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# Comparative study of three analysis methods (TTGE, flow cytometry and HPLC) for xenobiotic impact assessment on phytoplankton communities

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**Abstract:**

The impacts of the fungicide Opus® (epoxiconazole) on marine phytoplankton communities were assessed in a 12-day field experiment using in situ microcosms maintained underwater at 6 m depth. Three community analysis methods were compared for their sensitivity threshold in fungicide impact detection. When phytoplankton communities were exposed to 1 µg $l^{-1}$ of epoxiconazole, no effects could be demonstrated using TTGE (Temporal Temperature Gradient gel Electrophoresis), flow cytometry or HPLC. When exposed to 10 µg $l^{-1}$, TTGE fingerprints from PCR amplified 18S rDNA of communities exhibited significant differences compared with controls (ANOSIM, $P = 0.028$). Neither flow cytometry counts, nor HPLC pigment profiles allowed to show significant differences in microcosms exposed to 10 µg $l^{-1}$ of epoxiconazole. When exposed to 100 µg $l^{-1}$, all three methods allowed to detect significant differences in treated microcosms, as compared to controls. The TTGE analysis appears in this study as the most sensitive method for fungicide impact assessment on eukaryote microbial communities.

**Keywords:** Microcosm - Phytoplankton community - Epoxiconazole - TTGE - Flow cytometry - HPLC

# 1. Introduction

The massive use of pesticides induces acute or chronic contamination of aquatic biotas, via spray drift, leaching and run-off from urban and agricultural treated areas. Such contaminants can generate adverse effects on non-target organisms, including phytoplankton, involved in the microbial loop. This can ultimately affect the workings of the ecosystem, as the microbial food web plays a critical role in nutrient cycling and transfer of nutrients to higher trophic levels (DeLorenzo et al. 1999; Downing et al. 2004).

Numerous studies have been published about pesticide toxicity assessment on phytoplankton, using single-species tests (DeLorenzo et al. 2004; Gatidou and Thomaidis 2007; Rioboo et al. 2002; Santin-Montanya et al. 2007; Weiner et al. 2004). But such tests are subjected to high variability in the sensitivity of tested species (Cairns, 1986). Furthermore, Bérard et al. (1999a) demonstrated that single-species tests may fail to predict indirect or system responses, such as changes in the population distribution overtime. Studies focusing on the whole natural community and based on biological parameters measured at the community level, provide more reliable predictions about pesticide safety in aquatic environments (Barry and Logan, 1998; Bérard et al. 1999a).

Studies on natural phytoplankton community can be carried out using in situ microcosms. Such systems give a view of the whole community, including populations that are hard to maintain; they can thus provide a wider survey of organism sensitivity for toxicants (Yasuno et al. 1993). Leboulanger et al. (2001) and Seguin et al. (2001) pointed out the need for reliable toxicity data from such microcosm systems.

As significant perturbations of communities may be difficult to detect in background variations (Barry and Logan, 1998), studies should deal with several community variables using various methods.

In the 1980s, the development of automated High Performance Liquid Chromatography (HPLC) methods for pigment separations allowed the first large scale studies of phytoplankton communities (Jeffrey et al. 1999). HPLC phytoplankton analysis was reported in studies involving spatial and/or temporal community dynamics (Furuya et al. 2003; Wänstrand and Snoeijs 2006) and in enrichment experiments (Suzuki et al. 2005; Wong and

Crawford 2006). This tool was also successfully applied in a water-quality monitoring study (Sherrard et al. 2006) and in ecotoxicological studies assessing herbicide effects on phytoplankton community (Devilla et al. 2005; Dorigo et al. 2004; Readman et al. 2004). Flow cytometry can also provide informations about the distribution of natural microbial populations (Marie et al. 1999; Rutten et al. 2005). Toxicity assessment using flow cytometry was reported in studies involving either phytoplankton cultures (Lage et al. 2001; Stauber et al. 2005; Yu et al. 2007) or natural photosynthetic communities (de la Broise and Palenik 2007; Readman et al. 2004; Stachowski-Haberkorn et al. 2008; Zamora-Ley et al. 2006). Community changes can also be assessed at the molecular level: genetic fingerprinting techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al. 1993) or Temporal Temperature gradient Gel Electrophoresis (TTGE) (Yoshino et al. 1991) provide an overview of the whole community composition, including unculturable strains, which can account for 90% of microorganisms (Ward et al. 1990). When DGGE is run using a denaturing chemical gradient in the gel space at a constant temperature, TTGE is run using a homogeneous denaturing concentration and the temperature is enhanced during migration to ensure DNA denaturation. In the present study, the TTGE was applied because, as there is no need for a chemical gradient in the gel, analyses are easier to perform (Marie et al. 2006). Both DGGE and TTGE methods have been useful tools to describe the diversity of the whole prokaryote and eukaryote microbial communities in aquatic environments (Díez et al. 2001; Marie et al. 2006; Schäfer et al. 2001; Van Hannen et al. 1998). More recently, these methods were successfully applied to detect disturbances in microbial communities exposed to pollution (Kostanjšek et al. 2005; Pesce et al. 2006, 2008; Petersen et al. 2004; Stachowski-Haberkorn et al. 2008).

Even though HPLC, flow cytometry and fingerprinting techniques have proven their efficiency and reliability in toxicity assessment on phytoplankton communities, there is a lack of knowledge about the sensitivity thresholds of such analyses. However, the detection of an effect, and thus the conclusion of a research study, depends on the method(s) applied. Therefore, comparative data are essential to avoid misleading conclusions.

Among studies of pesticide toxicity assessment, fungicide impact studies on phytoplankton are very scarce. However, some species can be highly sensitive to fungicides (Abdel-Hamid et al. 1996). As far as we know, only one study reported adverse effects of epoxiconazole on a phytoplankton species: after a 24 h exposure to 100 µg $L^{-1}$ of its commercial formulation Opus, the dinoflagellate Karenia mikimotoï had significantly increased extent of DNA strand breaks (Akcha et al. 2008). This also highlights the potential genotoxicity of this fungicide.

No data on epoxiconazole concentration in marine waters was available. However, pesticide concentrations encountered in estuarine areas are usually below 1 µg $L^{-1}$ (Lehotay et al. 1999; Oros et al. 2003; Steen et al. 2001).

In the present study, natural marine microbial communities were exposed to the fungicide Opus at 1, 10 and 100 µg$L^{-1}$ of the active ingredient epoxiconazole, using in situ microcosms, in order to compare three analytical methods (TTGE, flow cytometry and HPLC analysis of pigments) for their sensitivity threshold in xenobiotic impact assessment.

## 2. Materials and Methods

In order to assess the effects of Opus on marine phytoplankton communities, a set of microcosms containing natural surrounding filtered seawater was exposed to the fungicide and immersed in situ. The genetic fingerprints of microcosm eukaryote communities were obtained from the whole community DNA amplified extracts, using Temporal Temperature Gradient Gel Electrophoresis (TTGE) analysis. Pigment analyses were made from biomass collected on filters, and microcosm subsamples were also analysed using flow cytometry, and microscopic species determination.

### 2.1. Fungicide

The pesticide tested in this study was the commercial formulation of the fungicide epoxiconazole, cis-1-[[3-(2-chlorophenyl)-2-(4-fluorophenyl)oxiranyl]methyl]-=1H-1,2,4-triazole, (Opus®, BASF). Concentration values represent those of the pure active substance. The fungicide stock solution (10 mg $L^{-1}$ epoxiconazole) was prepared in 0.22 µm filtered seawater, previously autoclaved for 20 min at 121°C. The solution was then agitated for 48 h using a magnetic stirrer, and stored at -24 °C.

Freshly melted stock solution was added directly into the microcosm bottles just before immersion. Fungicide concentrations were analyzed on the last day of the experiment, on pooled water samples from all the replicate bottles of each treatment. Samples were frozen until analysis. Fungicide analyses were performed by the Idhesa Laboratory (Brest, France), using on-line solid-phase extraction coupled with liquid chromatography and electrospray ionisation-tandem mass spectrometry (HPLC Waters separation module 2690, Waters photodiode array 996 detector; Micromass® Quattro Ultima™ mass spectrometer). The detection limit was 0.005 µg $L^{-1}$.

## 2.2. Microcosms

The outdoor experiment took place in summer 2006, in Port-la-Forêt Bay, south Brittany, France (47°52'12"N, 03°58'35"W), from 16th to 28th June, over a 2-week period (de la Broise and Palenik 2007). Microcosms consisted of hermetically closed 2.3 L glass bottles. These bottles were filled with 2 L of 200 µm filtered fresh seawater from the surface layer at the field site, containing natural microbial communities, and enclosed with about 300 mL of air (de la Broise and Palenik 2007).

Sixteen microcosms were prepared: four replicates for the control and each of the three different treatments (Opus 1 µg $L^{-1}$, 10 µg $L^{-1}$ and 100 µg $L^{-1}$). In order to validate the three analytical methods, it was decided to increase the concentrations using a factor of ten, as did Bengtson Nash et al. (2005).

Microcosms were hung on a 3 m diameter circular stainless steel frame that was anchored to the sea floor, on a 400 kg concrete block, and suspended from a surface buoy that allowed the bottles to remain at 6 m depth (mid depth of the water column), regardless of the tide. The replicate microcosm bottles of each treatment were distributed alternately all around the frame.

On days 2, 4, 7, 9 and 11, the frame was hauled out of the water and 200 mL (except for day 7, 300 mL) of the 2 litre-seawater content of each bottle were collected in a tank for proper disposal, replaced with the same volume of fresh 200 µm-filtered surrounding seawater, and the frame was re-immersed (de la Broise and Palenik 2007). No analysis was carried out on these 200 mL samples. Bottles were finally collected on day 12 for analyses.

One litre of water from each microcosm was filtered through a 0.22 µm polysulfone filter, that was then stored at -80°C until DNA extraction and HPLC pigment analyses. For cytometry analyses, 1.5 mL from each microcosm were fixed into a cryotube with glutaraldehyde (final concentration 0.25%). Cryotubes were vortexed and left 15 minutes at room temperature before freezing in liquid nitrogen. Samples were stored at -80°C until analysis.

## 2.3. DNA extraction and purification

The extraction and purification protocol was previously described in Stachowski-Haberkorn et al. (2008).

### 2.4. PCR

Eukaryotic 18S rDNA primers were used. The primers were Euk1A and Euk516r-GC (Díez et al. 2001), which amplify a fragment of approximately 560 bp. The PCR program (Díez et al. 2001) included an initial denaturation step at 94℃ for 130 s and 35 cycles as follows: denaturation at 94°c for 30 s, annealing at 56°C for 45 s and extension at 72°C for 130 s. After the last cycle, a final extension step was performed for 7 min at 72°C.

Approximately 15 ng of extracted DNA (estimated using spectrophotometry) were used as a template: the reaction mixture (50 µL) contained sterile nuclease-free water, each primer at a final concentration of 0.3 µM, Bovine Serum Albumin (final concentration 0.2 µg $\mu L^{-1}$) and Promega PCR Master Mix, (in which the final concentrations were: deoxynucleoside triphosphates (dNTPs) 200 µM each; $MgCl_2$ 1.5 mM; and Taq polymerase 25 units $mL^{-1}$) supplied in a reaction buffer (pH 8.5).

PCR products were checked on a 1% agarose gel before loading on TTGE gel.

### 2.5. TTGE

Denaturing gels (30 mL) were prepared composed of 6% polyacrylamide (stock solution: acrylamide/bis-acrylamide 37.5:1), 7 M urea, 1.25×TAE and 2 % glycerol. The stacking gel (5 mL) contained only TAE 1.25× and 6% polyacrylamide. Ammonium persulfate 10% (respectively 150 and 25 µL for the denaturing and the stacking gels) and TEMED (respectively 30 and 5 µL) were added before casting and the gel was left to polymerize at 20°C for 3-4 h. The gel dimensions were 16 cm×16 cm×1mm.

The TTGE was performed by using a Dcode universal mutation system (Bio-Rad), modified to improve accuracy and repeatability of the temperature gradient kinetics: the electrophoresis part of the Dcode system was immersed in a modified buffer tank (15 L). The tank was connected to an external temperature control unit (Cryostat Julabo, Seelbach, Germany) using 20 mm diameter tubing. Electrophoresis was performed for 18 h at 70 V in 1.25× TAE buffer. Gradual temperature increase was 57 to 62 °C. Gels were stained with SYBR Gold 1×, rinsed in 1× TAE buffer, and photographed on a UV transillumination table. Band detection on TTGE gel images was performed using the LabImage software v2.7 (Kapelan GmbH, Halle, Germany). On the basis of a band presence/absence matrix from each gel, a dendrogram was constructed applying the Dice coefficient and the unweighted pair group method of averages (UPGMA) using the Matlab software v6.1 (The Mathworks, Natik, MA).

### 2.6. Flow cytometry

Samples were run using a FACSort flow cytometer equipped with a 488-nm argon laser and standard filter setup (Becton Dickinson, San Jose, CA). Two populations of photosynthetic organisms were discriminated in microcosms samples, on the basis of their scatter signals and their natural red (>630 nm) and orange fluorescence (580 ± 20 nm): Synechococcus and small eukaryotes (<20 µm). Usually, picoplankton is considered to range from 0.2 µm to 2 µm, and nanoplankton from 2 µm to 20 µm. For more precise analysis, a clear sub-population of pico-eukaryotes was detected as part of the overall eukaryotic distribution (Fig. 1). Cells belonging to this sub-population have been identified by flow cytometric sorting in marine samples from various geographic locations, as prasinophytes (D. Marie, unpublished data) and for this reason, they were cited as "prasinophyte-like" in the following text. The other eukaryotes are nano-eukaryotes. Data were analysed using the WinMDI v2.9 software (J. Trotter, http://facs.Scripps.edu/).

### 2.7. HPLC of pigments

Pigments from half of each frozen 0.22 µm polysulfone filter were extracted in the dark, in 1 mL of 95% methanol as follows: the half-filter was ground for 3 min using a stainless steel spatula and sonicated on ice for 30 s. The solution was then filtered through a 0.22-µm PVDF syringe filter to remove debris. Aliquots (200 µL) were injected into the HPLC system (Waters 600 S controller, 616 pump, 600 column heater and 996 diode-array detector). Pigments were separated through a Waters Symmetry-C8 reverse-phase column maintained at 30°C (150×4.6 mm, 3.5 µm particle size, 100 Å pore size). The mobile phase was a gradient mixture of 4 solvents: methanol (100%), acetonitrile (100%), acetic acid (100%) and aqueous pyridine solution (0.25M). Whereas Zapata et al. (2000) used two eluents each constituted of two solvents, in the present study the four single solvents were directly used for gradient elution. This allowed an improved analysis repeatability.

The peaks were identified based on their retention time and absorption spectra compared with those of published pigments spectra (Jeffrey et al. 1997) and with those of pigments extracts prepared from clonal cultures with known pigment composition, obtained from the Roscoff Culture Collection (Vaulot et al. 2004): the dinoflagellate Amphidinium carterae (RCC 88), the chlorophyte Dunaliella tertiolecta (RCC 6), the prymnesiophytes Emiliania huxleyi (RCC 174), Pavlova lutheri (RCC 180) and Phaeocystis globosa (RCC 187), the chrysophyte Pelagococcus subviridis (RCC 98), the diatom Skeletonema costatum (RCC 70) and the cyanophyte Synechococcus sp. (RCC 752).

The pigment profile of a sample was defined as the set of sample pigment ratios (sample-pigment $X_{area}$ / sample-chl $a_{area}$) at 440 nm. Chl a concentration was also calculated in every sample, using a chl a standard solution (SIGMA, Chlorophyll a from spinach for HPLC Ref 10865).

The pigments detected and the associated abbreviated names are listed in Table 1.

### 2.8. Species determination

In order to provide an insight into the phytoplankton community before exposure to the fungicide, 100 mL of the surrounding seawater used to fill microcosms on the first day of experiment were fixed using acid Lugol's iodine solution (2%, final concentration) and kept in the dark.

Determination and quantification of micro- and nano-phytoplankton cells were carried out at the species level as follows: a sub-sample of 50 mL was settled into an Utermöhl settling chamber (Hasle 1978) and counted using a Wild M40 phase contrast inverted microscope. Counts were carried out on partial or whole bottom surface of the chamber, depending on the size and the abundance of the species (Lund et al. 1958), at × 200 to × 400 magnification. When possible, 400 cells were counted to ensure that the error in estimation of cellular abundance remained within the limits of ± 10% (Uehlinger 1964).

### 2.9. Statistical analysis

The pigment and flow cytometry data were first tested for homogeneity of variances (Bartlett's test) and normal distributions (Kolmogorov-Smirnov test). As several variables did not fulfill both conditions, a parametric analysis could not be used. Therefore, following the statistical procedures given in Sokal and Rohlf (1995), a Kruskal-Wallis rank test was used to check for significant differences in multiple treatment sets, and when the answer was positive a Mann-Whitney test was run for pairwise comparisons. Statistical tests were performed using the Statgraphics® Plus v5.1 software.

In order to test for significant differences between the genetic fingerprints obtained from different treatments, analyses of similarity (Clarke 1993) were performed on binary matrixes from gel image analysis using the one-way ANOSIM function in the Past v1.77 software (Hammer et al. 2001).

## 3. Results

### 3.1. Fungicide analysis

The surrounding water used to prepare the microcosms on the first day of experiment did not contain detectable epoxiconazole concentration (data not shown), neither did the control on the last day of experiment. As medium renewal was 10% every other day, the remaining fungicide concentration after twelve days should be approximately 56% of the nominal amount added.

On the last day of experiment, epoxiconazole value in 100 µg $L^{-1}$ treated microcosms was 44 µg $L^{-1}$, close to the expected value (44% instead of 56%). For the 10 µg $L^{-1}$ treatment, this value was much lower than expected (24%) at 2.4 µg $L^{-1}$, and higher (84%) at 0.84 µg $L^{-1}$ for the 1 µg $L^{-1}$ treatment.

### 3.2. Opus impacts on eukaryote and photosynthetic communities

#### 3.2.1. TTGE of the eukaryote community

Image analysis of the TTGE gel from control and 1 µg $L^{-1}$ Opus treated microcosms (Fig. 2a) allowed the detection of 34 different bands on the whole gel. 15 to 20 bands were detected for the control microcosms and 16 to 24 for 1 µg $L^{-1}$ Opus treated microcosms.

The cluster analysis (Fig. 2a) did not point out any grouping of the patterns in relation with the treatment, and the ANOSIM results did not indicate significant difference between control and treated microcosms.

These data illustrate that 1 µg $L^{-1}$ of Opus does not induce detectable effects on the eukaryotic communities in microcosms.

Image analysis of the TTGE gel from control, 10 µg $L^{-1}$ and 100 µg $L^{-1}$ Opus treated microcosms (Fig. 2b) allowed the detection of 45 different bands on the whole gel. 18 to 25 bands were detected for the control microcosms, 14 to 19 for microcosms treated with 10 µg $L^{-1}$ of Opus and 8 to 15 for microcosms treated with 100 µg $L^{-1}$ of Opus. The band counts were significantly different between the latter and the control microcosms (Mann-Whitney, $p = 0.03$). No difference in these counts was demonstrated for the Opus 10 µg $L^{-1}$ treatment as compared with controls ($p = 0.11$).

The cluster analysis (Fig. 2b) showed 3 groups of fingerprints: the first group includes three control replicates, the second group includes the four Opus 10 µg $L^{-1}$ replicates and the third group includes the four Opus 100 µg $L^{-1}$ replicates and one control replicate. The ANOSIM p-values point out significant differences between the control and both Opus treatments ($p = 0.028$ and $p = 0.029$ for Opus 10 and 100 µg $L^{-1}$ treatments, respectively). The R values

of the treatments versus control (R = 0.58 and R = 0.67 for Opus 10 and 100 µg $L^{-1}$, respectively) illustrate that similarities within each condition are higher than similarities between conditions (Clarke 1993).

These data illustrate that microcosms exposed to 10 and 100 µg $L^{-1}$ of Opus exhibit a modification of the community fingerprints.

### 3.2.2. Flow cytometry

Three populations were discriminated using flow cytometry: the pico-eukaryotes mostly composed by species belonging to the class Prasinophyceae, the nano-eukaryotes and the prokaryote Synechococcus. The cumulative counts in control microcosms, were not significantly different from the ones in treated microcosms.

Counts of the nano-eukaryotes for 1 µg $L^{-1}$, 10 µg $L^{-1}$ and 100 µg $L^{-1}$ Opus treatments were not significantly different from control (Fig. 3).

The prasinophyte-like counts of microcosms exposed to 100 µg $L^{-1}$ of Opus dropped dramatically to less than 2% of the control values ($p = 0.012$), whereas no significant effect could be shown on this population exposed to 1 and 10 µg $L^{-1}$. The Synechococcus counts were not significantly higher in Opus 1 µg $L^{-1}$ and 10 µg $L^{-1}$ treatments. However, when exposed to 100 µg $L^{-1}$, the Synechococcus counts were significantly ($p = 0.02$) enhanced, to approximately 260 % of the control values.

### 3.2.3. HPLC

HPLC analysis of phytoplankton pigments allowed to detect 17 different pigments (Table 1): 8 chlorophylls: chl a, b, $c_1$, $c_2$, $c_3$, chlide a, MgDVP, MV chl $c_3$; and 9 carotenoids: but-fuco, diadino, fuco, hex-fuco, neo, perid, pras, viola and zea.

Among these 17 pigments, chl a was quantified using a standard, when the others were analysed using their area ratios relative to chl a.

#### *Pigment ratios*

The Principal Component Analysis (PCA) of pigment ratios shows that about 74% of the total variance is explained by the first two axes (Fig. 4).

The projection of observations on the two axes exhibits two clearly separated groups: the first group of observations includes Control, Opus 1 µg $L^{-1}$ and Opus 10 µg $L^{-1}$, and can be projected on the positive side of the first axis. The second group, Opus 100 µg $L^{-1}$ replicates, can be projected on the negative part of the first axis with values lower than -3.

For each pigment, pairwise comparisons of pigment ratios were carried out between control and Opus treatments. The comparisons of pigment ratios between controls and microcosms exposed to 1 µg $L^{-1}$ and 10 µg $L^{-1}$ of Opus did not point out any significant difference (Mann-Whitney, $p>0.05$, data not shown). However, the comparison of pigment ratios between the control and the microcosms exposed to 100 µg $L^{-1}$ of Opus (Fig. 5) showed important differences: six of the 16 pigments observed in controls were not detected when phytoplankton was maintained with 100 µg $L^{-1}$ of Opus: pras, neo, viola, perid, chl b and chlide a.

Among the 10 other pigments, 7 were shown to have significantly different ratios (Mann-Whitney, $p<0.05$) in microcosms exposed to 100 µg $L^{-1}$ of Opus:

– hex fuco, chl $c_3$ and MV chl $c_3$ ratios were enhanced 2.5 times, 2 times and 10 times, respectively,

– fuco, but-fuco and MgDVP were half the control value,

– chl $c_1$ ratio was 5-fold lower compared to the control.

***Chlorophyll a quantification***

When microcosms were exposed to 1 µg $L^{-1}$ and 10 µg $L^{-1}$ of Opus, no significant difference in chl a concentration could be demonstrated, as compared with control ($p = 0.06$ and $p = 0.07$, respectively) (Fig. 6). Nonetheless, the chl a concentration in microcosms treated with 100 µg $L^{-1}$ of Opus was significantly increased to 177% of the control value ($p = 0.037$).

### 3.2.4. Species determination

The microscopic species determination allowed to identify 28 species in the surrounding water on day 0. Among them, Chaetoceros sp., nano-flagellates and cryptophytes accounted respectively for 59.2%, 36% and 4.4% of the total cell counts (data not shown).

## 4. Discussion

The microcosm system used here, with partial medium renewal and an air overlay, was previously validated as representative of the natural surrounding water, for Synechococcus and picoeukaryote counts (de la Broise and Palenik 2007; Stachowski-Haberkorn et al. 2008).

### 4.1. Fungicide exposure

Epoxiconazole analyses in 100 µg $L^{-1}$ treated microcosms on the last day of experiment showed a concentration in the range of the one expected. For the lower Opus concentrations,

analyses exhibited lower or higher concentrations than expected, for 10 µg $L^{-1}$ and 1 µg $L^{-1}$ respectively. This could be due to the possible adsorption/desorption during the dilution process, on bottle walls and/or on the organic matter present in seawater, and to accuracy of analysis at such low values.

No data on epoxiconazole concentration in marine waters was available. However, as pesticide concentrations encountered in estuarine areas are usually below 1 µg $L^{-1}$ (Lehotay et al. 1999; Oros et al. 2003; Steen et al. 2001), we can consider the lowest concentration tested to be likely representative of peak pollution in coastal areas. The 10 µg $L^{-1}$ value would perhaps be encountered in freshwater or estuarine areas in case of exceptional and extreme pollution. The higher concentration tested is obviously non realistic for a marine environment. It was however included in this experiment to insure detectable adverse effects using the methods assessed here.

**4.2. TTGE**

The TTGE was based on 18S rDNA amplification from the whole eukaryotic community present in microcosms. These eukaryotic organisms include photosynthetic phytoplankton, but also heterotrophic unicellular eukaryotes and pluricellular grazers.

The observation of TTGE gels reveals two kinds of variability:

– On the first gel (Fig. 2a), controls displayed 15 to 20 distinct bands, whereas there are 18 to 25 bands for controls on the second gel (Fig. 2b). These 2 gels were simultaneously run on the two sides of the Dcode system, showing that even with an improved control system for temperature kinetic, migrations were not rigorously identical. So it is recommended to load control samples on each gel and to avoid gel to gel comparisons. The whole sample set could not be run simultaneously on the same gel. Therefore the same controls were run on both gels, allowing data treatment using samples/control comparisons.

In spite of this drawback, several studies reported the high sensitivity and reliability of this method that yields a picture of the genetic diversity of the community as a whole (Gillan 2004; Muyzer and Smalla 1998), both in prokaryote (Kostanjšek et al. 2005; Pesce et al. 2006; 2008) and eukaryote (Doaré-Lebrun et al. 2006; Nieguitsila et al. 2007) community studies. Furthermore, only species at low relative abundance (<1%) are usually undetected on such fingerprints (Muyzer et al. 1993).

– when replicates were submitted simultaneously to similar experimental conditions, they exhibited differences in band intensities and/or locations. We can hypothesize a

non-deterministic community structuring process as the cause of such difference, as already reported in Drake et al. (1996). Variability between replicates was also reported in studies including phytoplankton communities (Caquet et al. 2001; Rosenzweig and Buikema 1994), and in an ecotoxicological study with aquatic microbial communities in microcosms analysed using DGGE (Brandt et al. 2004).

For data analysis of such diverging microcosms, it is recommended to use multivariate techniques on multiple replicate experiments (Kedwards et al. 1999; van Wijngaareden et al. 1995). These allow to reveal toxicant effects even with a small number of replicates (Kedwards et al. 1999). In that aim, we applied on a four replicate data set, the Dice/UPGMA cluster analysis on the presence/absence binary matrix, supported by the ANOSIM test. In this study, R values obtained from Opus 10 and 100 µg $L^{-1}$ treatments (0.58 and 0.67 respectively) illustrate that similarity within treatment replicates is higher than similarity between treatments and controls (Clarke 1993). Besides, the ANOSIM p-values <0.05 indicate the significance of the cluster analysis results, in other words communities exposed to 10 and 100 µg $L^{-1}$ of Opus display significantly different fingerprints compared to the control ones. When microcosms were exposed to 100 µg $L^{-1}$ of Opus, significantly fewer bands were detected in the TTGE fingerprints. Even though a band can consist of more than one sequence (Eichner et al. 1999; Muyzer et al. 1993; Muyzer and Smalla 1998), and can thus correspond to more than one species, a significant drop in the number of bands at 100 µg $L^{-1}$ can anyway be interpreted as a loss of diversity in relation with fungicide exposure. Besides, this kind of response is considered as a direct toxic effect (Brandt et al. 2004) of the fungicide on the community.

The use of 18S rDNA sequences appears as relevant to detect shifts in communities exposed to a fungicide.

### 4.3. Flow cytometry

Autofluorescence of phytoplankton cells allows their analysis using flow cytometry, directly after sampling or later, after adequate fixing and conservation of samples (Vaulot et al. 1989). This method, as applied in the present study, allows to show only photosynthetic phytoplankton populations, including eukaryote and prokaryote organisms. Flow cytometry analyses allow a rapid count of cells (a few minutes) and are run on approximately 250-300 µL of sample. Thanks to these two main assets, a large number of replicates can be considered, along with the statistical validation of results; and the small sample volumes needed allow simplified protocol design.

However, as the flow cytometer used in this study only allows the analysis of cells with diameter below 80 µm, small cells (<20 µm) at high concentrations can be discriminated, which usually correspond to picophytoplankton (0.2-2 µm) and nanophytoplankton (2-20 µm). The larger planktonic cells (20-200 µm) are not detected using flow cytometry (Collier and Campbell 1999). Thus, flow cytometry provides only a partial view of the community. Even though flow cytometry allows rapid acquisition of data about photosynthetic populations, until now its application to ecotoxicological studies on phytoplankton communities is still limited. A few studies involving flow cytometry reported the toxicity of Irgarol 1051® on phytoplankton communities (Devilla et al. 2005; Readman et al. 2004; Zamora-Ley et al. 2006).

In the present study, this tool, applied to natural phytoplankton communities, allows to point out significant effects of the fungicide Opus at 100 µg $L^{-1}$ on the prasinophyte-like and the Synechococcus populations, with opposite effects. When prasinophyte-like counts dropped dramatically, Synechococcus were enhanced, whereas no change was observed in the larger eukaryote counts. The Synechococcus may either be stimulated by the fungicide, or take advantage of the ecological niche left by the prasinophyte-like population.

**4.4. HPLC**

Nowadays, HPLC is a commonly used method for separation, identification and quantification of photosynthetic pigments from phytoplankton communities (Furuya et al. 2003; Seoane et al. 2006; Wänstrand and Snoeijs 2006). This method focuses on photosynthetic pigments of the phytoplankton community, as does flow cytometry. But whereas flow cytometry allows to analyse only small cells at relatively high concentrations, HPLC results are obtained from pigments of the whole photosynthetic community, including eukaryotes and prokaryotes. The HPLC analysis of pigments is a method of choice, due to its high sensitivity for detection and quantification of pigments (Jeffrey et al. 1999; Kwan Wong and Kim Wong, 2003; Zapata et al. 2000). However, its application to ecotoxicological studies is still scarce: to our knowledge only three studies involving HPLC were reported in works on herbicide effects on phytoplankton communities (Devilla et al. 2005; Dorigo et al. 2004; Readman et al. 2004).

In this ecotoxicological study, as the aim was to compare control and treated samples, we chose to work on relative data sets. Pigments were extracted and identified from strain cultures. And only chl a of the samples was quantified from standard injection. The peak area values from other pigments were divided by the chl a peak area value, for each sample. This

allows ratio comparisons between samples, in order to assess their variation in relation to chl a.

Applying this data treatment, we showed changes in several pigment ratios of communities, when exposed to 100 µg $L^{-1}$ of Opus. As a change in the ratio value could hide a chl a variation, a distinct statistical treatment was applied on chl a concentrations, in order to highlight and quantify the significant variations of this pigment, and to avoid misleading conclusions. As chl a concentration almost doubled in 100 µg $L^{-1}$ treated microcosms, we considered, when pigment ratio were two-fold lower than controls (fuco, but-fuco and MgDVP), that the corresponding pigment concentration was not different from control. Then, compared to the controls, communities exposed to 100 µg $L^{-1}$ of Opus still exhibited two kinds of changes:

– six pigments observed in control were not detected anymore in treated microcosms. Two of them, chl b and chlide a were already very close to the detection threshold in controls. The four other pigments were: prasinoxanthin, a prasinophyte marker (Jeffrey et al. 1997); neoxanthin, a marker of chlorophytes, prasinophytes and euglenophytes (Jeffrey et al. 1997); violaxanthin, a marker of chlorophytes, prasinophytes and eustigmatophytes (Jeffrey et al. 1997); and peridinin, a dinoflagellate marker (Jeffrey et al. 1997). Among these pigments, the prasinoxanthin, neoxanthin and violaxanthin reductions tend to confirm the drop in prasinophyte counts observed using flow cytometry.

– four other pigments exhibited significant variations in their ratios as compared with controls: chl $c_1$ dropped and the 3 others were enhanced (hex fuco, chl $c_3$ and MV chl $c_3$).

When chl $c_1$ dropped 5-fold, this may suggest an impact of the fungicide on diatoms, prymnesiophytes or raphidophytes (Jeffrey et al. 1997). Pigments for which ratios where enhanced in the presence of 100 µg $L^{-1}$ of Opus, reveal an increase of their concentrations: hexfuco which is a marker of prymnesiophytes and some dinoflagellates, like chl $c_3$ which is also found in some diatoms (Jeffrey et al. 1997).

Chl a concentration almost doubled: as chl a is ubiquitous in phytoplankton taxa, except in prochlorophytes (Jeffrey et al. 1997), its increase could be attributed, either to an enhancement of its intracellular concentration and/or to the enhanced cellular density of some taxa.

These information, arising from chemotaxonomy, provide an approximation, only at the class level, of the taxa possibly impacted by the fungicide and do not allow to determine more

precisely the organisms impacted. Furthermore, as some pigments are found across several classes (Jeffrey et al. 1999), and as these taxa do not inevitably occur in the environment during the experiment, hypotheses should be considered cautiously.

Using for each pigment, ratio related to chl a, we demonstrated significant fungicide effects on the phytoplankton community exposed to 100 µg $L^{-1}$ of Opus. These results clearly indicate a perturbation of the community, and the "loss" of several pigments suggests a lower diversity, probably in favor of taxa more tolerant to the fungicide.

**4.5. Comparison of TTGE, flow cytometry and HPLC**

Among the three overlapping methods applied here on community analysis, the genetic fingerprints revealed significant differences between controls and microcosms for Opus concentration as low as 10 µg $L^{-1}$, whereas flow cytometry and HPLC only revealed significant differences when microcosms were exposed to 100 µg $L^{-1}$ of Opus.
The comparison of the 18S rDNA fragment fingerprints appears thus as a more sensitive indicator of community disturbance, when compared to flow cytometry and pigment analysis. As a matter of fact, 18S rDNA amplified fragments, visualized using fingerprinting techniques, can be related to species (Kostanjšek et al. 2005) whereas cytometry analysis and pigment contents data can only be related to the class level or higher. This could explain that, when microcosms are exposed to 10 µg $L^{-1}$ of Opus, an effect on several species can be detected using TTGE, whereas no effect can be seen on the community using flow cytometry or HPLC. However, phytoplankton are not the only target of 18S rDNA analysis, as heterotrophic eukaryotes and/or grazers can also be present in microcosms, and thus appear in the genetic fingerprints.
Two main hypotheses could explain community modification when exposed to 10 µg $L^{-1}$ of Opus:

- the selection pressure exerted by the fungicide could reduce the population of some photosynthetic taxa, what can benefit to the more tolerant ones (Bérard et al. 1999a; 1999b; Bérard and Benninghoff 2001)
- as 18S rDNA fingerprints possibly include some grazers, the fungicide could also act on some grazer taxa, and thus disturb the complex microbial ecosystem interactions.

Brandt et al. (2004) compared, on microbial prokaryotic communities exposed to linear alkylbenzene sulfonates, the sensitivity of DGGE with other structural and functional analytical methods. The structural methods were community-level physiological profiling,

using Biolog Eco-Plate™ and Pollution-induced community tolerance, and the functional ones were the dynamics of inorganic nutrients N and P and the incorporation of [$^3$H]leucine. They also found the fingerprinting technique to be the most sensitive-one, and recommended the use of such techniques for community-level toxicity assessment.

## 5. Conclusion

The aim of this study was the comparison of the relative sensitivity of three overlapping methods for fungicide impact assessment on phytoplankton community. The detection methods tested here were focussed on the detection of modification in the community composition, as such changes are believed to be more sensitive to toxic stress than functional ones (Petersen and Gustavson 2000).

This work highlighted the sensitivity of photosynthetic organisms to the fungicide epoxiconazole when exposed to 100 µg $L^{-1}$, whether this effect is direct or indirect.

When the first step in ecotoxicological studies is usually the characterization of an impact threshold, no method can provide a full overview of structural changes induced after exposure to xenobiotics. Among the three methods tested here for community analysis, TTGE appeared the most sensitive for impact detection: only TTGE allowed to show community modifications when exposed to Opus concentration as low as 10 µg $L^{-1}$. TTGE focussed on the whole eukaryotic community, including non-photosynthetic organisms when flow cytometry and HPLC of photosynthetic pigments focussed only on phytoplankton.

In the search for improved tools for the evaluation of impact threshold, the present work underlines the fact that the definition of a threshold value depends not only on the significant effect of the toxicant, but also on the sensitivity of the analytical method applied.

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**Figure 1**

Figure 1

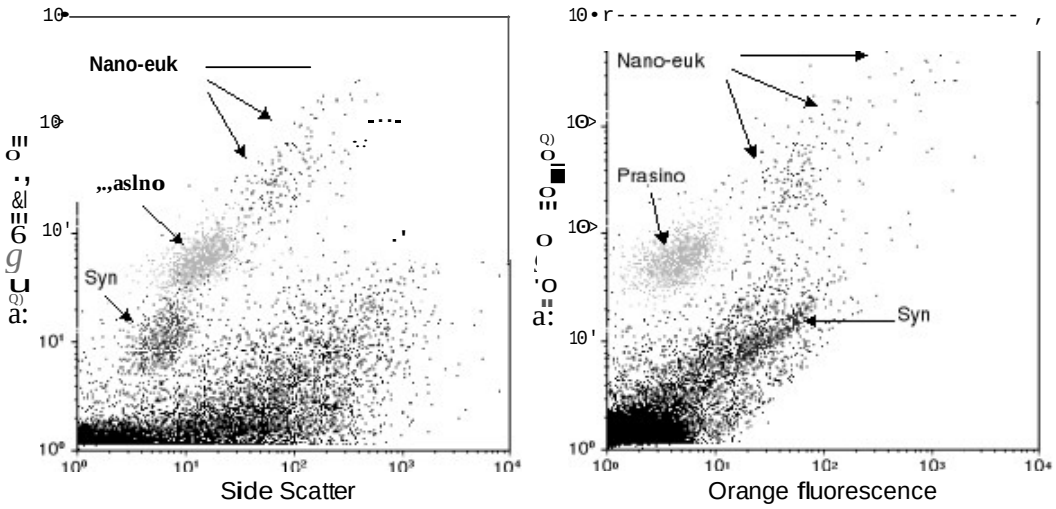
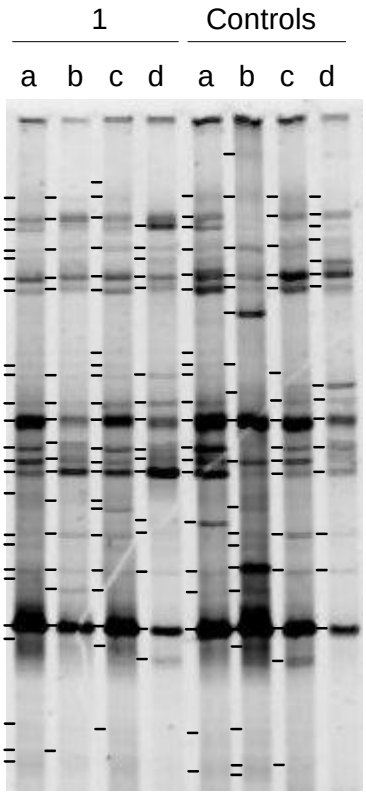


**Figure 2**

Figure 2a



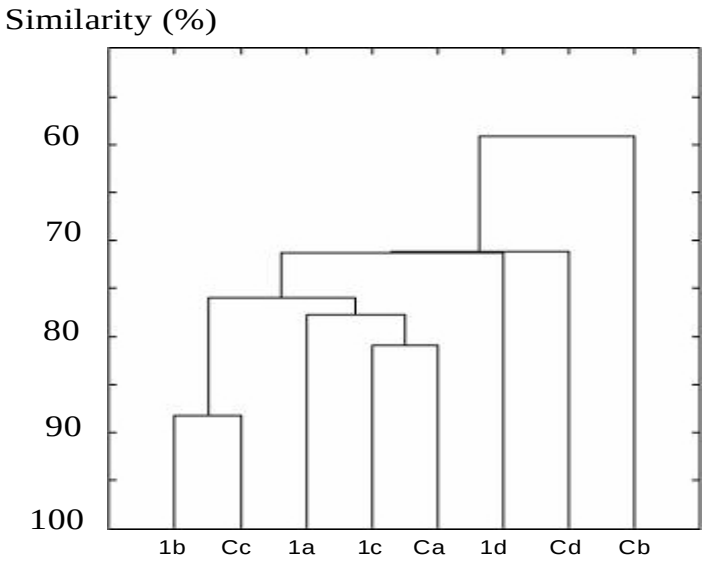
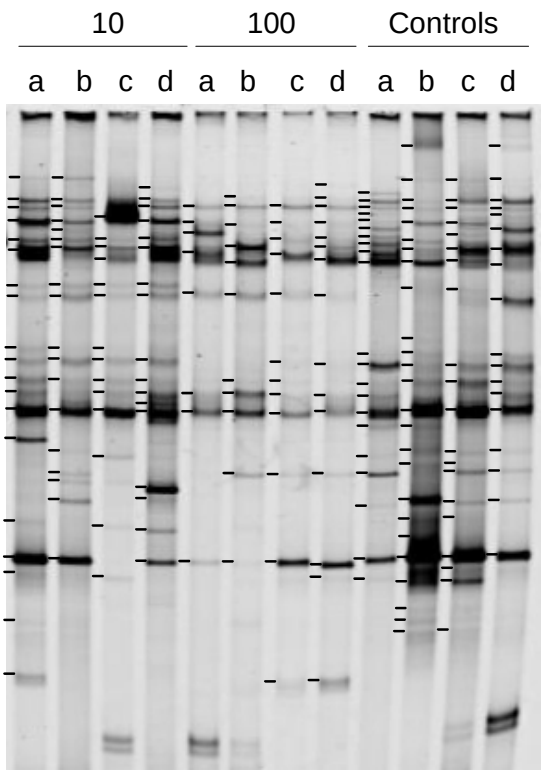




Figure 2b



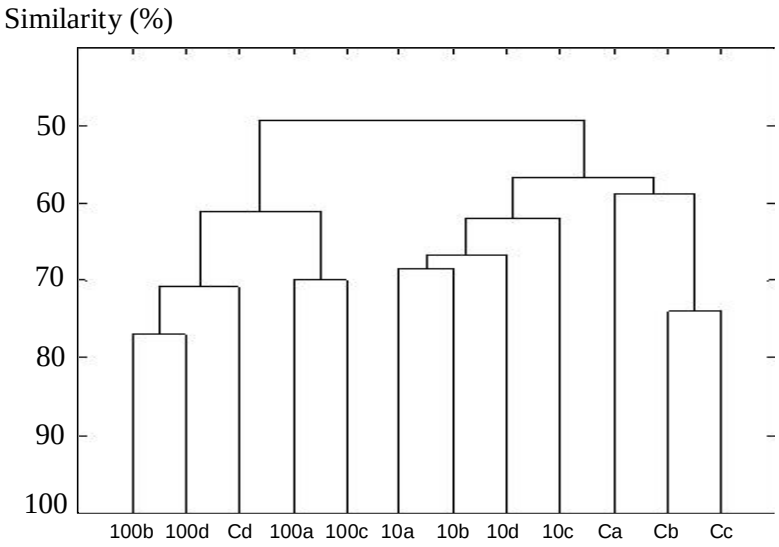



**Figure 3**

Figure 3

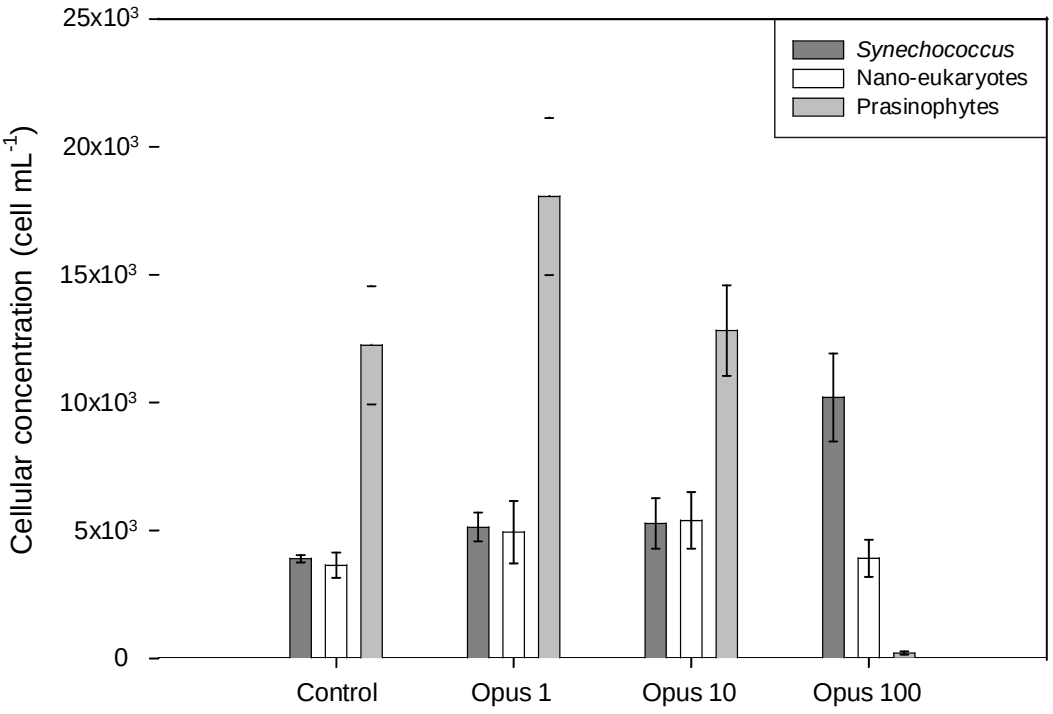


**Figure 4**

Figure 4

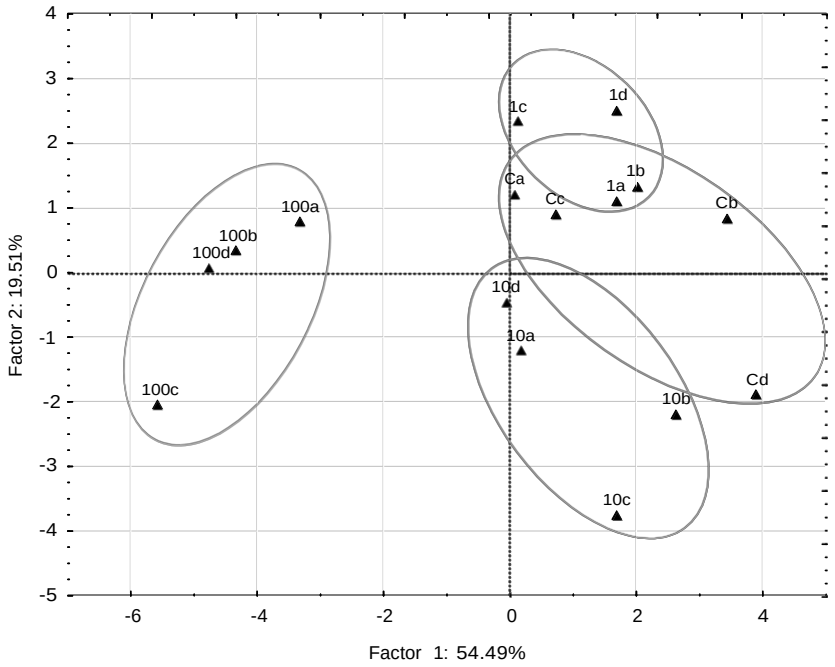


**Figure 5**

Figure 5

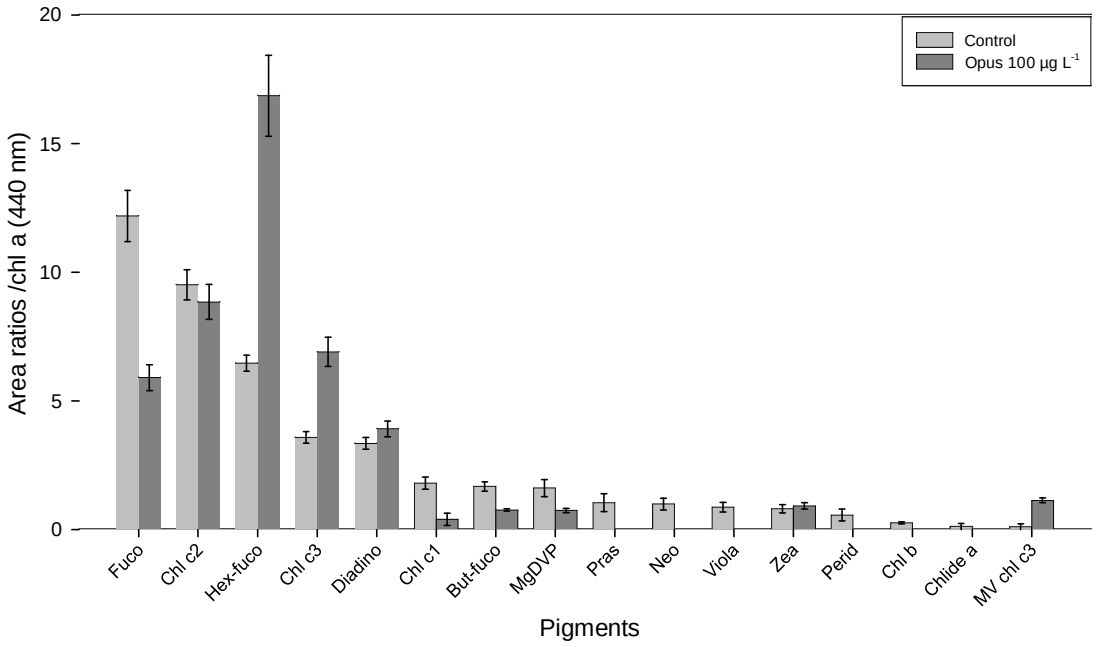


**Figure 6**

Figure 6

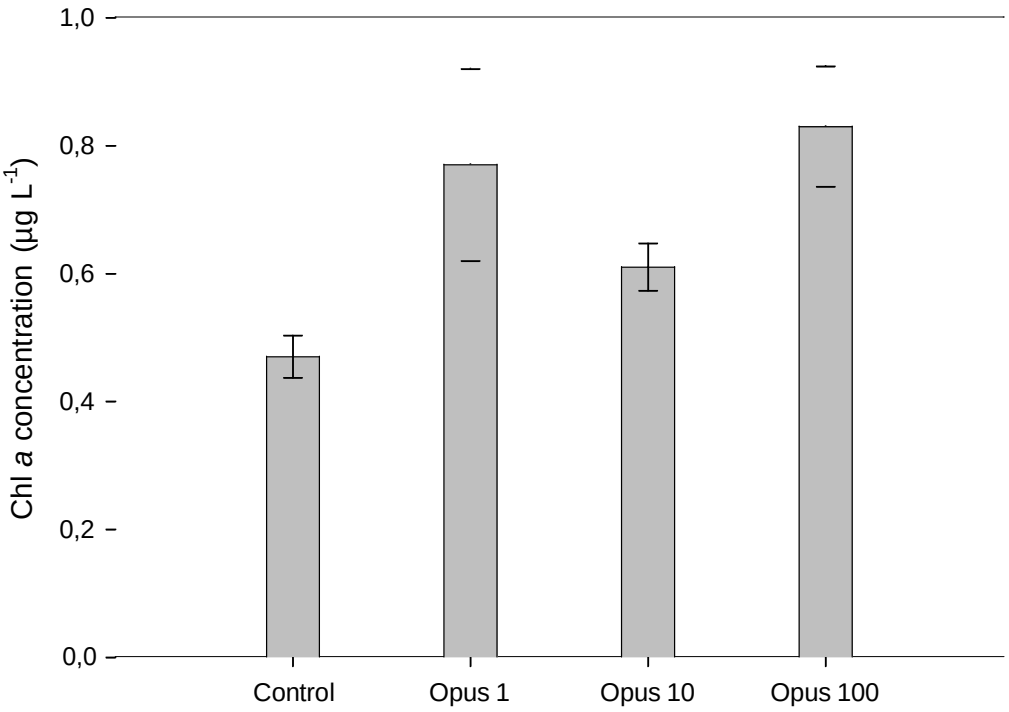


**Table 1**

Table 1

| Chlorophylls | |
|---|---|
| Chlorophyll a, b, $c_1$, $c_2$, $c_3$ | Chl a, b, $c_1$, $c_2$, $c_3$ |
| Chlorophyllide a | Chlide a |
| Mg 2,4-divinyl pheoporphyrin $a_5$ monomethyl ester | MgDVP |
| monovinyl chlorophyll $c_3$ | MV chl $c_3$ |

| Carotenoids | |
|---|---|
| 19'-Butanoyloxyfucoxanthin | But-fuco |
| Diadinoxanthin | Diadino |
| Fucoxanthin | Fuco |
| 19'-Hexanoyloxyfucoxanthin | Hex-fuco |
| Neoxanthin | Neo |
| Peridinin | Perid |
| Prasinoxanthin | Pras |
| Violaxanthin | Viola |
| Zeaxanthin | Zea |

**figure captions**

Table 1 List of pigments detected – abbreviated names

Figure 1 Cytograms of side scatter and fluorescence of a seawater sample, showing the populations of Synechococcus, prasinophyte-like, and nano-eukaryotes

Figure 2a TTGE gel profiles from 18S rDNA PCR products and UGPMA cluster analysis of Dice similarity matrix calculated from the banding patterns. C = Control; 1 = Opus 1 µg $L^{-1}$; a,b,c,d = replicates (on the left of each lane, dashes indicate the bands detected)

Figure 2b TTGE gel profiles from 18S rDNA PCR products and UGPMA cluster analysis of Dice similarity matrix calculated from the banding patterns. C = Control; 10 = Opus 10 µg $L^{-1}$; 100 = Opus 100 µg L-1; a,b,c,d = replicates (on the left of each lane, dashes indicate the bands detected)

Figure 3 Cellular concentrations (cell $mL^{-1}$) of the photosynthetic populations in microcosms on the last day of experiment, discriminated using flow cytometry (mean ± standard error, SE)

Figure 4 PCA analysis of pigment ratios in microcosms: projection of the observations (microcosms).

Figure 5 Pigment ratios in controls and in 100 µg $L^{-1}$ Opus treatments (mean ± standard error, SE).

Figure 6 Chl a concentrations (µg $L^{-1}$) in microcosms on the last day of experiment (mean ± standard error, SE)