ % in December greater than that of the controls, respectively.

**Discussion**

Unicellular algae are ideal for the study of responses to different environmental factors and free from complications inherent in research with more complex higher plants (McCormick and Cairns 1994). Furthermore, assays using microalgae are generally sensitive, rapid and a cost-effective means to evaluate changes in environmental conditions, especially with respect to water pollution (Sosak-Swiderska et al. 1998) and provide a potentially useful early warning signal of deteriorating conditions and their possible causes. Previous studies have shown that most microalgae display relatively higher sensitivity to changes in environmental conditions, especially with respect to water pollution. For these reasons, algae are being widely used as ecological indicators and phyto-remediation organisms in polluted aquatic environments (Kelly et al. 1998).

Inhibition of photosynthesis and concomitant changes in concentrations of photosynthetic pigments has been suggested as the most integrative measure of effects of pollutants on microalgae (Franqueira et al. 2000). In the present study, organic pollutants caused significant increases in concentrations of Chl *a*, Chl *b* and carotenoid when *E. gracilis* was exposed to lesser concentrations of organic extract, especially in June. However, exposure to greater concentrations of the organic extracts resulted in lesser concentrations of these pigments, which suggests a hormetic effect. Although the reason for the observed hormesis is unknown, the lesser concentrations of organic extract stimulated accumulation of chlorophyll, and it is...
consistent with other results where the exposures to lesser concentrations of chemical stressors have caused results in stimulation (Stebbing 1982). This effect has been suggested to be due to adaptive responses of organisms to stressors (Giesy 2001). At greater concentrations the organisms can no longer respond to the stress and damage accumulates (Giesy et al. 1988; Giesy and Graney 1989). The lesser content of chlorophyll observed when E. gracilis was exposed to greater concentrations could be due to peroxidation of lipid in membrane of chloroplasts. Destabilization of chloroplast membranes is attributed to increased peroxidation of the membranes via increased production of free radicals (Aust et al. 1985). In microalga cells, formation and accumulation of free radicals can result in membrane lipid peroxidation leading to structural alterations in chloroplast and decreased chlorophyll contents, which can hinder growth of cells. Chl a was more sensitive to the effects of organic pollutants than was Chl b, and carotenoids, and these results corroborate well with those reported by Couderchet and Vernet (2003).

Cells have evolved efficient strategies to counteract oxidative stress. Antioxidant enzymes are one of the components of the systems that prevent oxidative stress in plants, with activity of one or more of these enzymes are generally greater when exposed to stressful conditions and the elevated activities correlate to increased stress tolerance (Allen 1995; Mazhoudi et al. 1997). Enzymatic and non-enzymatic reactions of free radical scavengers minimize the cellular oxidations. The enzymes SOD, CAT and POD can control cellular levels of reactive oxygen species (ROS) (Weckx and Clijsters 1996). All aerobic organisms possess the means to protect themselves from the toxic effects of reduced oxygen species. The significant changes in SOD and POD activities observed in this study are consistent with the micro-alga having been under oxidative stress as the result of exposure to chemicals in the organic extract. Different micoralga species exhibit different responses of antioxidant among seasons (Butow et al. 1997). In the case of the green alga Monostroma aff. arcticum, there is significantly greater SOD activity throughout summer (Aguilera et al. 2002). In the current study, changes in SOD of E. gracilis in response to exposure to organic extracts of water were more sensitive in June than in other seasons, while POD activity was less in June and greater in September.

Genotoxicity has also been measured directly in evaluated drinking water for environmental biomonitoring or clinical applications (Singh et al. 1988; Tice et al. 2000). The comet assay, is now widely used to measure DNA damage, during biological surveillance, and genotoxicity evaluation for different kinds of field studies, and it is becoming a primary tool for pollutant biomonitoring in the aquatic environment as well (Mitchelmore and Chipman 1998; Klobuear et al. 2003; Akcha et al. 2008). Eukaryotic cells are generally used in the comet assay and thus few reports describe the applicability of microorganisms. The comet assay has been used to determine damage to DNA in the algae, Chlamydomonas reinhardtii (Erbes et al. 1997) and E. gracilis (Watanabe and Suzuki 2002; Aoyama et al. 2003). Therefore, due to its ease of culturing, standardization of inoculum, availability in micro-alga, and its wide distribution in aquatic ecosystems E. gracilis was used as an ecological indicator to detect genotoxicity of organic extracts in the drinking water source. The results of the comet assay showed that the organic extracts of water collected during all four seasons could induce DNA damage to the micro-alga E. gracilis, which might affect long-term survival. Damage to DNA in June and September was greater than that in March and December, when relatively little damage was observed regardless of whether the endpoint monitored was OTM or TM. While the algae cells with fast growth, rapid reproduction and metabolism activity in June and September, which is relatively sensitive, were easy to be damaged. The other reason may be due to the greater concentrations of BTEX in June and September, which could produce oxidative DNA damage in human lymphocytes (Chen et al. 2008). However, compared with other assays, the micro-alga E. gracilis is more sensitive than human peripheral blood (Wu et al. 2008), mouse spermatid cells (Chen et al. 2007) and zebra fish embryos (Chen et al. 2007) and it can be used as water environment monitoring and evaluation.

Conclusions

In this study, organic extracts of water used for drinking water, collected in all four seasons from Taihu Lake could affected activities of antioxidant enzymes (SOD and POD) and induce DNA damage in E. gracilis. The results indicated that the comet assay of E. gracilis could be a biomarker for environmental monitoring and early warning, especially for evaluating mutagenic/carcinogenic changes of individuals exposed in organic pollutants, and microalga E. gracilis could be used as a bioindicator for testing genotoxic potentials of organic pollutants in Taihu Lake.

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Microalga Euglena as a bioindicator

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Conflict of interest The authors declare that they have no conflict of interest.

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