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The Combined Toxic and Genotoxic Effects of Chromium and Volatile Organic Contaminants to *Pseudokirchneriella subcapitata*

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Abstract In this report, the toxic effect of TCE (trichloroethylene), PCE (tetrachloroethylene), and potassium dichromate on *P. subcapitata* was investigated. The test was conducted at different concentrations of pollutants, starting from the European Community limit values defined for each analysed contaminant. Mixtures of pollutants were also tested to verify the combined effect of algae cells. Results suggest that both TCE and PCE were able to reduce *P. subcapitata* growth and metabolism starting from 0.05 and 0.02 mg L^{-1} of contaminant, respectively. PCE seems to be substantially more toxic than TCE. Chromium produces a clear effect on algae growth and esterase activity only starting from 1 mg L^{-1} of potassium dichromate; this result confirms the suitability of EU limit value. AFLP analysis showed that all tested pollutants produce DNA mutations probably due to oxygen radicals. Generally, chromium, at high concentrations, is more toxic and genotoxic that TCE or PCE. Test performed with a mixture of pollutants showed a synergic effect of chromium and organic compounds suggesting that the membrane damage induced from organic substances should increase the chromium cellular access.

Keywords AFLP · Potassium dichromate · *Pseudokirchneriella subcapitata* · Tetrachloroethylene · Trichloroethylene

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1 Introduction

Toxicity of metals has received considerable attention in recent years because industrial effluents frequently contain metals that can have adverse effects on human health and on the environment (Paine 2001; Valitutto et al. 2007). Other antropic contaminants frequently

found in waters are volatile organic contaminants (VOCs) such as trichloroethylene, tetrachloroethylene, dichloroethylene, vinyl chloride, and benzene and their degradation products. VOCs are largely used in the industry as a polymer precursor, dry cleaning agents, and degreasing agent for metals and for these reasons they are present in contaminated water in combination with different heavy metals such as chromium, lead, and arsenic (Suk and Olden 2005; Barata et al. 2006; Hsieh et al. 2006). All these water contaminants were demonstrated to be carcinogenic for human beings and animals and in some aquifers they persist for decades causing environmental and ecosystem alterations (Dekant et al. 1990; Channel et al. 1998; Hu et al. 2008; Cho et al. 2008).

Toxicity tests were performed on each of these single contaminants and provided fundamental information for the chemical risk assessment. However, taking into account that aquatic organisms are not exposed to a single chemical substance, but rather to mixtures of organic and inorganic chemicals, it is clear that an analysis of the effect of the chemical mixtures is necessary to better define the risk for living organisms and environment (Shehata et al. 1999; Barata et al. 2006; Kontas 2008). Specifically, the speciation due to the interactions between heavy metals and organic chemicals in natural waters is important in the toxicity to aquatic organisms and for water quality criteria (Burton et al. 2002; Kontas 2008). Analyses of chemical mixtures indicate that the toxicity may be equal to the sum of the fractional toxicities of individual components, or higher/lower than the sum due to synergistic/antagonistic interactions (Altenburger et al. 2000; Suk and Olden 2005).

Based on these data, the purpose of our study was to investigate the single and combined effects of metals and VOCs pollutants on aquatic organisms. Chromium, trichloroethylene (TCE), and tetrachloroethylene (PCE) were selected as metal and VOCs pollutants, respectively because, as widely reported, they enter waters from industrial discharges. In addition, they are all carcinogens and produce a wide array of toxicological effects on animals and humans (Wang et al. 2001; Hu et al. 2008).

The unicellular algae *Pseudokirchneriella subcapitata* was selected as a test organism, since some studies on water pollution showed that it is the most broadly sensitive test organism for assays of elutriates as well as surface waters and ground samples (Hsieh et al. 2006; Labra et al. 2007; Marques et al. 2008). Synergism or antagonism among chromium, TCE and PCE were evaluated by measuring growth rate and metabolic activity of *P. subcapitata* by fluorescein diacetate (FDA) esterase test. In addition, DNA damage induced by single and mixed chemical compounds was estimated by the amplified fragment length polymorphism (AFLP) technique (Labra et al. 2007). The results obtained were statistically elaborated and discussed.

2 Materials and Methods

2.1 Algal Material and Test Condition

P. subcapitata (Chlorophyceae) is a non-motile, unicellular, crescent-shaped (40 to 60 μm^3) green alga common to most fresh waters. Clumping seldom occurs in it because it is free of complex structures and does not form chains. *P. subcapitata* is diploid with modest DNA content (C value 0.2 pg), and its growth is sufficiently rapid with a duplication time of about 18–20 h.

The *P. subcapitata* strain (SAG 61.81) used in this study was obtained from the Collection of Algal Cultures, Göttingen, Germany (SAG). The algae were cultured in static and axenic condition in Bristol's medium solution (Nichols 1979). Algae were periodically transferred in a new medium (every 7 days) to obtain viable cell in log phase. These cultures were used to inoculate Erlenmeyer flasks containing 100 mL of solution to a final concentration of about 1×10^5 cell mL^{-1}. The toxicity tests were performed using chromium prepared from reagent-grade $Cr_2K_2O_7$ (Sigma-Aldrich, St. Louis, USA), TCE (Applichem, Germany) and PCE (Carlo Erba, Italy).

Two independent sets of experiments were performed. For single substances, five different concentrations were tested for each contaminant. In the case of TCE and PCE, the analysis was conducted starting from the 0.01 mg L^{-1} (Council Directive 1998/83/EC 1998) to 0.5 mg L^{-1}. Specifically, solutions containing 0.01; 0.02; 0.05; 0.2, and 0.5 mg L^{-1} of TCE or PCE were tested (Table 1).

In the case of chromium, the analysis was performed on 0.05 (European Limit value—Council Directive 1998/83/EC 1998), 0.5, 1.0, 2.5, and

Table 1 Composition of different solutions and mixtures used in the toxicological tests

Solution	Trichloroethylene (mg L^{-1})	Tetrachloroethylene (mg L^{-1})	Potassium dichromate (mg L^{-1})
T1	0.01	–	–
T2	0.02	–	–
T3	0.05	–	–
T4	0.2	–	–
T5	0.5	–	–
P1	–	0.01	–
P2	–	0.02	–
P3	–	0.05	–
P4	–	0.2	–
P5	–	0.5	–
C1	–	–	0.05
C2	–	–	0.5
C3	–	–	1.0
C4	–	–	2.5
C5	–	–	5.0
TP1	0.005	0.005	–
TP2	0.05	0.02	–
TP3	0.05	0.05	–
TP4	0.2	0.02	–
TP5	0.2	0.05	–
TC1	0.01	–	0.05
TC2	0.02	–	1
TC3	0.02	–	2.5
TC4	0.05	–	1
TC5	0.05	–	2.5
PC1	–	0.01	0.05
PC2	–	0.02	1
PC3	–	0.02	2.5
PC4	–	0.05	1
PC5	–	0.05	2.5

5.0 mg L^{-1} of potassium dichromate corresponding to 0.017, 0.17 0.35, 0.87, and 1.75 mg L^{-1} of chromium, respectively (Table 1). Basing on the toxicological effect observed for each tested substance, the composition of the 15 binary mixtures described in Table 1 was defined.

Bioassay was performed in a phytotron room under controlled condition: 23±2°C and 40.5 µmol photons m^{-2} s^{-1}. All test sets were performed in triplicate.

2.2 Microscopy Analysis

The algal cells were detected in a Bürker chamber by light microscopy, and a total count was made. Counts were performed after 24, 48, and 72 h of incubation and were repeated almost three times for each sample. Tests were conducted with a single chemical contaminant as well as with mixtures.

Membrane integrity and viability of algal cells were estimated using the fluorescein diacetate (FDA) method (Mayer et al. 1997). This is based on the incubation of 100 µL of the analysed sample with 2 µL of FDA solution (1 g L^{-1}) for 5 min, in darkness at room temperature. FDA is a lipophilic, non-fluorescent molecule that is readily taken up by cells. In the viable cells non-specific esterases cleave the ester bonds, producing fluorescein, which presents green fluorescence under blue light excitation. The fluorescence intensity depends on the cell esterase activity and on the membrane cell integrity, both these elements reveal the physiological state of the algae. The percentage of metabolic viable cells, namely FDA-positive cells, was estimated by counting in a Bürker chamber using a standard fluorescence microscope (magnification ×400) equipped with epi-illumination (Axioplan, Zeiss, Germany), 100 W halogen bulb, band pass 450–490 nm (blue) excitation filter, 510 nm chromatic bean splitter and 520 nm long-pass filter. This filter combination allows the simultaneous visualisation of FDA-green and red chlorophyll fluorescence.

2.3 AFLP Analysis

DNA was extracted from samples after 72 h treatment (including the control) using the Plant Genomic DNA Miniprep Kit (Sigma-Aldrich), starting from 100 mg of fresh algae material.

AFLP was performed according to Labra et al. (2007). The selective amplification was carried out using primer pairs E01-M01, E01-M02, E33-M01, E34-M01 E34-M02, E35-M01, E35-M02, E38-M01, E39-M01 and E39-M02 (Labra et al. 2007). A total of 1.5 µL of the PCR-amplified mixture was added to an equal volume of loading buffer (80% formamide, 1 mg mL^{-1} xylene cyanol FF, 1 mg mL^{-1} bromophenol blue, 10 mM EDTA, pH 8.0), denatured for 5 min at 92°C, loaded onto a 5% denaturing polyacrylamide gel and electrophoresed in TBE buffer for 3 h at 80 W.

The gel was fixed in 10% (*v*/*v*) acetic acid and exposed to X-ray film for 24 h. Polymorphic bands were scored by visual inspection of the resulting autoradiogram.

2.4 Statistical Analysis

The range of pollutant concentrations producing growth inhibition and cell damage was evaluated by counting the total algal cells and fluorescent cells at different times from the beginning of the treatment. Three independent experiments were performed for each treatment. Data were used to calculate mean values ± standard deviations. Data were statistically analysed by Statistica for Windows v. 6.0 (StatSoft Italia srl 2001) and StatGraphics programme for Windows (version 5.0, Manugistic, MD, USA). The Duncan test for a multiple sample comparison was applied when normality and homogeneity of variance were satisfied.

First, we analysed the response to single contaminants and mixes testing the hypothesis of no difference between percentages of live cells with respect to the control groups. Low dosages showed no effect, while a strong evidence of growth reduction was shown for higher dosages (see sections below for details). In order to analyse joint doses response, we followed the statistical approach of Gennings et al. (1997). We used data from single chemicals exposure to build a linear model where the percentage of live cells is an affine function of the chemicals quantities. The model showed a good fit to data. Then, we produced prediction intervals for the mean percentage of live cells under the hypothesis of additivity of the effects. Several departures from additivity were observed: if the observed mean was larger than the largest predicted response, we inferred an antagonistic effect of the two chemicals; if the observed mean was smaller than the smallest predicted response, we inferred a synergism between the two chemicals.

In the case of the AFLP analysis, each band detected after electrophoresis was scored as a binary character for absence (−) or presence (+). All the analysed samples were compared with the control sample. Similar genotypes were defined as “a”, with a = ++, or “d”, with d = −−; dissimilar genotypes were defined as “b”, with b = +−, or “c”, with c = −+. The algorithm used to define genetic similarity was the simple matching (SM) coefficient:

$$SMij = a + d/a + b + c + d,$$

where a and d are the numbers of bands present or absent, respectively. Data were analysed by the NTSYS-pc computer software and the matrices of similarity were then analysed using the UPGMA cluster method.

3 Results

3.1 Algae Growth Inhibition in Response to Single Contaminants

We first investigated the effects of the tested contaminants on *P. subcapitata* after 24, 48, and 72 h of treatment. For TCE and PCE contaminants, the analyses were performed at 0.01, 0.02, 0.05, 0.2, 0.5 mg L^{-1}, while for chromium, solutions containing 0.05, 0.5, 1.0, 2.5 and 5.0 mg L^{-1} of potassium dichromate were tested. Results are described in Fig. 1a, b, c. There was no statistical evidence of algae growth disruption at concentrations of 0.01 and 0.02 mg L^{-1} of TCE or PCE (Fig. 1a, b). Evidence of growth inhibition was detected at 0.05 mg L^{-1} of TCE; the value is borderline after 24 h (the p value equals 0.05) but becomes increasingly clear the following days: after 72 h the p value is of order 10^{-4}. Clear growth reduction was observed in the presence of 0.2 and 0.5 mg L^{-1} of TCE (the p values being from 0.001, at 0.2 and after 24 h, to 10^{-6} at 0.5 after 72 h). The PCE treatment at 0.02 mg L^{-1} showed a growth reduction already after 24 h (p value equal to 0.02) but was much more clear after 48 and 72 h (p values of order 10^{-3} and 10^{-4}). Higher dosages of PCE were clearly effective starting after 24 h (p values at most 10^{-4}), while at the dosage of 0.01 no significant effects were detected (Fig. 1b).

A clear effect of growth inhibition was shown also after exposure to potassium dichromate (Fig. 1c), but only starting from 1 mg L^{-1}, (p value 0.03 after 24 h, afterwards it is lower than 10^{-3}). Higher concentrations are even more effective. All significant concentrations have effects that become increasingly significant as time progresses.

3.2 Algae Membrane Integrity and Metabolism Activity in Response to Single Contaminants

Cell metabolism and membrane integrity have been evaluated using FDA test. The number of algae with intact membrane and able to cleave the FDA ester bonds

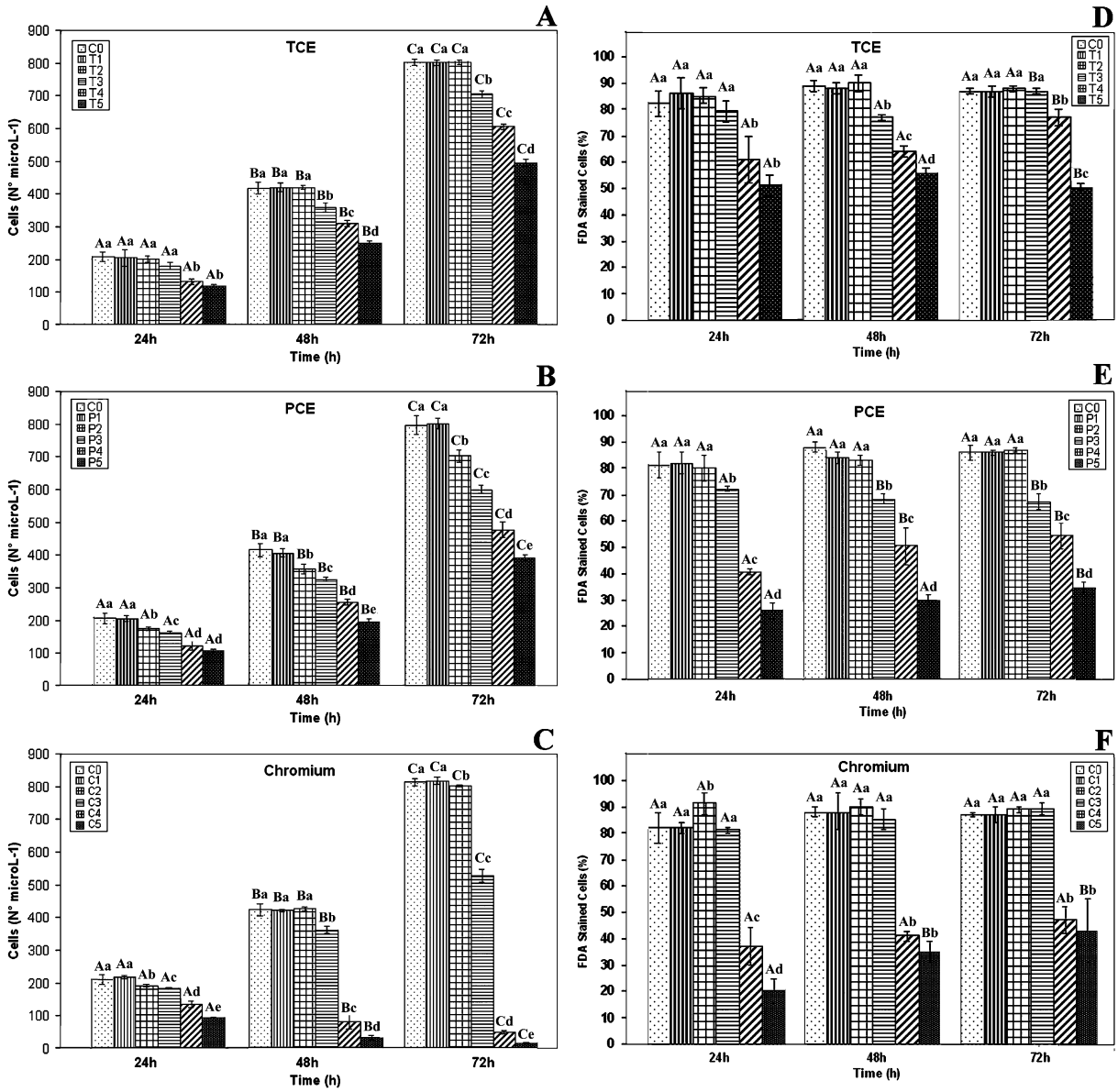

Fig. 1 Total algal number and percentage of algae stained with FDA detected by microscopy analysis in response to TCE (**a** and **d**), PCE (**b** and **e**) and potassium dichromate (**c** and **f**) at different concentrations and times (24, 48 and 72 h). The experiments were conducted in triplicate and results are shown as the mean with standard deviations. The letters indicate the results of Duncan's multiple range test ($p<0.05$). Means with standard deviations followed by the *same letters* are not significantly different. *Uppercase letters* for comparisons among different treatment times, *lowercase letters* for comparisons among different pollutant concentrations

producing green fluorescent fluorescein was used to score the results. Data obtained from tests conducted with single contaminants are shown in Fig. 1d, e, f.

There was no statistical evidence of a reduction of FDA fluorescence at concentrations of 0.01 and 0.02 mg L^{-1} of TCE or PCE (Fig. 1d, e). Evidence of fluorescence reduction was first detected in samples treated with 0.05 mg L^{-1} of TCE, only after 48 h of treatment (the p value being of order 10^{-4}). A clear decrease in fluorescein fluorescence was observed with higher TCE concentrations (0.2 and 0.5 mg L^{-1}) with the p values being from 0.02, at 0.2 mg L^{-1} and after 24 h, to 10^{-6} at 0.5 mg L^{-1} after 72 h. The PCE treatment at 0.05 mg L^{-1} showed effects on fluores-

cein fluorescence already after 24 h (the p value equals 0.04) but was much more clear after 48 and 72 h (p values of order 10^{-4}). Higher dosages of PCE were clearly effective starting after 24 h (p values at most 10^{-4}), dosages of 0.02 and lower produced no significant effects. A clear effect was shown also after exposure to potassium dichromate (Fig. 1c), but only starting from 2.5 mg L^{-1} (p values at most 10^{-3}), while there was no evidence at concentrations of 1 mg L^{-1} and lower.

The linear regression model (percentage of live cells equalling an affine function of the chemicals' concentrations) showed a good fit: its R^2 ranges from 0.839 to 0.863. All predictors (TCE, PCE, and chromium concentrations) resulted highly significant in the regression. Nevertheless, PCE and chromium are more relevant (the hypothesis of no relevance is refused even at a significance level of 10^{-14}) while the significance of TCE has p value 10^{-8}.

3.3 Algae Growth Inhibition in Response to the Chemical Mixtures

Based on the results of toxicological tests conducted with TCE, PCE, and chromium at different concentrations (Fig. 1) the binary mixtures of the three contaminants were defined to investigate the combined effect. A total of 15 mixtures (Table 1) were tested at different times (24, 48, and 72 h) on *P. subcapitata*. Data on growth inhibition are described in Fig. 2a, b, c. Very low dosage mixtures such as TP1, TC1, and PC1 did not show clear evidence of growth inhibition. All other tested mixtures induced a clear growth reduction (the p values are at most 10^{-3} after 48 h). Lower concentrations (TP2, TC2, TC4) tend to show their full strength only after 48 h: the p value after 24 h is about 0.02, while significance increases after 48 and 72 h. All mixtures of PCE and chromium, besides PC1, show an effect (the p values are at most 10^{-3}), while a concentration of 1 mg L^{-1} of chromium in association with TCE needs time to be effective, the combination with PCE proved to be more dangerous: the significance is already about 10^{-3} after 24 h.

3.4 Algae Membrane Integrity and Metabolism Activity in Response to the Mixtures

The effect of different mixtures on algae metabolism and membrane integrity at different times was described in Fig. 2d, e, f. Very low dosage mixtures such as TP1, TC1, and PC1 did not show clear evidence of reduction of fluorescein fluorescence. All other tested mixtures induced a clear reduction (the p values are at most 10^{-3}); a clear dose/effect relationship was observed with these tested mixtures (TP2–TP5 of Table 1), especially after 48 and 72 h of exposure. We remark though that a concentration of 1 mg L^{-1} of chromium in association with TCE or PCE produced a clear effect only after 48 and 72 h. The combination of chromium with PCE proved to be more dangerous than the mixture consisting of chromium and TCE (Fig. 2 e, f). Moreover, we note that no evidence of response to single chemicals was detected at the concentrations of TP2, TC2, TC4, PC2, but there was a clear effect of combined pollutants.

The interest arisen by mixtures became evident also with a linear regression: the linear model involving both single chemicals and mixtures showed a worse fit to data than the model with only single contaminants (the R^2 at 72 h is 0.661), indicating departure from additivity. Moreover, the observed means of percentages of live cells where clearly smaller than the predicted means, under the additivity hypothesis (see Table 2). From the linear regression, we also deduce that potassium dichromate is more toxic in presence of other pollutants such as TCE and PCE, since the data analysis using also mixtures shows an increased significance of chromium concentrations (its p values are of some orders of magnitude smaller than the ones of PCE, while TCE remains the less toxic of the three—its p value being of order 10^{-9}). We remark that the synergistic effect of mixtures involving potassium dichromate and TCE or PCE (namely TC2, TC3, TC4, and PC2) has an antagonistic effect within the 24 h and only later shows a synergistic toxicity, suggesting that any similar study should be conducted for at least 72 h.

3.5 AFLP Data

To verify the genotoxic effect of the analysed organic and inorganic contaminants and quantify the DNA damage, AFLP analysis was performed on DNA extracted from the control and the 72 h treated algae. Ten different primer combinations were used to estimate the genetic differences between the control and each analysed sample. Table 2 summarises data

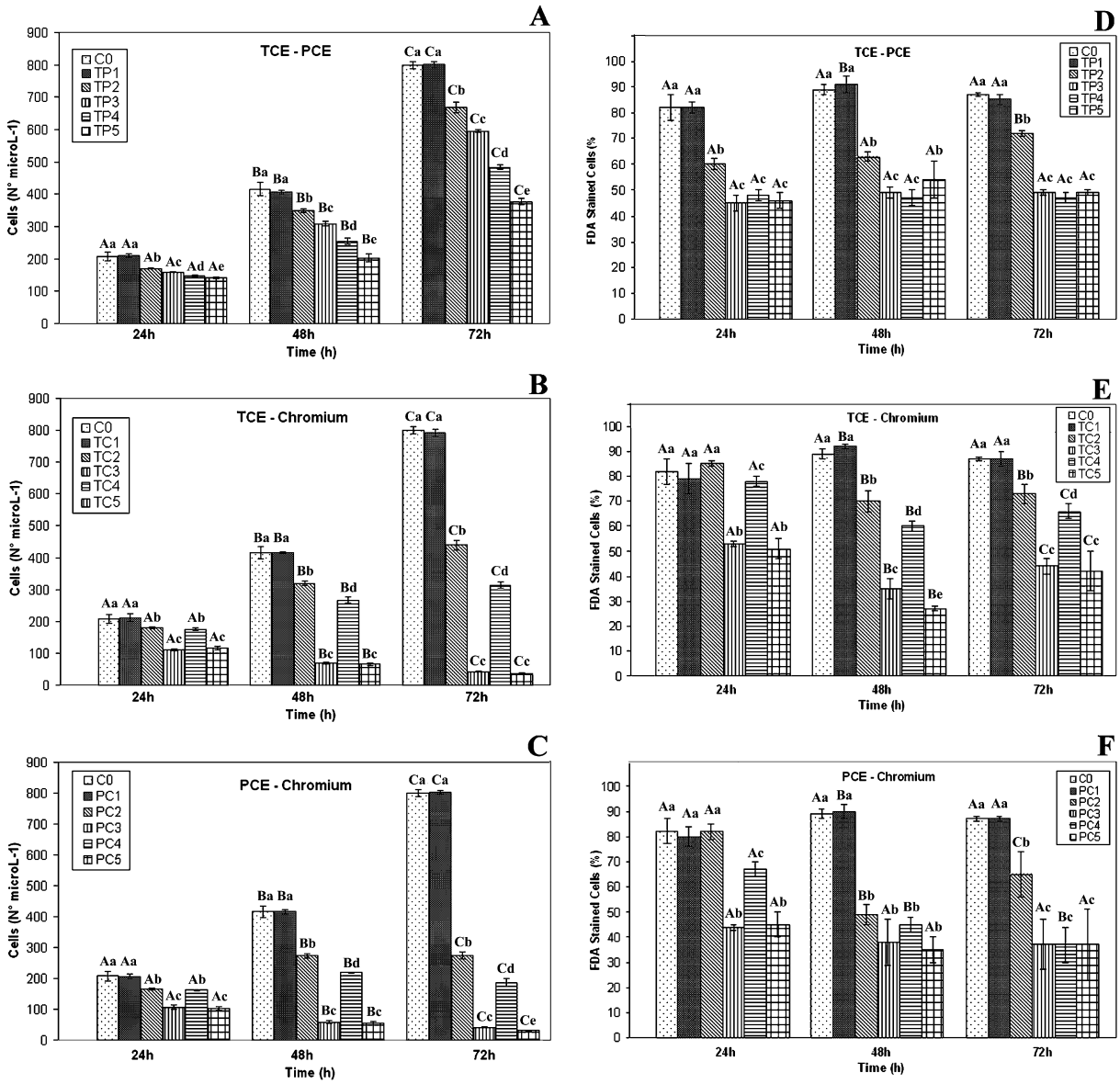

Fig. 2 Total algal number and percentage of algae stained with FDA detected by microscopy analysis in response to different mixtures, TCE-PCE (**a** and **d**), TCE–potassium dichromate (**b** and **e**) and PCE–potassium dichromate (**c** and **f**) after different times of treatment (24, 48 and 72 h). The experiments were conducted in triplicate and results are shown as the mean with standard deviations. The *letters* indicate the results of Duncan's multiple range test ($p<0.05$). Means with standard deviations followed by the *same letters* are not significantly different. *Uppercase letters* for comparisons among different treatment times, lowercase letters for comparisons among different mixtures

obtained for every AFLP primers pair. A total of 252 bands were detected; of these, 148 were polymorphics. The value of polymorphism detected for each primer combination (Table 2) demonstrated the usefulness of the ten selected primer pairs, with E33-M01 and E39-M01 showing the highest and the lowest percentage of polymorphism, respectively.

Data of similarity matrix produced by measuring the proportion of shared bands between the control samples and each of the analysed samples are shown in Table 3. The SM similarity index among accessions varies from 0.978 (high genomic similarity with the control) to 0.676. AFLP markers were found to be highly reproducible with an overall error rate of 2–3%

Table 2 Number of total bands, polymorphic bands and percent of polymorphism of the 10 primer combinations used for AFLP analysis

Primer combinations	Total band	Polimorphyc band	Percentage of polymorphism
E01-M01	41	22	54.65
E01-M02	37	21	56.75
E33-M01	14	5	35.71
E34-M01	16	10	62.50
E34-M02	21	13	61.90
E35-M01	19	12	63.15
E35-M02	23	14	60.86
E38-M01	30	18	60.00
E39-M01	29	19	65.51
E39-M02	22	14	63.63
Total	252	148	

(Vos et al. 1995). Based on this data, we suggest that algae exposure to low concentration of TCE (T1, T2, and T3), PCE (P1 and P2) and potassium dichromate (C1 and C2) did not show a significant degree of DNA polymorphism in comparison to the control sample (Table 3). Significant levels of genomic modification were detected in the algae treated with consistent concentrations of both organic contaminants. In comparison to TCE or PCE, a stronger genotoxic effect was revealed for the potassium dichromate, especially at the highest concentration (C5 sample).

Table 3 Genetic distances between control and treated samples defined basing on similarity matrices computed by simple matching (SM) coefficient

Single contaminants	Control	Mixtures	Control
T1	0.977	TP1	0.978
T2	0.978	TP2	0.976
T3	0.978	TP3	0.952
T4	0.948	TP4	0.937
T5	0.936	TP5	0.917
P1	0.976	TC1	0.972
P2	0.976	TC2	0.908
P3	0.948	TC3	0.754
P4	0.924	TC4	0.901
P5	0.909	TC5	0.746
C1	0.976	PC1	0.974
C2	0.977	PC2	0.900
C3	0.897	PC3	0.742
C4	0.809	PC4	0.853
C5	0.676	PC5	0.738

Dendrogram of Fig. 3 is a graphical representation of the obtained AFLP data. A dendrogram consists of many U-shaped lines connecting objects in a hierarchical tree; the height of each U represents the distance between the two objects being connected and the differences between each other. In our analyses, the dendrogram showed the relationship between treated samples in comparison with the control one. This statistical analysis allows the quantification of the damage on DNA induced by different substances or mixtures.

A correlation between the frequency of polymorphic bands and the increasing potassium dichromate or organic pollutants was observed, as shown in the dendrogram.

Different polymorphic bands were detected in each of the algae treated with VOC or potassium dichromate; this indicates an absence of preferential mutation sites in the genome. In all cases, polymorphisms were due to the loss of amplified bands in the treated samples compared with the control one. Few polymorphisms (seven to 17 bands, data not shown) were detected in the algae treated with TP3, TP4, and TP5 mixtures. Plants exposed to mixtures constituted by TCE or PCE and chromium show a significant degree of polymorphism if compared to the control sample, with the exception of TC1 and PC1. Generally, the genotoxic effect was evident in the mixture consisting of VOC and high chromium concentration (2.5 mg L^{-1} of potassium dichromate) such as TC3, TC5, PC3, and PC5 (Table 3 and Fig. 3). These mixtures showed a stronger genotoxic effect than C4 (2.5 mg L^{-1} of potassium dichromate), indicating a synergic genotoxic effect of the mixtures compose with organic

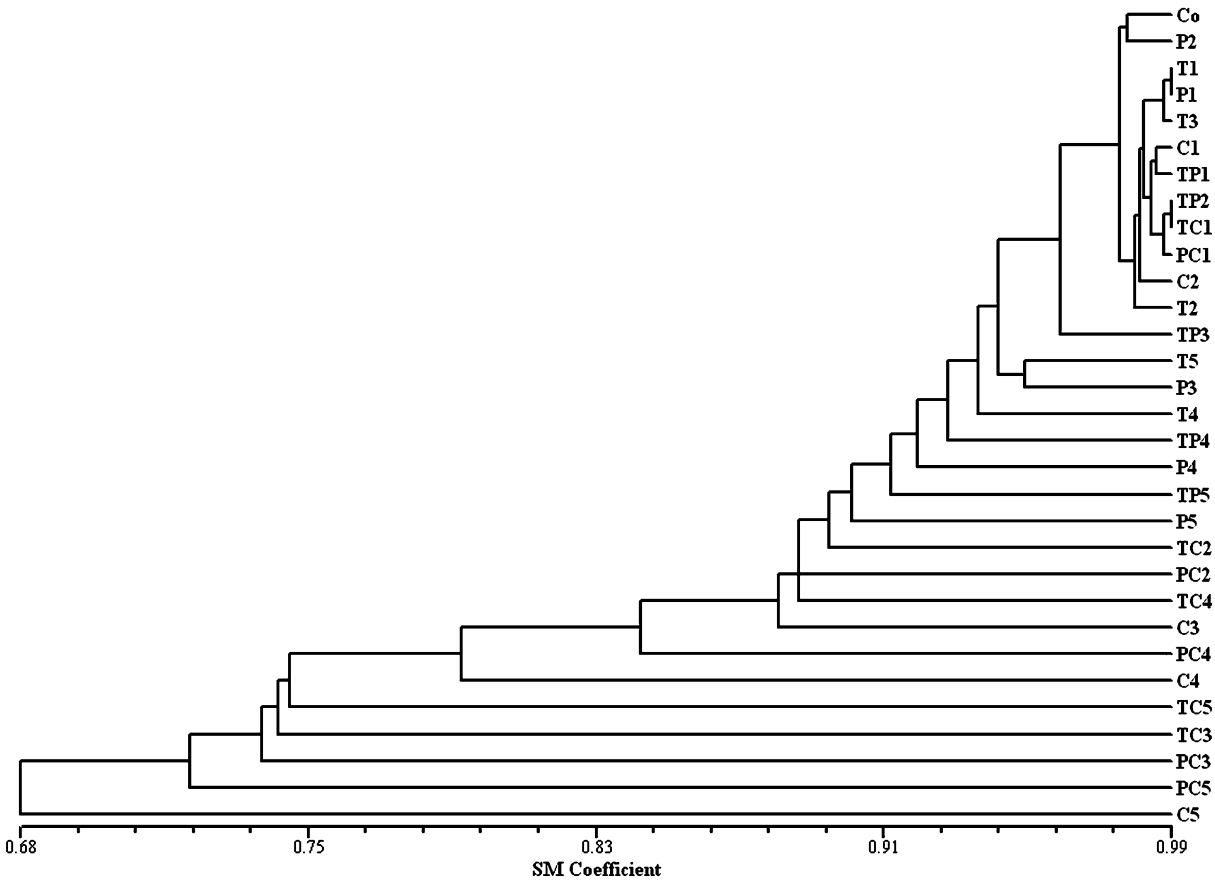

Fig. 3 Dendrogram, produced with the UPGMA method, showing SM coefficient distance among the control and treated samples. The dendrogram was based on a total of 252 scorable AFLP bands

pollutants and chromium. However, C5 results as the most genotoxic solution, able to induce about 30% of DNA polymorphism in comparison to the control samples (Table 3 and Fig. 3).

4 Discussion

4.1 Toxic Effect of Single Contaminant and Mixtures

Our work showed that *P. subcapitata* is a sensitive organism to test organic and inorganic contaminants in the water and represents a suitable bio-indicator (Hsieh et al. 2006; Labra et al. 2007). Toxicological tests led with increasing concentrations of contaminants and mixtures showed the dose–response for each tested substance. In the case of TCE and PCE our analysis confirmed the suitability of European Community limit values (0.01 mg/L) described for drinking water (Council Directive 1998/83/EC 1998); the toxic effect on algae was detected only starting from the concentration of about five times higher than the EU limit value. In the case of potassium dichromate, a strong growth inhibition effect was observed at 0.5 mg L^{-1}, while at the EU limit value (0.05 mg L^{-1}), no significant effect was observed.

Comparing the effect of PCE and TCE, the first seems to be substantially more toxic to *P. subcapitata* cells than TCE; since two- to threefold of TCE was required to have the same effect on algae. This data agree with the test conducted on CHO-K1 cells (Wang et al. 2001) or in animal models (Lash et al. 1998a, b). This higher toxicity of PCE in comparison to TCE can be attributable to the higher number of chloride atoms characterising PCE.

TCE and PCE are known to produce membrane damage through increased lipid peroxidation (Channel et al. 1998). Our test, conducted by FDA method,

showed a clear perturbation in the membrane integrity in presence of single and combined organic pollutants resulting in a reduced or a lack of fluorescence. A dose-dependent decrease in fluorescein fluorescence was observed after exposing the algae to consistent TCE and PCE concentrations and a synergic effect was also detected for most of the tested organic mixtures (Table 4), starting from 24 h of treatment. It is generally considered that the potential toxic effect of PCE and TCE on living organisms results from biological active metabolites rather than from the parental compounds themselves (Dekant et al. 1990; Lash et al. 1998, b). Glutathione (GSH) is known to protect cells from pollutants through conjugation with the toxin, resulting in a lower amount of toxic intermediates and thus reducing the injury effect. The *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) and *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC) are two metabolites in kidney derived from GSH conjugation of TCE and PCE, respectively (Lash et al. 1998, b). Both DCVC and TCVC induce cell toxicity and apoptosis but the cellular response is quite different. Previous investigations conducted on rat cells showed that DCVC sulfoxide had been shown a rapid addition with GSH and DCVC sulfoxide administered to rat resulted in hepatic and renal GSH depletion (Sausen and Elfarra 1991). TCVC sulfoxide reacts rapidly with GSH, although not as rapidly as DCVC sulfoxide, whereas the parent compound TCVC was much more stable and toxic. Additionally, in animals, TCE and PCE is also metabolised by cytochrome P450-dependent oxidation. The first step in TCE and PCE oxidation may involve the formation of an unstable epoxide (Miller and Guengerich 1982) or the generation of a TCE/ PCE -P450 intermediate (Wu and Berger 2007). Experimental data showed that the reactive metabolites derived from cytochrome P450-dependent oxidation of TCE or PCE bind to proteins and enzymes (Halmes et al. 1996) and have been directly associated with cell toxicity. Basing on these data, we assume that in algae, the toxic effect induced from TCE or PCE is related to the chemical structure and stability of each intermediate compound derived from the cell detoxification process. The simultaneous presence of the TCE and PCE in plant cells could trigger both common and individual cellular detoxification responses with the consequent production of biological active degradation products causing synergistic toxic effects.

A clear interaction was also observed for mixture composed from potassium dichromate and TCE or PCE: negative synergic effects on cell viability at 48–72 h, followed by antagonism or additivity at 24 h of treatment were observed. Chromium exhibits different physiologic and toxicologic effects depending on its oxidation state. Chromium metal is generally inert and non-toxic; Cr(VI) is more soluble than Cr(III), it is also more bioavailable for living organisms. Although it is proved that trivalent chromium is essential for humans and animals (Labra et al. 2004), researchers still debate whether or not trivalent chromium is essential for plants (Sharma et al. 2003). At high concentrations, Cr(III) may inhibit plant growth (Sharma et al. 2003) and hexavalent chromium is toxic for both plants and animals (Paine 2001). Generally, Cr(VI), in contrast to Cr(III), can readily cross algae cellular membranes via non-specific anion carriers (Paine 2001); however, when the biological membrane is damaged by TCE or PCE, all the chromium oxidation forms could move in the cell (Speranza et al. 2007). Basing on this, we conclude that after 48 and 72 h of organic and chromium mixture treatments, the algae membrane is clearly compromised; thus the uptake of chromium in the cell reaches high concentrations producing a drastic toxic effect.

4.2 Genotoxic Effect of Single Pollutants and Mixtures

Genotoxicological studies conducted with TCE and PCE suggested controversial results; tests on human cell with both these compounds were negative for sister chromatid exchange (Seiji et al. 1990) and chromosomal aberrations (World Health Organization, WHO 2006). It was demonstrated that PCE itself does not have significant genotoxic potential; however, some degradation products of PCE, such as TCVC, showed a powerful mutagenic effect (Dreeßen et al. 2003; World Health Organization, WHO 2006). In the case of TCE, DNA strand breaks and chromosome damage were observed in CHO-K1 (Wang et al. 2001) and HepG2 cells (Hu et al. 2008). Our analysis showed that consistent concentrations of both TCE and PCE are able to induce DNA polymorphisms. Basing on the analysis of AFLP profiles, we assumed that polymorphisms induced by TCE or PCE are due to mutations in the AFLP enzymes restriction sequences

Table 4 Observed mean algae metabolic activity and change in algae viability and their associated 95% prediction intervals under the assumption of additivity

Mixture	Observed mean (% of alive)	Predicted mean	Deviation from prediction mean	95% Prediction interval under additivity	Interaction effect
TP1-24 h	81.96	82.51	−0.55	[79.18, 85.84]	Additivity
TP2-24 h	60.35	77.32	−16.97	[68.16, 86.48]	Synergism
TP3-24 h	44.66	73.43	−28.77	[64.28, 82.59]	Synergism
TP4-24 h	48.58	66.51	−17.93	[57.24, 75.78]	Synergism
TP5-24 h	45.79	62.62	−16.83	[53.35, 71.90]	Synergism
TP1-48 h	90.83	84.52	6.31	[81.38, 87.66]	Antagonism
TP2-48 h	63.38	79.69	−16.31	[76.89, 82.49]	Synergism
TP3-48 h	49.40	76.05	−26.65	[73.36, 78.74]	Synergism
TP4-48 h	47.25	69.66	−22.41	[65.77, 73.55]	Synergism
TP5-48 h	54.52	66.02	−11.50	[62.12, 69.92]	Synergism
TP1-72 h	85.45	86.45	−1.00	[83.43, 89.47]	Additivity
TP2-72 h	71.85	81.51	−9.66	[78.82, 84.20]	Synergism
TP3-72 h	49.50	78.06	−28.56	[75.47, 80.65]	Synergism
TP4-72 h	47.00	70.81	−23.81	[67.07, 74.55]	Synergism
TP5-72 h	48.88	67.36	−18.48	[63.61, 71.11]	Synergism
TC1-24 h	79.36	82.13	−2.77	[78.84, 85.42]	Additivity
TC2-24 h	85.07	68.66	16.41	[65.71, 71.61]	Antagonism
TC3-24 h	53.28	48.55	4.73	[43.88, 53.22]	Antagonism
TC4-24 h	77.64	66.50	11.14	[63.61, 69.39]	Antagonism
TC5-24 h	51.50	46.39	5.11	[41.68, 51.10]	Antagonism
TC1-48 h	92.42	84.24	8.18	[81.14, 87.34]	Antagonism
TC2-48 h	69.90	73.13	−3.23	[70.35, 75.91]	Synergism
TC3-48 h	35.41	56.64	−21.23	[52.24, 61.04]	Synergism
TC4-48 h	60.28	71.12	−10.84	[68.40, 73.84]	Synergism
TC5-48 h	27.47	54.63	−27.16	[50.19, 59.07]	Synergism
TC1-72 h	86.74	86.18	0.56	[83.20, 89.16]	Additivity
TC2-72 h	72.98	76.19	−3.21	[73.52, 78.86]	Synergism
TC3-72 h	43.99	61.56	−17.57	[57.33, 65.79]	Synergism
TC4-72 h	66.42	74.05	−7.63	[71.44, 76.66]	Synergism
TC5-72 h	41.50	59.42	−17.92	[55.15, 63.69]	Synergism
PC1-24 h	79.74	81.55	−1.81	[78.26, 84.84]	Additivity
PC2-24 h	82.02	67.51	14.51	[64.56, 70.46]	Antagonism
PC3-24 h	44.36	47.39	−3.03	[42.72, 52.06]	Synergism
PC4-24 h	66.52	63.62	2.90	[60.73, 66.51]	Additivity
PC5-24 h	44.60	43.50	1.10	[38.79, 48.21]	Additivity
PC1-48 h	90.06	83.70	6.36	[80.60, 86.80]	Antagonism
PC2-48 h	49.36	72.04	−22.68	[69.26, 74.82]	Synergism
PC3-48 h	38.38	55.55	−17.17	[51.15, 59.95]	Synergism
PC4-48 h	45.48	68.41	−22.93	[65.69, 71.13]	Synergism
PC5-48 h	34.64	51.92	−17.28	[47.48, 56.36]	Synergism
PC1-72 h	86.63	85.74	0.89	[82.76, 88.72]	Additivity
PC2-72 h	65.05	75.32	−10.27	[72.65, 77.99]	Synergism
PC3-72 h	37.36	60.69	−23.33	[56.46, 64.92]	Synergism
PC4-72 h	36.80	71.88	−35.08	[69.27, 74.49]	Synergism
PC5-72 h	37.29	57.24	−19.95	[52.97, 61.51]	Synergism

or to random breaks in DNA (Walles 1986; Hu et al. 2008). Our analyses exclude the formation of new EcoRI and MseI restriction sites induced by TCE or PCE. Indeed, in all cases, the mutations do not lead to new amplified bands in the treated samples when compared with the control and it support DNA strand breaks effect.

DNA damage and in particular random breaks in DNA could be induced from oxygen radicals formed during the biotransformation of PCE and TCE (Walles 1986; Dreeßen et al. 2003; Hu et al. 2008). Generally, DNA is subject to continuous oxidative damage from oxygen radicals generated during normal cellular metabolism and unrepaired DNA damage can lead to mutation (Labra et al. 2004). We suppose that the TCE and PCE in the algae cell increased the rate of damage above background levels and thus enlarge the potential for unrepaired lesions to become permanent mutations. Our hypothesis is supported by an investigation conducted previously on rat liver (Toraason et al. 1999) and on human cells (Hu et al. 2008).

Oxidative damage seems to be a major mechanism involved in DNA toxicity induced by organic and inorganic pollutants (Toraason et al. 1999; Hu et al. 2008) and our test conducted with potassium dichromate supports this consideration. Chromium is able to produce DNA damages in algae cells (Labra et al. 2007) probably by some of the products of DNA-oxidative damage such as modified bases, primarily 8-oxoguanine, abasic sites, base adducts of carbon-centred radicals and single or double strand breaks in the phospho-sugar back-bone of DNA (Wise et al. 2008). These mutations were observed in our AFLP analysis and data suggest that potassium dichromate is a strong mutagenic agent, especially at high concentrations.

In conclusion, AFLP analysis showed that mixtures containing TCE and PCE could enhance the toxic effect of chromium in cells increasing the mutagenic effect on algae genome (Fig. 3).

5 Conclusion

In conclusion, although our data support the suitability of the EU limit values, not only for a single pollutant but also in mixtures, results showed that VOCs are able to increase the toxicological effect of heavy metals. The chemistry of VOCs and the interaction between different water contaminants in the environmental conditions are much more complex than those highlighted in this work, conducted under controlled conditions. However, the importance of this work is to evaluate what are the mechanisms of action of different molecules, alone and combined together, on living organisms. Our tests analysed not only the effect of pollutants on cell viability but also the genotoxicity by using innovative approaches such as AFLP useful to evaluate DNA damage. Starting from our data, we could introduce different environmental variables to test the biological response of bio-indicators both in vitro and in vivo. Only the exhaustive study of chemical processes and mechanisms of action of different molecules in the environmental condition could determine the appropriate limit value for contaminants in water.

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References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., & Grimme, L. H. (2000). Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: mixtures composed of similar acting chemicals. *Environmental Toxicology and Chemistry, 19*(9), 2341–2347.

Barata, C., Baird, D. J., Nogueira, A. J. A., Soares, A. M. V. M., & Riva, M. C. (2006). Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. *Aquatic Toxicolology, 78*(1), 1–14.

Burton, D. T., Di Lorenzo, J. L., Shedd, T. R., & Wrobel, J. G. (2002). Aquatic hazard assessment of a contaminated surficial aquifer discharge into the bush river, Maryland (U.S.A.). *Water, Air, and Soil Pollution, 139*(1–4), 159–182.

Channel, S. R., Latendresse, J. R., Kidney, J. K., Grabau, J. H., Lane, J. W., Steel-Goodwin, L., et al. (1998). A subchronic exposure to trichloroethylene causes lipid peroxidation and heptocellular proliferation in male B6C3F1 mouse liver. *Toxicological Sciences, 43*(2), 145–154.

Cho, C., Corapcioglu, M. Y., Park, S., & Sung, K. (2008). Effects of grasses on the fate of VOCs in contaminated soil and air. *Water, Air and Soil Pollution, 187*(1–4), 243–250.

Council Directive 1998/83/EC. (1998). On the quality of water intended for human consumption. *Official Journal of the European Communities L, 330*, 32–54.

Dekant, W., Koob, M., & Henschler, D. (1990). Metabolism of trichloroethene: in vivo and in vitro evidence for activation by glutathione conjugation. *Chemico-Biological Interactions, 73*(1), 89–101.

Dreeßen, B., Westphal, G., Bünger, J., Hallier, E., & Müller, M. (2003). Mutagenicity of the glutathione and cysteine S-conjugates of the haloalkenes 1,1,2-trichloro-3,3,3-trifluoro-1-propene and trichlorofluoroethene in the Ames test in comparison with the tetrachloroethene-analogues. *Mutation Research, 539*(1-2), 157–166.

Gennings, C., Schwartz, P., Carter, J., & Simmons, J. E. (1997). Detection of departure from additivity in mixture of many chemicals with a threshold model. *Journal of Agricultural, Biological, and Environmental Statistics, 2*(2), 198–211.

Halmes, N. C., McMillan, D. C., Oatis, J. E., & Pumford, N. R. (1996). Immunochemical detection of protein adducts in mice treated with trichloroethylene. *Chemical Research in Toxicology, 9*(2), 451–456.

Hsieh, S. H., Tsai, K. P., & Chen, C. Y. (2006). The combined toxic effects of nonpolar narcotic chemicals to *Pseudokirchneriella subcapitata*. *Water Research, 40*(19), 1957–1964.

Hu, C., Jiang, L., Geng, C., Zhang, X., Cao, J., & Zhong, L. (2008). Possible involvement of oxidative stress in trichloroethylene-induced genotoxicity in human HepG2 cells. *Mutation Research, 652*(1), 88–94.

Kontas, A. (2008). Trace metals (Cu, Mn, Ni, Zn, Fe) Contamination in marine sediment and Zooplankton samples from Izmir Bay. (Aegean Sea, Turkey). *Water, Air, and Soil Pollution, 188*(1–4), 323–333.

Labra, M., Grassi, F., Imatio, S., Di Fabio, T., Citterio, S., Sgorbati, S., et al. (2004). Genetic and DNA-methylation changes induced by potassium dichromate in *Brassica napus* L. *Chemosphere, 54*(8), 1049–1058.

Labra, M., Bernasconi, M., Grassi, F., De Mattia, F., Sgorbati, S., Airoldi, R., et al. (2007). Toxic and genotoxic effect of potassium dichromate in *Pseudokirchneriella subcapitata* detected by microscopi and AFLP analysis. *Aquatuatic Botany, 86*(3), 229–235.

Lash, L. H., Qian, W., Putt, D. A., Desai, K., Elfarra, A. A., Sicuri, A. R., et al. (1998). Glutathione conjugation of perchloroethylene in rats and mice in vitro: sex-, species-, and tissue-dependent differences. *Toxicology and Applied Pharmacology, 150*(11), 49–57.

Lash, L. H., Qian, W., Putt, D. A., Jacobs, K., Elfarra, A. A., Krause, R. I., et al. (1998). Glutathione conjugation of trichloroethylene in rats and mice: sex-, species-, and tissue-dependent differences. *Drug Metabolism and Disposition, 26*(1), 12–19.

Marques, C. R., Abrantes, N., Figueiredo, D. R., Pereira, M. J., & Goncalves, F. (2008). Are *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* affected by environmental samples from a rice field? *Water, Air, and Soil Pollution, 189*(1–4), 49–59.

Mayer, P., Kuhel, R., & Nyholm, N. (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research, 31*(10), 2525–2531.

Miller, R. E., & Guengerich, F. P. (1982). Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. *Biochemistry, 21*(5), 1090–1097.

Nichols, H. W. (1979). Growth media-freshwater. In J. R. Stein (Ed.), *Handbook of phycological method, culture methods and growth measurement* (pp. 7–24). Cambridge: Cambridge University Press.

Paine, A. J. (2001). Mechanisms of chromium toxicity, carcinogenicity and allergenicity: review of the literature from 1985 to 2000. *Human & Experimental Toxicology, 20*(9), 439–451.

Sausen, P. J., & Elfarra, A. A. (1991). Reactivity of cysteine S-conjugate sulfoxides: formation of *S*-[1-chloro-2-(*S*-glutathionyl)vinyl]-L-cysteine sulfoxide by the reaction of *S*-(1, 2-dichlorovinyl)-L-cysteine sulfoxide with glutathione. *Chemical Research in Toxicology, 4*(6), 655–660.

Seiji, K., Jin, C., Watanabe, T., Nakatsuka, H., & Ikeda, M. (1990). Sister chromatid exchanges in peripheral lymphocytes of workers exposed to benzene, trichloroethylene, or tetrachloroethylene, with reference to smoking habits. *International Archives of Occupational and Environmental Health, 62*(2), 171–176.

Sharma, D. C., Sharma, C. P., & Tripathi, R. D. (2003). Phytotoxic lesions of chromium in maize. *Chemosphere, 51*(1), 63–68.

Shehata, S. A., Lasheen, M. R., Kobbia, I. A., & Ali, G. H. (1999). Toxic effect of certain metals mixture on some physiological and morphological characteristics of freshwater algae. *Water, Air, and Soil Pollution, 110*(1–2), 119–135.

Speranza, A., Ferri, P., Battistelli, M., Falcieri, E., Crinelli, R., & Scoccianti, V. (2007). Both trivalent and hexavalent chromium strongly alter in vitro germination and ultrastructure of kiwifruit pollen. *Chemosphere, 66*(7), 1165–1174.

Suk, W., & Olden, K. (2005). Multidisciplinary research: strategies for assessing chemical mixtures to reduce risk of exposure and disease. *Human and Ecological Risk Assessment, 11*(1), 141–151.

Toraason, M., Clark, J., Dankovic, D., Mathias, P., Skaggs, S., Walker, C., et al. (1999). Oxidative stress and DNA damage in Fischer rats following acute exposure to trichloroethylene or perchloroethylene. *Toxicology, 138*(11), 43–53.

Valitutto, R. S., Sella, S. M., Silva-Filho, E. V., Pereira, R. G., & Miekeley, N. (2007). Accumulation of metals in macrophytes from water reservoirs of a power supply plant, Rio de Janeiro State, Brazil. *Water, Air, and Soil Pollution, 178*(1–4), 89–102.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407–4414.

Walles, S. A. S. (1986). Induction of single-strand breaks in DNA of mice by trichloroethylene and tetbachloroethylene. *Toxicology Letters, 31*(1), 31–35.

Wang, J. L., Chen, W. L., Tsai, S. Y., Sung, P. Y., & Huang, R. N. (2001). An in vitro model for evaluation of vaporous

toxicity of trichloroethylene and tetrachloroethylene to CHO-K1 cells. *Chemico-Biological Interactions, 137*(2), 139–154.

Wise, S. S., Holmes, A. L., & Wise, J. P., Sr. (2008). Hexavalent chromium-induced DNA damage and repair mechanisms. *Reviews on Environmental Health, 23*(1), 39–57.

World Health Organization (WHO). (2006). *Tetrachloroethylene in: Concise International Chemical Assessment Document 68, International Programme on Chemical Safety (IPCS)*. Geneva: Monograph, WHO.

Wu, K. L., & Berger, T. (2007). Trichloroethylene metabolism in the rat ovary reduces oocyte fertilizability. *Chemico-Biological Interactions, 170*(1), 20–30.