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Application of methods for assessing the geno- and cytotoxicity of Triclosan to *C. ehrenbergii*

Claudia Ciniglia, Carmela Cascone, Roberto Lo Giudice, Gabriele Pinto, Antonino Pollio *

Dipartimento di Biologia Vegetale, "Università Federico II", via Foria 223, Naples 80139, Italy

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Abstract

The toxic effects of Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) on morphology and sexual and asexual reproduction of *C. ehrenbergii* were examined. In addition, the genotoxic effects of Triclosan were evaluated on the same alga using the microgel electrophoresis test, also-called Comet assay. Increasing Triclosan concentrations in the range 0.125–5 mg L^{-1} did not affect size and shape of the cells but had relevant effects on both chloroplast morphology and dimension. Triclosan inhibited the vegetative growth of *C. ehrenbergii* at 0.5 mg L^{-1}. The effects on sexual reproduction indicate that the number of *C. ehrenbergii* zygospores was significantly reduced by the application of 0.937 mg L^{-1}. The Comet assay showed that Triclosan treatments led to a dose-dependent DNA damage of *C. ehrenbergii*; 0.25 mg L^{-1} caused significant genotoxic effects and higher concentrations irreversibly altered the DNA strands. These results suggest that *C. ehrenbergii* could represent a useful organism to evaluate the whole toxicity of pharmaceuticals and personal care products (PPCPs), giving valuable information for a risk assessment.

Keywords: *Closterium ehrenbergii*; Triclosan; Sexual reproduction; Vegetative reproduction; Comet assay

1. Introduction

The occurrence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment has been worldwide reported in the last years [1]. To produce data for risk assessments of these substances, laboratory acute and chronic toxicity assays have been carried out on different organisms, ranging from bacteria to microalgae, invertebrates and fishes. As far as concern microalgae, conventional toxicity tests on PPCPs have been mainly performed on *Psuedokirchneriella subcapitata* (formerly named *Selenastrum capricornutum*), the standard test microalga recommended by US EPA and OECD guidelines [2,3]. Recently [4], it has been shown that the extention to other algal strains belonging to Cyanophyta and Bacillariophyceae enhances the possibility of generating a sound risk assessment procedure for PPCPs, as already reported for other toxic compounds [5]. However, standardized protocols for microalgae, so far used in toxicity assessments for PPCPs, are limited to inhibition tests on asexual reproduction, with 72–96 h exposure time.

The aim of this study deals with the combined application of algal growth and zygospore inhibition test (AGZI) and Comet assay on *Closterium ehrenbergii* to estimate cytotoxic and genotoxic potential of PPCPs. The so-called AGZI assay has been specifically developed in the past decade [6] for the desmidiacean unicellular alga *C. ehrenbergii*. This alga reproduces with two modes: asexually and sexually. The AGZI test is actually composed by two tests, the growth inhibition (GI) test, a standard assay on asexual reproduction, and the zygospore inhibition (ZI) test, planned to reveal interferences of xenobiotics on sexual reproduction. In this way, the AGZI test fulfils the requirement of focusing on the entire life cycle of the test organism, giving additional information for a risk assessment. This assay was developed to evaluate the effect of the exposure to microcontaminants of wastewaters

* Corresponding author. Tel.: +39 081 2538537; fax: +39 081 450165.
E-mail addresses: ciniglia@unina.it (C. Ciniglia), carcasco@unina.it (C. Cascone), rolog@libero.it (R.L. Giudice), gabpinto@unina.it (G. Pinto), anpollio@unina.it (A. Pollio).

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from sewage treatment plant (STP), such as surfactants or mutagenic compounds [7,8], but it has never been carried out on PPCPs. In addition to AGZI test, we have also performed on *C. ehrenbergii* the single cell gel electrophoresis (SCGE), or Comet assay, which is used to assess the genotoxic potential of single substances, or mixtures from different sources (chemical industries, agricultural drainage, municipal wastewaters). The Comet assay allows the estimation of the genotoxic potential of a compound also in cells which do not show proliferative activity [9], and for this reason it is preferred to other genotoxicity assays. The occurrence of genotoxicity was also compared to the results of GI and ZI assays, to evaluate the relative sensitivity to toxicants of each test.

As a model, PPCPs was selected the bisphenol known as Triclosan, a broad-spectrum disinfectant, possessing not only mostly antibacterial but also antifungal and antiviral properties [10]. As a result of its bacteriostatic activity against a wide range of both gram-negative and gram-positive bacteria it has found increasing and recent popular use in personal care products (i.e. toothpaste, deodorant soaps, deodorants, antiperspirants, detergents, cosmetics and anti-microbial creams, lotions and hand soaps). It is also used as an additive in plastics, polymers and textiles to give these materials antibacterial properties [10]. The progressively increasing use of Triclosan during the last two decades has led to its detection in the environment, inducing bacterial resistance, with very similar mechanisms to those involved in antibiotics resistance, and some of these mechanisms can also generate cross-resistance with antibiotics [10]. Estimates of predicted environmental concentration (PEC) values of Triclosan in surface waters were 0.009–0.303 μg/L for high technology plants, and 0.149–1.26 μg/L for low technology plants [11], values which are among the highest PEC estimated for a PPCPs in surface waters. On the basis of aquatic toxicity tests performed with the three main groups of aquatic organisms (algae, crustaceans and fishes), the resulting PEC/PNEC ratios for surface waters were ≥1 for low technology plants and for part of the modern plants [11]. Data on Triclosan toxicity towards microalgal strains available in literature [11,12], shall allow us a sound comparison with the results obtained with the *C. ehrenbergii* toxicity tests and to evaluate the potentiality of *C. ehrenbergii* as an assay organism complementary to the standard algal test.

2. Materials and methods

Both plus and minus strains of *C. ehrenbergii*, respectively nos. 228 and 229 of National Institute of Environmental Studies, Tsukuba, Japan, were grown into 100mL flasks containing *Closterium* medium [13]. The flasks were placed at 20±1 °C under illumination of light of 80 μE m^{-2} s^{-1} for 24 h. A logarithmic growth phase preculture was applied to the asexual and sexual reproduction tests and to the Comet assay.

2.1. Preparation of tested stock solution

For all the experiments, Triclosan (CAS nos. 3380-34-5, purity >97%) was purchased from Sigma–Aldrich. The stock solutions of Triclosan were prepared by dissolving a known quantity of compound in 80% of a specified volume of *Closterium* medium. Then, solutions were stirred for 48 h in the dark at room temperature. After this time, solutions were adjusted at the specified volume with the same medium and stored at 4 °C in the dark for 2 weeks at the most. These stock solutions (pH 7.5) were used for experiments. A wide range concentration was developed for asexual reproduction, in order to find the adequate range of toxicity for *Closterium*. For all the experiments, the toxic Triclosan concentrations fell in the range 0.125–5 mg L^{-1}.

2.2. Asexual reproduction test (GI test)

Aliquots of preculture of *C. ehrenbergii* strain 228 containing around 2000 cells were inoculated in 50 mL of MIH medium with Triclosan at each established concentration. Each test, performed in three replicates, was incubated at room temperature under a 16:8 h light/dark cycle. Observations on cell morphology were registered after 2, 24, 48 and 96 h. The cell number was counted after 5 days.

2.3. C. ehrenbergii cell size measurement

A minimum of 20 cells from an exponential-phase of growth control culture were randomly selected to determine mean cell volume and surface area. An appropriate geometric shape was selected and cell volume (*V*) and surface area (*S*) were calculated using the formulae from Hillebrand et al. [14]. Cellular dimensions were measured at light microscope using the software package LUCIA measurement version 4.80.

2.4. Sexual reproduction test (ZI test)

Aliquots of each preculture were first washed three times in MIH medium [13] lacking in nitrogen source and inoculated with the same medium. After 2 weeks, aliquots containing about 2000 cells of both mating type of *Closterium* were inoculated in 20 mL of MIH medium containing Triclosan at each established concentration. This test also included three replicates at each test concentration. The tests were carried out at 24 ± 1 °C under continuous light. The number of normal zygotes was counted using an inverted microscope (Leitz Diavert) after 5 days.

2.5. Comet assay

The Comet assay with *Closterium* was performed on cells from GI test (see Section 2.4), after 5 days, according to the modified method of Singh et al. [9]. Treated and untreated cells were collected by precipitation and embedded in a three-

layered microgel, on a fully frosted microscopic slide, composed as following: (1) a bottom layer of 1% normal melting agarose; (2) a second layer of 0.5% low-melting agarose containing 50 μL of tested and untested algal solutions; (3) an upper layer of 0.7% low-melting agarose. The slides were dipped into a lysis solution containing 300 mM NaOH, 30 mM Na_2EDTA and 0.01% sodiumdodecylsulphate (SDS) for 1 h and then in an electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA) for 15 min at 4 °C, to allow unwinding of DNA. Electrophoresis was carried out using the same buffer at 4 °C for 20 min at 25 V and 300 mA. Then, the gels were neutralized embedding the slides twice in 400 mM Tris buffer (pH 7.5); DNA molecules were stained with ethidium bromide. The DNA images were observed using epifluorescent microscopy (Nikon Eclipse E800) equipped with a digital camera. Tail length, head DNA% and tail moment were measured from 30 comets per sample, with an automatic analyzing system (TriTek CometScore version 1.0.1.1). The viability of *C. ehrenbergii* cells was assessed along the treatment, by chlorophyll autofluorescence, and after staining with neutral rot and metylene blue. The first method has revealed to be more useful for differentiating living and dead cells. The numbers of live cells (autofluorescent) and dead cells were counted in approximately 100 cells, using epifluorescent microscopy (Nikon Eclipse E800).

2.6. Statistical analysis

Data were represented by box and whiskers plots as followings: measured values for the tested compound were shown by a box including 50% of the data. The top and bottom of the box marked 75th and 25th percentiles, respectively, and the inner line the median value; the whiskers marked the 90th and 10th percentile values. To determine significant differences among each treatment group and the control group, data were assessed by one-way ANOVA when groups were homogeneous, and by ANOVA Kruskall–Wallis when groups were not homogeneous (SPSS Software Inc., Chicago). When significant differences ($P < 0.05$) appeared, Dunnet's multiple comparison test was used to isolate the group(s) apart from a control group. Growth rate inhibition was also calculated, as following: (1 − growth rate of treatment/growth rate of control) × 100 [15].

3. Results and discussion

Triclosan had toxic effect on *C. ehrenbergii* cells, as well as on asexual and sexual reproduction. Autofluorescence viability tests (Table 1) and observations on cellular morphology were performed after 2 h of exposure to Triclosan, and daily until the end of the experiments (96 h). At Triclosan concentration 0.125 mg L^{-1}, shape and size of the cells were never affected and viable cells were around 95–100% during all the experiments. At 0.187 and 0.25 mg L^{-1}, cell viability decreased after 48 h from 100 to 60%; the nucleus tended to occupy a peripheral position, and irregular-shaped chloroplasts were frequently observed, with dark green colour, more intense than in chloroplasts of control cells (Fig. 1a and b). During the following 2 days, around 20% of the affected cells showed a tendency to resume the normal morphology, and at the end of the test they were quite similar to the control ones (not shown).

Table 1
Cell viability (autofluorescence) of *C. ehrenbergii* during the treatment with Triclosan

Triclosan concentration (mg L^{-1})	Viability (percentage of living cells)			
	2 h	24 h	48 h	96 h
0	100	100	100	100
0.125	100	98	98	92
0.187	98	75	75	80
0.25	98	60	60	80
0.5	98	12	14	2
1	98	10	10	2

At 0.5–1 mg L^{-1}, 2 h of exposure to Triclosan caused effects on chloroplast morphology but not on cell viability. After 48 h, only 10% of the cells was still viable; these cells showed a smaller and shapeless chloroplast, in which several narrowings were evident (Fig. 1c).

The modifications of cell morphology observed in the experiments with Triclosan seem to be comparable to those observed with toxic compounds as Mitomicine C and other mutagens [7]. However, also at the highest concentrations tested Triclosan did not cause increasing of cell dimension and abnormal cell shape, which were observed in the case of mutagenic compounds.

In Fig. 2a the effects of Triclosan on the asexual reproduction of *C. ehrenbergii* (GI test) are shown. The NOEC corresponded to 0.25 mg L^{-1} and the LOEC to 0.5 mg L^{-1} ($P = 0.007$); the EC_{50} was found at 0.62 mg L^{-1}.

These data indicate that Triclosan affected to a much larger extent the growth rate of the standard organism *P. subcapitata*, which showed an EC_{50} of 4.46×10^{-3} mg L^{-1} [11]. The different sensitivity to Triclosan of these two algae is probably related to their different size. According to Kent and Currie [16], a good prediction of sensitivity to a toxicant is based on the surface/volume ratio: the smaller the size the higher the sensitivity. The S/V ratio of *C. ehrenbergii* (0.44), measured according to the formulae proposed by Hillebrand et al. [14], was four times higher than that of *P. subcapitata* (1.64), as reported by Kent and Currie [16].

According to EU TGD on risk assessment [17] a predicted no effect concentration (PNEC) was calculated by applying an assessment factor of 1000 on the EC_{50} for Triclosan found in GI tests on *C. ehrenbergii*. A tentative risk assessment for Triclosan was subsequently attempted by dividing the highest predicted environmental concentration (PEC) of Triclosan (1.2 μg/L) by the PNEC previously calculated. The risk quotient (RQ) was higher than 1, suggesting that the risk of Triclosan to the environment might be not negligible.

Fig. 1. (a) Vegetative cell of *C. ehrenbergii*; (b) effects of Triclosan on the vegetative cell of *C. ehrenbergii* after 2 h; and (c) effects of Triclosan on the vegetative cell of *C. ehrenbergii* after 48 h.

This result is in agreement with a risk assessment on Triclosan based on tests performed with a battery of organisms belonging to algae, crustaceans and fish [11]. According to this study, a RQ > 1 in surface waters was predicted in the case of Triclosan discharges from low technology plants to waters with low dilution.

Triclosan also influenced the sexual reproduction of *C. ehrenbergii* (Fig. 3). The effect was calculated counting the number of zygospores at the end of the tests (96 h), in control and Triclosan-treated cells (ZI test). Triclosan concentrations from 1.25 to 5 mg L^{-1} inhibited completely the sexual reproduction. At these values, many gametes apparently of normal size and shape were found in culture, but no fusion of opposite gametes occurred. At 0.9375 mg L^{-1} Triclosan caused 84% reduction of zygospore number, whereas cells exposed to 0.625 mg L^{-1} of Triclosan gave no significant reduction of zygospore number.

The similarity observed between the results from the tests on Triclosan inhibition of vegetative or sexual reproduction of *C. ehrenbergii* has been shown also by Okamura et al. [8], who reported similar values of sensitivity for GI and ZI tests on the antifouling agent Irgarol 1051. On the other hand,

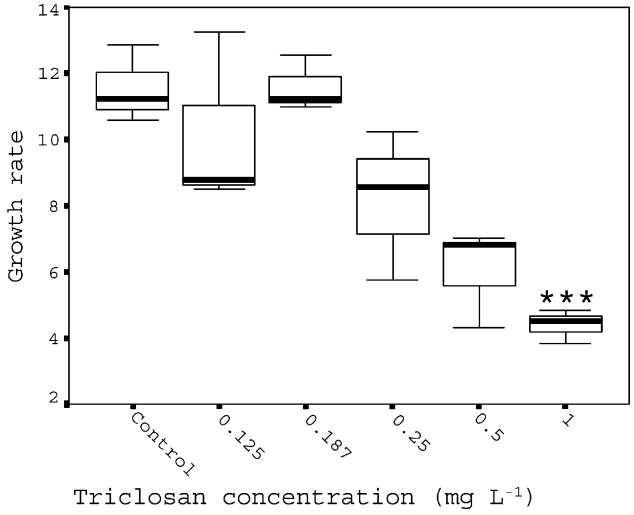

Fig. 2. Effects of Triclosan on vegetative reproduction of *C. ehrenbergii*. Data are presented in box- and whiskers-plot. Significance: $^{***}P < 0.001$.

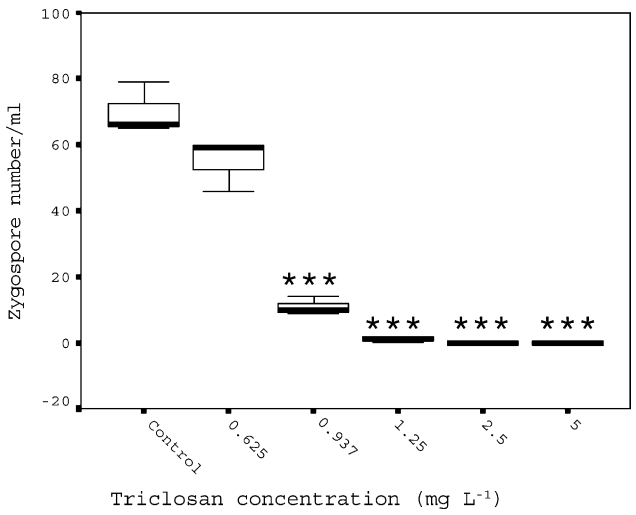

Fig. 3. Effects of Triclosan on zygopore production of *C. ehrenbergii*. Data are presented in box- and whiskers-plot. Significance: $^{***}P < 0.001$.

Kim et al. [6] found that the zygospore inhibition test on non-ionic surfactants was at least 20 folds more sensitive than the assay on growth inhibition. It is well known that sexual reproduction in *C. ehrenbergii* is mediated by the release of a pheromone [18]. Triclosan could be specifically effective as inhibitor of synthesis and/or release of this pheromone. It is interesting to note that Foran et al. [19] reported a weak androgenic activity for this compound, and more recently Ishibashi et al. [20] demonstrated that Triclosan could also act as an estrogenic compound with the potential to induce vitellogenin in male medaka *Oryzias latipes*.

The results obtained with the Comet assay indicate that the Triclosan can genetically impact *C. ehrenbergii*. Cells were exposed for 96 h to Triclosan concentrations ranging from 0.125 to 1 mg L^{-1}. Comet assays were performed at the end of the experiments to evaluate the chronic genotoxic effects of Triclosan. The DNA damage was measured as tail moment of the comet (TM = tail length × % tail DNA)/100).

Control nuclei of *C. ehrenbergii* consisted of a compact nucleus, and only occasionally were observed comets, whereas on other algae, such as *Chlamydomonas reinhardtii*, the presence of comets in untreated cells was constantly found [21]. The exposure to 0.125 mg L^{-1} of Triclosan for 96 h was uneffective on *C. ehrenbergi* nuclei. Concentrations of 0.187 mg L^{-1} did not cause a significant DNA damage. Triclosan concentration of 0.25 mg L^{-1} caused a significant increase ($P = 0.001$) of tail moment (Fig. 4). At 0.5 and 1 mg L^{-1}, the complete dissolution of nuclei occurred in all the performed assays. The dose-dependent effect observed with Triclosan-treated *C. ehrenbergii* cells was found also on *Chlamydomonas reinhardtii* [21] and *Euglena gracilis* [22], with several mutagenic compounds causing DNA damage.

Comet assay on *C. ehrenbergii* was the most sensitive test, providing valuable informations on chronic genotoxicity of Triclosan. However, it is well known that the genetic damage occurs immediately after the addition of a toxic compound. Tests carried out during the first hours from the exposure to the toxicant, coupled with specific analyses to evaluate DNA repairing, are necessary to better define the extent of damage on DNA strands.

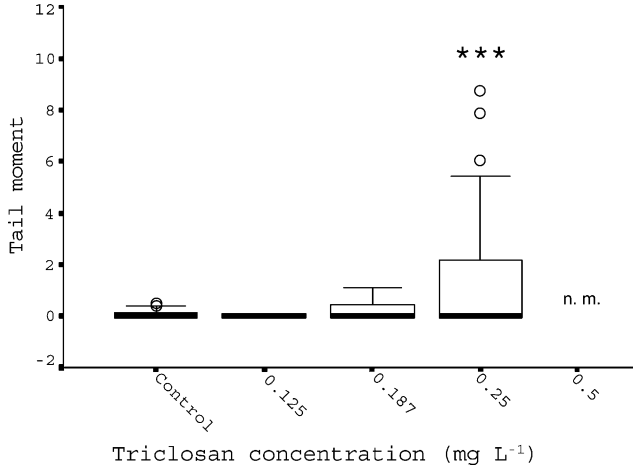

Fig. 4. Comet assay of *C. ehrenbergii* treated with Triclosan. Data are presented in box- and whiskers-plot of the comet parameter tail moment; n.m., not measurable automatically by image analysis system. Significance: *** $P < 0.001$.

4. Conclusions

Based on the observations made from this research, the following conclusions are drawn:

1. *C. ehrenbergii* is an ideal organism to apply the Comet assay, being provided of a large nucleus easily detectable and amenable to the routine protocol of single cell gel electrophoresis.
2. Comet assay has evidenced a potential genotoxicity of Triclosan on *C. ehrenbergii*, although at concentrations higher than those measured in STP effluents.
3. Sexual reproduction in *C. ehrenbergii* is sensitive to Triclosan, as already reported for other types of organisms [19].
4. The growth rate of *C. eherenbergii* is affected only by high Triclosan concentrations; the comparison with the results obtained with *P. subcapitata* confirms that this latter is a very sensitive organism as far as concerns the effects of xenobiotics on asexual reproduction.
5. The conventional set of bioassays with the standard recommended organisms can identify the toxicity of a compound along the food-net, and still play a key role in the development of a risk assessment for a xenobiotic. The application of the above-described three bioassays on *C. ehrenbergii* does not replace the use of conventional battery of test organisms, but provides an overall evaluation of the toxic effects of a chemical on the same organism, giving responses which are immediately comparable. This is not easy to obtain when different tests organisms are used to assess cyto- and genotoxicity of a compound.

References

[1] C.G. Daughton, T.A. Ternes, Pharmaceuticals and personal care products in the Environment: agents of subtle change? Environ. Health Perspec. 107 (1999) 907–938.
[2] U.S. EPA, Algal (*Selenastrum capricornutum*) growth test. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, Environmental Monitoring Systems Laboratory, Environmental Protection Agency, Cincinnati, Ohio 1989.
[3] Organization for Economic Co-operation and Development (OECD). OECD guidelines for testing of chemicals 201-alga, growth inhibition test. OECD guidelines for the testing of chemicals, vol. 1, Paris 1993.
[4] B. Ferrari, R. Mons, B. Vollat, B. Fraysse, N. Paxéus, R. Lo Giudice, A. Pollio, J. Garric, Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environ. Toxicol. Chem. 23 (2004) 1344–1354.
[5] R. Rojíčková-Padrtová, B. Maršálek, I. Holoubek, Evaluation of alternative and standard toxicity assay for screening of environmental

samples: selection of an optimal test battery, Chemosphere 37 (1998) 495–507.
[6] S- G. Kim, J. Hamada, S. Matsui, H-K. Park, Ecotoxicological assessment of non-ionic and ionic surfactants to *Closterium ehrenbergii* using algal growth and zygospore inhibition (AGZI) test, in: Proceedings of the 3rd IWA Specialized Conference on Hazard Assessment and Control of Contaminants, Ecohazard 99 (1999) 387–392.
[7] J. Hamada, S.G. Kim, S. Matsui, Morphological and lethal effects of mitomycin C, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, Benzo(a)pyrene and 4-nitroquinoline 1-oxide on a large unicellular indicator organism, *Closterium ehrenbergii* (green alga), Water Sci. Tech. 33 (1996) 305–312.
[8] H. Okamura, I. Aoyama, T. Takami, T. Maruyama, Y. Suzuki, M. Matsumoto, I. Katsuyama, J. Hamada, T. Beppu, O. Tanaka, R..J. Magiure, D. Liu, Y.L. Lau, Phytotoxicity of the new antifouling compound Irgarol 1051 and a major degradation product, Mar. Pollut. Bull. 40 (2000) 754–763.
[9] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[10] H.P. Schweizer, Triclosan: a widely used biocide and its link to antibiotics, FEMS Microbiol. Lett. 202 (2001) 1–7.
[11] L. Samsøe-Petersen, M. Winther-Nielsen, T. Madsen, Fate and effects of Triclosan, environmental project no. 861, Danish Environ. Prot. Agency (2003).
[12] D.R. Orvos, D.J. Versteg, J. Inanuen, M. Capdevielle, A. Rothenstein, V. Cunningham, Aquatic toxicology of Triclosan, Environ. Toxicol. Chem. 21 (2002) 1338–1349.
[13] T. Ichimura, Hybrid inviability and predominant survival of mating type minus progeny in laboratory crosses between to closely related mating groups of *Closterium ehrenbergii*, Evolution 37 (1983) 252–260.
[14] H. Hillebrand, C.D. Dürselen, D. Kirschtel, U. Pollinger, T. Zohary, Biovolume calculation for pelagic and benthic microalgae, J. Phycol. 55 (1999) 403–424.
[15] W.F. Blankley, Toxic and inhibitory materials associated with culturing, in: J.R. Stein (Ed.), Handbook of Phycological Methods, Cambridge University Press, 1973, pp. 207–232.
[16] R.A. Kent, D. Currie, Predicting algal sensitivity to a pesticide stress, Environ. Toxicol. Chem. 14 (1995) 983–991.
[17] European Commission 2003: Technical guidance document on risk assessment in support of commission directive 93/67/EEC on risk assessment for new notified substances commission regulation no. 1488/94 on risk assessment for existing substances. Part II. European Chemicals Bureau, Institute for Health and Consumer Protection, Ispra, Italy.
[18] R. Fukumoto, N. Dohmae, K. Takio, S. Satoh, T. Fujii, H. Sekimoto, Purification and characterization of a pheromone that induces sexual cell division in the unicellular green alga *Closterium ehrenbergii*, Plant Physiol. Biochem. 40 (2002) 183–188.
[19] C.M. Foran, E.R. Bennett, W.H. Benson, Developmental evaluation of a potential non-steroid estrogen: Triclosan, Mar. Environ. Res. 50 (2000) 153–156.
[20] H. Ishibashi, N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao, K. Arizono, Effects of Triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin, Aquatic Toxicol. 67 (2004) 167–179.
[21] M. Erbes, A. Wessler, U. Obst, A. Wild, Detection of primary DNA damage in *Chlamydomonas reinhardtii* by means of modified microgel electrophoresis, Environ. Mol. Mutagen. 30 (1997) 448–458.
[22] K. Ayoama, K. Iwaori, N. Miyata, Application of *Euglena gracilis* cells to Comet assay: evaluation of DNA damage and repair, Mutat. Res. 538 (2003) 155–162.