Automated image analysis of *Euglena gracilis* Klebs (Euglenophyta) for measuring sublethal effects of three model contaminants

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**ABSTRACT**

The short-term impacts of atrazine (herbicide), tributyltin (organometal) and copper on the behaviour of *Euglena gracilis* Klebs (Euglenophyta) were assessed. First, the ECOTOX automated image analysis system was used, which measured swimming velocity, cell shape, percentage of cells swimming upwards, and randomness of swimming. Next, visual observation by microscopy was used to measure percentage of cell motility and cell shape. Behavioural changes can be used as an indicator of stress in less than 24 h, potentially making them suitable for inclusion in early-warning systems for water quality. Findings indicate that *E. gracilis* is a very sensitive organism to copper, showing inhibition of motility with visual observation at 0.8 μmol/L within 1 h. The image analysis system was in general less sensitive than visual observation for detecting behavioural changes after incubation in copper. In contrast, after exposure to organic contaminants atrazine and tributyltin, the ECOTOX system detected small changes in the number of cells swimming upwards (antigravitactic behaviour) at higher concentrations.

**INTRODUCTION**

The potential to include automated systems in an early-warning water quality monitoring program is of great importance and must be considered as a cost- and time-saving alternative to chemical testing. It is essential to consider whether the system can promptly and reliably detect behavioural changes in the species studied after exposure to environmentally relevant concentrations of contaminants. This study aims to determine the suitability of the ECOTOX image analysis system in assessing behavioural parameters of *Euglena gracilis* under toxicant stress in comparison to visual observation methods, in view of its integration in an early-warning system for drinking water quality.

Biomonitoring uses organisms to obtain information about the quality of their environment using measured toxicological responses (Kettrup & Marth 1998; Kieu et al. 2001; Gerhardt et al. 2006). Advances in biomonitoring technology in the past few decades, such as the development of automated systems, have led to their increased use in early-warning systems to monitor water quality and safety (LAWA 1996; Kieu et al. 2001; Lechelt et al. 2006). Automated behavioural analysis systems can be used in conjunction with chemical analyses to supplement information for drinking water quality monitoring and ecosystem health. They do this by providing almost immediate results which include information on the bioavailability of contaminants for organisms, adding ecologically relevant and timely responses to available chemical analyses.

Automated systems measure small changes in the organisms’ behaviour, which are indicative of stress and may provide several advantages over using survival or lethality as an endpoint. Changes in locomotory behaviour are environmentally relevant, as they can provide information about the general physiological condition of organisms. They are also associated with reproduction, foraging, predation and escape, all of which can change the community structure in aquatic ecosystems (Baillieul & Scheunders 1998; Charoy & Janssen 1999; Jeon et al. 2008). Behavioural...
responses are also readily visible and faster than mortality studies, and can be detectable in much less than 24 h. Automated biomonitoring may be capable of detecting small concentrations of contaminants, synergistic effects between contaminants, and contaminants which may not be tested for in a battery of chemical tests (Maradona et al. 2012). They also provide a large cost advantage, as test organisms are readily available and systems ideally run with minimal supervision.

The system used in this study, ECOTOX (D.P.P. Häder, University of Erlangen-Nuremberg), is an automated real-time image analysis system that tracks several locomotory behaviour parameters of the single-celled flagellate *Euglena gracilis* Klebs (phylum Euglenophyra). This widely distributed species possesses both plant and animal characteristics, is genetically stable due to asexual reproduction and easily cultured, making it an ideal model organism (Tahedl & Häder 2001). *E. gracilis* exhibits a variety of behaviours such as flagellum-assisted swimming and altering cell shape between round and elongated spindle, achieved through a contractile mechanism caused by microtubule depolymerisation in the cytoskeleton (Lachney & Lonergan 1985). Cells orient themselves in the water column via phototaxis and gravitaxis, moving towards the light source under low light, away from the light source under strong light, and upwards (anti-gravitactic) in dark conditions (Lebert & Häder 1999; Häder et al. 2003; Streb et al. 2006; Richter et al. 2007). *E. gracilis* cell growth, measured by both growth rate and population density, was found to be a sensitive parameter to organophosphate pesticides (Moore 1970) as well as the heavy metals mercury, cadmium, lead (Navarro et al. 1997), nickel (Ahmed & Häder 2010), and chromium (Rochetta et al. 2006). Acute toxicity tests measuring parameters such as photosynthetic capacity have found *E. gracilis* to be sensitive to several contaminants (Danilov & Ekelund 2001).

The ECOTOX system automatically pumps together the cell culture and water sample, or contaminant solution, in fixed proportions. It performs a series of short-term static tests (10–20 seconds) whereby test organisms and water samples are incubated and cells are tracked by a camera attached to a microscope for a period of time. The images are sent to a computer, where the system’s software calculates a set of behavioural parameters. These include cell swimming velocity, cell shape (compactness), direction of swimming, and precision of orientation (r-value). Previous studies using automated image analysis systems have found that gravitactic and phototactic movement and orientation responses of *E. gracilis* are sensitive to several heavy metals (Tahedl & Häder 1999; de Kuhn et al. 2006; Streb et al. 2006; Richter et al. 2007). The system calculates direction of swimming as a percentage of tracked cells which are moving upwards, within a 120° range. It also calculates the randomness of swimming, or precision of orientation (r-value), as cells are thought to swim more uniformly under control conditions, and more randomly under stress conditions (Tahedl & Häder 1999). *E. gracilis* cell shape is measured by the system as cell form factor, or compactness, and is calculated as a ratio of circumference to area of the cell. This variety of behavioural endpoints makes *E. gracilis* an ideal test organism for an automated behavioural analysis system. Further, it has been shown to exhibit different behavioural reactions to different contaminants (Tahedl & Häder 2001), increasing the potential for response sensitivities to be used to identify categories of contaminants present in water samples tested.

To test the applicability of the ECOTOX automated image analysis system within a water quality setting, three model contaminants of concern were chosen that are ubiquitous in the Great Lakes region, have known toxicity to aquatic invertebrates, and for which maximum concentration guidelines exist in Ontario. The contaminants chosen represent different categories of contaminants: copper (heavy metal), atrazine (organochlorine), and tributyltin (organometal). Responses were measured and the sensitivity of *E. gracilis* to a variety of concentrations was assessed.

**MATERIALS AND METHODS**

**Test organisms**

*E. gracilis* strain Z was obtained from Ward’s Natural Science (86W 2650) and grown in mineral medium (Checucci et al. 1976) at a ratio of 1:5. Cells were grown in 100 mL axenic batch cultures in 250 mL Erlenmeyer flasks at 24 °C under constant light at 4000 lux. Flasks were kept on a shaker at 100 rpm, and cultures between 7 and 10 days old were used for all experiments (Tahedl & Häder 2001).

**Contaminant stock solutions**

Concentration ranges of contaminants for these experiments were chosen based on previously detected concentrations of these contaminants in the environment. High concentrations of atrazine have been found in drinking water...
(81 µg/L) and surface water in North America following spring application (Graymore et al. 2001). Prior to being banned, TBT concentrations in freshwater were found at the highest recorded concentration of 1 µg/L in several heavily-travelled harbours (Alzieu 1998). Copper is found in drinking water at concentrations of up to 1 mg/L, and was found to be toxic to aquatic invertebrates at concentration ranges between 5 and 86 µg/L (Hodson et al. 1979).

Stock solutions were made for atrazine, tributyltin (TBT), and copper. Main stock solutions were made of 500 mg/L atrazine and 100 mg/L TBT dissolved in 0.1% dimethyl sulfoxide (DMSO) as a carrier solvent. A stock solution of 5 g/L of copper sulfate was prepared in deionized water. Dilutions from the stock were made to be 1,000× concentrated for each concentration used in these experiments, so that in each experiment the contaminant solution would be added at a 1:1,000 v/v ratio to the E. gracilis-containing medium. For the contaminants being used with DMSO as a carrier, the final concentration of DMSO after this method would remain at 0.1%, a concentration which has been found to be nontoxic to algae and invertebrates (Bowman et al. 1981; LeBlanc & Surprenant 1985), which was further confirmed in the laboratory.

**Visual observation experiment – motility**

Twenty millilitres of E. gracilis cell suspension in medium were transferred into 30 mL glass vials, and 20 µL of contaminant-containing solution was added to each vial. Preliminary trials were performed with coarse concentration series of 5, 50, and 500 µg/L atrazine in 0.1% DMSO was added, as well as 1, 10, and 100 µg/L TBT in 0.1% DMSO, and 50, 100, 500, and 5,000 µg/L of CuSO₄. Cells were incubated under a constant light source for 1 h, 2 h, and 24 h. Vials were then gently shaken before a sample was taken and placed on a haemocytometer. A Canon PowerShot A710IS camera was attached to the eyepiece of the microscope and picture stills were taken from different areas of the haemocytometer within the counter grid. Each cell was assigned a number value for shape category: 0, for round cells; 1, for oval cells; and 2, for elongated cells. This experiment was repeated in triplicate.

**Image analysis system experiment – contaminant incubation**

20 mL of E. gracilis cell suspension in medium were transferred into 30 mL glass vials, and 20 µL of contaminant-containing solution was added to each vial. A concentration series of 5, 50, and 500 µg/L atrazine in 0.1% DMSO was added, as well as 1, 10, and 100 µg/L TBT in 0.1% DMSO, and 50, 100, 500, and 5,000 µg/L CuSO₄. Cells were then placed under a constant light bank with the lids loosely fitted for an incubation period of 1 h and 2 h for copper, and 2 h and 24 h for atrazine and TBT based on results from motility experiments. The ECOTOX image analysis system was then used for measurement of the following behavioural endpoints: cell shape, swimming velocity, and gravitactic orientation. The system was set to control mode, where contaminant solution and culture medium are pumped into a mixing chamber at a ratio of 1:1.

**Image analysis system experiment – controlled trials**

To test for the appropriate functioning of the ECOTOX equipment, cell readings were taken using the control mode, diluting cell cultures in a 1:1 ratio with sample water. The final concentrations of controls used after dilutions are as follows: 100% growth medium, 0.1% DMSO, 25 g/L NaCl, and 50% ethanol. The NaCl and ethanol treatments were included as positive controls. All experiments were repeated in triplicate.

**Statistical analysis**

To determine whether there were differences in E. gracilis behaviour between treatments and reference conditions after incubation in contaminants, a one-way analysis of variance (ANOVA) was carried out, and each parameter was tested against reference conditions at a particular sampling...
time. EC50 values were calculated using ED50plus v1.0, created by M. H. Vargas, Mexico, 2000.

RESULTS

Visual observation of organism’s motility

For the visual observation experiments looking at motility, no differences were detected from reference conditions in the cells exposed to atrazine or TBT, even after 24 h incubation. When looking at copper exposure, the visual observation experiment showed that *E. gracilis* cell motility is sensitive at the two higher concentrations measured (Figure 1). After 1 h, there was a drop in motility at 500 μg/L (2.0 μM Cu2+), averaging at 7.3% after 1h (p = 0.004) compared with 45.9% motile cells in reference conditions and 45.1% motile cells in 100 μg/L (0.4 μM Cu2+). After 2 h incubation, 8.5% cells were motile at 500 μg/L (p = 0.004) compared with 47.8% motile cells at reference conditions and 47.5% in 100 μg/L. At 5,000 μg/L, cell motility was found to be almost entirely inhibited, at 0.75% after 1h (p = 0.004) and 1.54% after 2 h (p = 0.004). The EC50 values for *E. gracilis* motility after copper exposure at 26 W°C were 0.947 μM after 1 h and 0.818 μM after 2 h.

However, after 24 h of exposure, no difference could be seen between control conditions and the treatments.

ECOTOX automated system

The system detected changes in the gravitactic behaviour (percentage of cells swimming upwards) after 24 h incubation in both atrazine (Figure 2), for concentrations of 50 μg/L (p = 0.003) and 500 μg/L (p = 0.005), and TBT (Figure 3), for all concentrations: 1 μg/L (p < 0.001), 10 μg/L (p < 0.001), and 100 μg/L (p = 0.005). Despite this, the number of cells swimming up remained stable and did not decrease as concentrations of atrazine and TBT increased.

After copper exposure for 1 h and 2 h, ECOTOX established a reduction in velocity at 5,000 μg/L (p < 0.001), a concentration much higher than was found with visual observation of motility. Differences in gravitactic behaviour were found after 2 h at 5,000 μg/L (p < 0.001), and after 1 h at 300 and 5,000 μg/L, but not at 500 μg/L, suggesting that this parameter does not provide consistent results. When the r-value was examined, results differed from reference conditions only at the lowest concentration of copper tested, 100 μg/L, after 1 and 2 h exposure (p = 0.24 and 0.23 respectively), while higher concentrations did not differ from the reference.

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**Figure 1** Average cell motility (visual observation) of *E. gracilis* after 1 h, 2 h, and 24 h exposure to copper. Asterisks indicate a statistically significant difference from control conditions. Bars indicate standard deviation.
Figure 2 | *E. gracilis* gravitactic orientation (automated system) after incubation in atrazine in 23.2, 231.8, and 2318.2 nmol/L. After 24 h incubation, the number of cells swimming up was significantly reduced. Asterisks indicate results significantly different from control conditions. Bars indicate standard deviation.

Figure 3 | *E. gracilis* gravitactic orientation (automated system) after incubation in TBT in 1.7, 16.8, and 167.9 nmol/L. Asterisks indicate results that are significantly different from control conditions. Bars indicate standard deviation.
DISCUSSION

There was a difference in the usability out of the endpoints measured (cell shape, gravitactic orientation, and velocity) for tracking responses after copper exposure versus exposure to the two organic compounds (TBT and atrazine). Results were not consistent when motility-related behavioural endpoints were assessed with the ECOTOX automated system. The results for the cell velocity and randomness of swimming ($r$-value) did not differ for either atrazine or TBT, even after 24 h incubation. Ohta & Suzuki (2007) had previously found cell immobilization after 5 minutes exposure to 50 μmol/L (29.8 mg/L) of TBT.

The fact that the automated system detected changes in gravitactic behaviour after 24 h incubation in both atrazine (Figure 2) and TBT (Figure 3) suggests that the automated system is capable of detecting changes that cannot be seen or measured through visual observation alone. However, based on the current set of experiments, the gravitactic response to contaminants is not following a typical sigmoid dose–response curve, and further assessment of this endpoint is necessary.

In the cases where there was a difference in $r$-value after copper exposure, the $r$-value in fact decreased, meaning that the cells were swimming more uniformly, opposite to what was previously found when looking at $r$-values under copper stress using the ECOTOX automated system (Tahedl & Häder 1999; Ahmed & Häder 2010). It is possible that this is a hormetic effect; however, at higher concentrations observed there was still no inhibition detected and $r$-values were very similar to reference conditions. This makes $r$-value an unreliable parameter when looking at copper toxicity at the concentration range examined.

To compare visual observations with parameters measured by the automated system, it was assumed that the visual observation of motility and the automated system’s measure of velocity would be equivalent, as the automated velocity value is given as an average of many cells over a 60 s period, and the lower the motility, the lower the overall velocity of cells would be. As visual observation determined that *E. gracilis* showed reduced motility under copper stress, with an EC50 of 236.8 μg/L after 1 h, it was expected that the automated system would detect a reduced swimming velocity at or near the same concentration. However, a drop in velocity was only detected by the automated system at 5,000 μg/L, a 20-fold concentration increase (Figure 4). Visual observation had determined that motility was almost entirely inhibited at 5,000 μg/L, at 0.75%, yet the same samples yielded a reduction in velocity of 46% from reference conditions. Observation of the image captured by the software suggests that the issue is not with the image analysis software or its ability to calculate parameters, but with the hardware component of the system.

![Figure 4](https://iwaponline.com/wst/article-pdf/66/8/1708/441964/1708.pdf)

**Figure 4** *E. gracilis* cell velocity after incubation in copper in 0.4, 1.2, 2, and 20 μmol/L. Asterisks indicate results significantly different from control conditions. Bars indicate standard deviation.
Gravitactic orientation and randomness of swimming are also behaviors which can be measured with ECOTOX, as it records both the percentage number of cells swimming upwards, and the randomness of cell swimming, or r-value. These cannot be measured by visual observation and therefore the sensitivity of the system for measuring these parameters cannot be determined. However, when the ECOTOX system was used in previous research, the r-value was found to be the most sensitive behavioral parameter for detecting stress induced by most of the contaminants tested by Tahedl & Häder (1999, 2001), including formaldehyde, ethanol, cyclohexim, Na-PCP, copper, cobalt, and silver, among others. Given these results, it was therefore expected that cells would swim more randomly under stress of contaminants used; however, in all trials including reference conditions the r-value was fairly low (more random swimming) and in fact increased after incubation in low doses of contaminants. These results would suggest an inconsistency in this parameter.

On the other hand, the percentage number of cells swimming upwards, as measured by ECOTOX, was sensitive enough to detect changes after 24 h incubation in atrazine and TBT. This parameter was not found to be the most sensitive for detecting any of the contaminants studied by Tahedl & Häder (1999, 2001). Changes detected after copper exposure showed some inconsistency at low concentrations, whereas at higher concentrations the lower percentage of cells swimming upwards was related to the overall decrease in the number of motile cells.

This study has reinforced the idea that *E. gracilis* is a very sensitive organism to the presence of copper, and changes in motility can be detected at concentrations between 200 and 300 μg/L in as little as 1 h. The visual observations of motility did not detect any changes after exposure to atrazine and TBT, suggesting that *E. gracilis* behavior parameters can be discriminating when exposed to different types of contaminants, such as organic contaminants and heavy metals.

The image analysis system ECOTOX was less sensitive than all visual observations for copper. Table 1 compares the LOEC between visual observations and the automated detection of the movement parameters of *E. gracilis*, showing that despite a clear impact of Cu on the swimming capacity of the organisms, ECOTOX was not able to detect any significant change in the swimming-related parameters. However, the system detected changes in gravitactic behavior after 24 h incubation in atrazine and TBT. The changes detected are inconclusive towards determining whether the ECOTOX system is sensitive enough to detect behavioral changes in *E. gracilis* caused by exposure to organic contaminants, but it may have the potential for doing so. A modification of the system to improve measures of cell motility and/or swimming velocity, such as including a horizontally placed cuvette, may improve the suitability of this system for use in a drinking water quality testing setting.

Automated image analysis systems are highly desirable for application in a water quality testing setting. However, the ECOTOX did not prove to be ready for inclusion in an early-warning biomonitoring system for water quality. Discrepancies between the automated results and visual observations indicate that more work is required to make ECOTOX a reliable instrument. When these issues are resolved, the ECOTOX system has the potential to be indicated for inclusion in a water quality biomonitoring program.

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>LOEC values obtained by visual observations of cell movement, and ECOTOX analysis of cell velocity and upward swimming</th>
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<tbody>
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<td><strong>Atrazine</strong></td>
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<tr>
<td>Visual observations: Cell movement</td>
<td>LOEC &gt; 500 μg/L</td>
</tr>
<tr>
<td>ECOTOX: Cell velocity</td>
<td>LOEC &gt; 500 μg/L</td>
</tr>
<tr>
<td>ECOTOX: Gravitactic swim</td>
<td>LOEC = 50 μg/L</td>
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</tbody>
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