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Evaluation of the partial renewal of in situ phytoplankton microcosms and application to the impact assessment of bentazon and dimethenamid

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Abstract:

Microcosms, each consisting of 2 L natural surface seawater maintained in 2.3-L glass bottles, were immersed at a depth of 6 metres. The renewal of ten percent of microcosm volumes was carried out every other day. Phytoplankton-containing seawater was used for renewal (previously filtered through 25-, 50- or 200- μ m cut-off). Phytoplankton community pigment analysis (by HPLC) and flow cytometry analysis were performed. After 13 days, data exhibited phytoplankton characteristics in microcosms in the same range as that of the natural surrounding sea water over the same period. Furthermore, in these microcosms, a negative correlation was observed between the filtration cut-off used for renewal water, and the eukaryote cell count. Herbicides were tested as commercial mixtures at 1, 10 and 100 μ g.L⁻¹ active principal. Both Frontier® (dimethenamid) and Basamais® (bentazon) induced significant modifications of the phytoplankton populations at every concentration tested. Such results suggest a possible disturbance in polluted coastal areas.

Keywords: phytoplankton; microcosm; herbicides; dimethenamid; bentazon

1. Introduction

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3 Pesticides are in widespread use, especially in intensive agriculture, but induce environmental
4 hazards such as acute or chronic contamination of aquatic biota via spray drift, leaching or
5 run-off from treated areas. In estuarine and coastal areas, herbicides have been reported at
6 concentrations ranging from ng.L^{-1} to $\mu\text{g.L}^{-1}$ (Chesworth et al. 2001; Steen et al. 2001; Oros et
7 al. 2003; Burgeot et al. 2007; Lewis et al. 2009; Añasco et al. 2010). These contaminants can
8 negatively impact non-target organisms including phytoplankton, which are potentially
9 vulnerable to herbicides due to their physiological similarities to terrestrial plants (DeLorenzo
10 et al. 2001; Dorigo et al. 2004). The impact of herbicides on phytoplankton communities
11 could seriously damage ecosystem function, as these primary producers are vital for nutrient
12 transfer to higher trophic levels.
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Phytoplankton toxicity assessments are usually run using monospecific microalgal bioassays
in controlled laboratory conditions. Many such studies have been conducted on pesticides
(Rioboo et al. 2002; DeLorenzo et al. 2004; Weiner et al. 2004; Gatidou and Thomaidis 2007;
Santin-Montanya et al. 2007; Galhano et al. 2009; Prado et al. 2009; Magnusson et al. 2010),
but the sensitivity threshold in such tests is highly dependent on the species tested (Cairns
1986). Furthermore, Bérard et al. (1999) demonstrated that single-species tests may fail to
predict indirect or system responses, such as changes in the population distribution over time.
Therefore, studies focusing on the whole natural community, 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. 1999).

The impact of toxicity on natural communities can be assessed in several ways: by sampling
at different natural sites and/or times to look for pollution-related characteristics (Kostanjšek
et al. 2005; Pesce et al. 2008), by sampling natural communities from the field and
maintaining them in microcosms under controlled laboratory conditions (DeLorenzo et al.
1999b; Seguin et al. 2001), or by conducting studies using *in situ* microcosms. We recently
showed that such microcosms immersed in “average water column conditions”, with partial
water renewal, can be considered as good experimental models for natural nano- and
picophytoplankton from coastal waters (de la Broise and Palenik 2007). Such *in situ* micro- or
mesocosm experiments offer the best aspects of both laboratory and open field systems, as
this experimental set-up allows the testing of multiple replicates in ecologically realistic
conditions (Van den Brink et al. 2002). Microcosms set up in natural environments allow a
wider species spectrum to be tested, including populations that are difficult to maintain *ex*

1 *situ*, and can thus permit a wider survey of the susceptibility of the microbial community to
2 toxicants (Yasuno et al. 1993). Seguin et al. (2001) and Leboulanger et al. (2001) pointed out
3 the need for such microcosm systems in order to obtain reliable toxicity data. In the present
4 study, we chose to develop small-volume high throughput *in situ* microcosms, allowing
5 routine testing of several substances, using up to 108 microcosms simultaneously.
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9 The aims of the present study were to:

- 10 – Investigate the need for partial renewal in microcosms;
- 11 – Compare microcosms with the natural surrounding surface water to evaluate whether they
- 12 support communities that are representative of the surrounding environment;
- 13
- 14 – Validate the microcosm experimental system for the impact assessment of herbicides on
- 15 natural phytoplankton communities.
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22 Pigment fingerprints are one of the tools that can be used to provide an overall view of
23 phytoplankton communities, as their profiles are related to community composition. They are
24 obtained from High performance liquid chromatography (HPLC) analysis and can be applied
25 in studies dealing with environmental diagnoses (Sherrard et al. 2006), as well as in
26 ecotoxicological studies to assess the effects of pesticides on the phytoplankton community
27 (Dorigo et al. 2004; Readman et al. 2004; Devilla et al. 2005; Stachowski-Haberkorn et al.
28 2009).

29
30 Flow cytometry can also provide information about the distribution and abundance of natural
31 photosynthetic populations (Marie et al. 1999; Rutten et al. 2005). Toxicity assessment using
32 flow cytometry has been used both in studies based on phytoplankton cultures (Lage et al.
33 2001; Stauber et al. 2005; Yu et al. 2007) and those examining natural photosynthetic
34 communities (Readman et al. 2004; Zamora-Ley et al. 2006; de la Broise and Palenik 2007;
35 Stachowski-Haberkorn et al. 2008, 2009).

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37 These two complementary techniques were combined in our sample analysis to increase the
38 sensitivity with which we could compare phytoplankton communities.
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53 **2. Materials and Methods**

54 A set of microcosms in bottles containing filtered surface seawater was placed *in situ* and
55 their contents partially replaced every two days during experiments. We designed the
56 experiments to test: i) the role of filtration cut-off level used on the renewal water and ii) the
57 impacts of Basamaïs® and Frontier®, on marine phytoplankton communities.
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1 Chlorophyll *a* (Chl *a*) measurements and pigment fingerprints were obtained from biomass
2 collected on filters, and microcosm subsamples were also analysed using flow cytometry.
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7 **2.1. *Microcosm apparatus***

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9 The outdoor experiments were conducted from mid June to early August 2006, in Port-la-
10 Forêt Bay, southern Brittany, France (47°52'12"N, 03°58'35"W). Microcosms consisted of
11 hermetically sealed 2.3-L glass bottles. These bottles were filled with 2 L of 200- μ m filtered
12 fresh seawater from the surface layer at the field site, containing natural zooplankton and
13 phytoplankton communities, enclosed with about 300 mL of air (de la Broise and Palenik
14 2007). Thirty-six microcosms were installed on a 3-m diameter circular stainless-steel frame
15 (a "wheel") that was anchored to the sea floor with a 400 kg concrete block, and suspended
16 from a surface buoy allowing the bottles to remain at 6 m depth (average mid-depth of the
17 water column), regardless of the tide (Stachowski-Haberkorn et al. 2008). Every other day,
18 the wheel was hoisted out of the water and positioned so that it encircled the boat (Fig. 1),
19 allowing bottle handling and partial medium renewal. Three wheels with microcosms were
20 installed for the summer.
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31 **2.2. *Renewal experiment***

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33 Microcosms were managed according to two protocols: either with no water renewal
34 (control), or a 10% renewal (200 mL) every other day, using ambient seawater filtered
35 through mesh of different sizes: 0.22, 25, 50 and 200 μ m. Thirty microcosms were prepared:
36 six replicates for each of the five treatments. Replicate microcosm bottles were distributed all
37 around the frame (one from each of the five treatments in succession). The experiment was
38 continued for 13 days. On the first, fourth and last days of experiment, five samples of
39 surrounding surface seawater (2 L) were also collected for analysis.
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49 **2.3. *Herbicide assessment***

50 **2.3.1. *Herbicides***

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52 The herbicides tested in this study are the commercial formulations of bentazon (Basamaï's®,
53 BASF) and dimethenamid (Frontier®, BASF). Concentration values represent those of the
54 pure active substances. The herbicide stock solutions (10 mg/L bentazon and 10 mg/L
55 dimethenamid) were prepared in 0.22- μ m filtered seawater, previously autoclaved for 20 min
56 at 121°C. After agitation for 48 h using a magnetic stirrer, the solutions were kept at -24°C.
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1 Freshly thawed stock solutions were added directly into the microcosm bottles just before
2 immersion. Herbicide concentrations were analyzed on pooled water samples from all the
3 replicate bottles of each treatment, collected on the last day of the experiment. Samples were
4 frozen until analysis.
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8 Herbicide analyses were performed by the IDHESA Laboratory (Brest, France), using on-line
9 solid-phase extraction coupled with liquid chromatography and electrospray ionisation-
10 tandem mass spectrometry (Waters 2690 HPLC Separation Module; Waters 996 Photodiode
11 Array Detector; Micromass® Quattro Ultima™ Mass Spectrometer). The detection limit was
12 0.005 µg.L⁻¹.
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18 19 20 *2.3.2. Herbicide experiments*

21 An experiment was carried out for each herbicide. In both cases, twenty microcosms were
22 prepared: five replicates for control and five for each of the three concentrations tested (1
23 µg.L⁻¹, 10 µg.L⁻¹ and 100 µg.L⁻¹). Replicate microcosm bottles were distributed all around the
24 frame (one from each of the four treatments in succession).
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27 Every other day, the frame was hauled out of the water and 10% (200 mL) of the 2 L seawater
28 in each bottle was collected in a tank for proper disposal, and then replaced with the same
29 volume of fresh 200-µm filtered surrounding seawater, before re-immersing the frame. All the
30 bottles were finally collected for analyses on the last day. They were kept protected from the
31 light in plastic boxes, under white PVC sheets, until treatment.
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40 *2.4. Analyses*

41 From each microcosm or surrounding seawater sample, 1 L water was filtered through 0.22
42 µm polysulfone filters. The filters were then stored at -80°C until pigment analysis using
43 HPLC. For cytometry analyses, samples (1.5 mL) were dropped into cryotubes and fixed with
44 glutaraldehyde (final concentration 0.25%). Cryotubes were vortexed and left 15 minutes in
45 the dark at room temperature before freezing in liquid nitrogen. Samples were stored at -80°C
46 until analysis.
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54 55 *2.4.1. HPLC pigment analysis*

56 Pigments were extracted from the polysulfone filter in a 95% methanol solution and were
57 separated in a mobile phase gradient (methanol, acetonitrile, acetic acid and aqueous pyridine
58 solution) through a Waters Symmetry®-C8 reversed-phase column and analysed through a
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1 diode array detector. These extraction and separation protocols, adapted from Zapata et al.
2 (2000), were previously described in Stachowski-Haberkorn et al. (2009).
3

4 *Pigment fingerprinting*

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6 Peaks were identified based on their retention time and absorption spectra compared with
7 those of published pigment spectra (Jeffrey et al. 1997) and with those of pigment extracts
8 prepared from clonal cultures with known pigment composition, obtained from the Roscoff
9 Culture Collection (Vaulot et al. 2004): the dinoflagellate *Amphidinium carterae* (RCC88),
10 the chlorophyte *Dunaliella tertiolecta* (RCC 6), the prymnesiophytes *Emiliana huxleyi*
11 (RCC174), *Pavlova lutheri* (RCC180) and *Phaeocystis globosa* (RCC187), the chrysophyte
12 *Pelagococcus subviridis* (RCC98), the diatom *Skeletonema costatum* (RCC70) and the
13 cyanophyte *Synechococcus sp.* (RCC752). If “Pigment X” represents the pigment name,
14 Pigment X ratio was defined as: pigment X area / sample-Chl *a* area. The pigment fingerprint
15 of a sample was defined as the set of all pigment ratios from this sample.
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26 *The Chl a quantification*

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28 Chl *a* concentration, measured at 440 nm, was calibrated against a Chl *a* standard solution
29 (SIGMA, chlorophyll *a* from spinach for HPLC Ref 10865), allowing calculation of Chl *a*
30 concentration in samples.
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36 *2.4.2. Flow cytometry*

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38 Samples were run using a FACSort flow cytometer equipped with a 488-nm argon laser and
39 standard filter set-up (Becton Dickinson, San Jose, CA). Data were analysed using WinMDI
40 v2.9 software (J. Trotter, <http://facs.Scripps.edu/>). Three populations of photosynthetic
41 organisms were discriminated on the basis of their scatter signals and their natural red (>630
42 nm) and orange fluorescence (580 ± 20 nm), as described in Stachowski-Haberkorn et al.
43 (2008).
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50 *2.5. Statistical and principal component analysis of data*

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

Principal Component Analysis (PCA) of pigment fingerprints was performed using Statistica 8 software.

3 Results

3.1 *Experimental set-up*

The stainless-steel wheel supporting the microcosm bottles was easily hoisted manually onto the boat due to its seemingly low weight in water. After the wheel had been installed encircling the boat (Fig. 1), the whole structure was stable enough to allow bottle handling and the partial renewal of their contents. Microcosms were maintained out of the water for handling during approximately 15 minutes.

Work was only possible when the average wave height was less than 0.5 metres.

Such conditions are rarely exceeded in a protected bay, during the summer season. However, very rough conditions were observed on the day before the end of the experiment, when some bottles were lost.

3.2 *Microcosm assessment*

In natural surface water, Chl *a* values (Fig. 2) decreased approximately 8-fold from day 0 to day 13.

In every microcosm, either control bottles or bottles with renewal, results on day 13 also showed a drastic Chl *a* reduction (Fig. 2). This illustrates an important decrease in photosynthetic biomass over the period, both in microcosms and in the surrounding surface water.

Pigment fingerprints, obtained from HPLC analysis of microcosms and surface water, were analysed by PCA (Fig. 3).

Pigment fingerprint replicates within a group from surface water samples on the same day (day 0, day 4 or day 13) showed close positions on the graph, but the three groups had distinctly separate positions. This illustrates the marked evolution of pigment fingerprints of the surface water over the period. After 13 days, the microcosms whose renewal was carried out with 25-, 50- or 200- μm filtered sea-water exhibited, an intermediate position (ellipse drawn in the centre) compared with those of natural surface water samples. Conversely, microcosms without renewal, or where renewal was made with water without living biomass (cut-off 0.22 μm), had values that were double those of any surface water sample (uppermost

1 ellipse), suggesting in these microcosms a phytoplankton structure different from the
2 surrounding waters over the period.

3 Analyses of these samples were also performed using flow cytometry. In surface water on the
4 first day of the experiment, the detected phytoplankton cells (Fig. 4a) were mainly larger
5 eukaryotes (about two thirds of the total cell count) followed by Prasinophytes, and then by a
6 minority of *Synechococcus*. After four days, although the total cell count was maintained in
7 the same range (Fig. 4b), a shift occurred in cell distribution, with the main cell population
8 becoming the Prasinophytes. On day 13, this distribution pattern of relative abundances was
9 maintained, but the total cell counts had dropped dramatically to a value about 3.5 times lower
10 than on the first and fourth days of experiment (Fig. 4b). This decrease of larger cells
11 (Prasinophytes + larger eukaryotes) observed throughout the experiment, is consistent with
12 the concomitant Chl *a* decrease (Fig. 2).
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21 In microcosms, on the 13th day of the experiment, flow cytometry distribution patterns
22 showed two trends:
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24 – Control and 0.22 μm microcosms were dominated by larger eukaryotes (more than two
25 thirds of the total cell count), while Prasinophytes and *Synechococcus* both stood at values
26 equal or lower than 20%. These microcosms appeared different from any of the surface water
27 samples or other microcosms, as the Prasinophyte counts in the samples were 4 to 5 times
28 lower than the counts for larger eukaryotes.
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34 – Microcosms with partial filtration at 25, 50 and 200 μm exhibited a pattern within the range
35 of surface waters over the period: *Synechococcus* were lower than 30%, Prasinophytes ranged
36 from 30 to 60%, and larger eukaryotes from 20 to 65%.
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40 Total counts in this second group of microcosms on day 13 were also at intermediate values
41 compared with the surface water over the same period.
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43 In these microcosms, total cell counts were significantly higher when filtration cut-off was
44 reduced from 200 to 50 and 25 μm (Fig. 4b; Spearman rank correlation $r = -0.60$, $p\text{-value} =$
45 0.016).
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51 **3.3 Pesticide impact assessment**

52 **3.3.1 Bentazon**

53 Bentazon was added to the microcosms at 1, 10 and 100 $\mu\text{g.L}^{-1}$ on the first day. Due to very
54 rough weather conditions, this exposure experiment was terminated after 7 days.
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1 The final bentazon concentrations measured in the microcosms after 7 days were 0.76, 5.67,
2 and 28.49 $\mu\text{g.L}^{-1}$, respectively, while the expected remaining herbicide concentrations,
3 calculated after 3 renewals, were 0.73, 7.3 and 73 $\mu\text{g.L}^{-1}$, respectively.
4

5 After 7 days, no difference in Chl *a* values was detected for any bentazon concentration
6 compared with the control (data not shown).
7

8 Using PCA of the pigment fingerprints from microcosm replicates (Fig. 5), 4 groups could be
9 detected that were related to the bentazon concentrations.
10

11 The 5 microcosms exposed to 1 $\mu\text{g.L}^{-1}$ were positioned far from those of the 5 control
12 microcosms. Furthermore, while the 10 $\mu\text{g.L}^{-1}$ group was closer to the control group, the 100
13 $\mu\text{g.L}^{-1}$ microcosm replicates were localised mainly (3 out of 4) distant from the control. Such
14 differences suggest a modification of the pigment fingerprints, starting at 1 $\mu\text{g.L}^{-1}$ bentazon.
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17 A further data analysis of the pigment fingerprints from microcosms showed, for 10 pigments
18 out of 17, a significant effect of the bentazon addition (Fig. 6). It must be noted that, although
19 the 1 $\mu\text{g.L}^{-1}$ and 10 $\mu\text{g.L}^{-1}$ induced similar effects on the pigment ratios (of the 6 impacted
20 pigments, 4 were simultaneously impacted), the 100 $\mu\text{g.L}^{-1}$ treatment had a different impact:
21 over the 5 impacted pigments for this concentration, only one was also impacted at 1 $\mu\text{g.L}^{-1}$,
22 and none were at 10 $\mu\text{g.L}^{-1}$.
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25 Flow cytometry analysis (Fig. 7) also showed these significant differences for every exposed
26 microcosm compared to the control. Furthermore, as for pigment analysis, flow cytometry
27 revealed two kinds of impact, one at 1 and 10 $\mu\text{g.L}^{-1}$, and the other at 100 $\mu\text{g.L}^{-1}$. In both
28 cases, among the three populations detected, only one was significantly impacted: the larger
29 eukaryote abundance was reduced by 23% in 1 $\mu\text{g.L}^{-1}$ and by 27% in 10 $\mu\text{g.L}^{-1}$, while the
30 Prasinophyte abundance was increased by 99% in 100 $\mu\text{g.L}^{-1}$ microcosms.
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33 3.3.2 Dimethenamid:

34 Dimethenamid was added to microcosms at 1, 10 and 100 $\mu\text{g.L}^{-1}$ on the first day. The final
35 concentrations measured in microcosms after 12 days were 0.61, 1.89 and 23.17 $\mu\text{g.L}^{-1}$,
36 respectively, while expected remaining herbicide concentrations, calculated after 5 renewals,
37 were 0.59, 5.9 and 59 $\mu\text{g.L}^{-1}$, respectively.
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40 After microcosms were exposed to dimethenamid for 12 days, Chl *a* concentrations (Fig. 8)
41 were significantly higher for all tested doses (p-value <0.05 Mann-Whitney). Furthermore, a
42 significant positive correlation (Spearman rank: 0.76; p-value <0.05) was observed between
43 dimethenamid and Chl *a* concentrations.
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1 Similarly, flow cytometry analysis (Fig. 9) showed a significant increase of 68% and 86% of
2 larger eukaryotes in microcosms exposed to 10 and 100 $\mu\text{g.L}^{-1}$ respectively, and a significant
3 correlation (Spearman rank: 0.64, $p < 0.05$) was also demonstrated. However, PCA did not
4 suggest any difference in pigment fingerprint compared with the control (Fig. 10).
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10 **4 Discussion**

11 ***4.1 Experimental set-up***

12 By running up to 108 independent microcosms, the experiment simultaneously tested multiple
13 replicates of phytoplankton communities exposed to different molecules at different
14 concentrations. The experiments, where three stainless-steel wheels were immersed and
15 hoisted out of the water every other day, could be efficiently run by only two persons.
16 However, working on a small (3.9 m) boat was quite challenging, and we would suggest, for
17 routine ecotoxicological work, to use a larger boat equipped with a hoist. Before the work
18 described here was performed, tests were carried out over the two previous summers. The
19 total work conducted over these three summer seasons represents approximately one hundred
20 immersion and hauling operations, and the wheels were maintained underwater for
21 approximately 5 months per year over this 3-year period, without significant problems except
22 the impact due to bad weather during the last experiment. The material was installed in a bay
23 open to northward wind and swell. A more protected area, which would also have offered the
24 required 8-metre depth for mooring, was not available at working distance from the
25 laboratory. Should a permanent experimental site be organised, the first selection criteria
26 would be to use an area that was both deep and protected.
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44 ***4.2 Microcosm assessment***

45 Chl *a* content of the surface water exhibited a marked decrease over the experimental period
46 (Fig. 2), and flow cytometry analysis showed large decreases in total cell number, particularly
47 the larger eukaryotes, over the same period (Fig. 4a, 4b). As the chlorophyll content is usually
48 higher in larger cells and as the cell fraction larger than 20 μm was not counted by flow
49 cytometry, the total cell count cannot be directly related to the chlorophyll value. However the
50 reductions observed in cell count could, at least partly, explain the Chl *a* decrease observed.
51 Such a reduction could be either due to the mixing or the displacement of the water masses by
52 currents and tides, or to the evolution of the phytoplankton populations within the water
53 column under physical and/or biological constraints (Cloern 1996; Pannard et al. 2008).
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1 Our goal was to maintain the phytoplankton community in microcosms as similar as possible
2 to the natural community in surrounding waters over the experimental period.

3 On day 13, when no renewal or renewal only with cell-free water was made, the microcosms
4 showed a drastic reduction in Prasinophyte/larger eukaryote ratios compared to natural waters
5 or to other microcosms. It should be noted that these differences in ratio would be even
6 higher, if the comparison was based on the (more ecologically significant) cumulative cell
7 surface or cell volume of each population. The PCA of pigment fingerprints also suggested
8 that populations of these microcosms were different from surface water samples.
9

10 In the 0.22 μm microcosms, the water renewal, using cell-free water, diluted the populations
11 compared with the control microcosms. Its effect can be estimated to reduce the cell counts by
12 47% over the experiment (6 times at a 10% rate). However, the total cell count remained
13 slightly higher in these microcosms than in the control. This result suggests that cell-free
14 water addition could help to maintain the phytoplankton population. It must be noted that if
15 cells were not discarded during the water renewal process, the calculated cell densities in
16 these 0.22 μm microcosms would be almost doubled, and would then be in the same range as
17 in the other renewal microcosms.
18

19 Chlorophyll *a* and total cell counts decreased in microcosms over the 13-day period.

20 A first cause could be, as hypothesized for the natural surrounding waters, a change in
21 physical environmental conditions (light, temperature, etc) that would modify the fate of
22 populations subject to growth, death and grazing.
23

24 Second, previous studies have demonstrated that in phytoplankton communities, the reduction
25 of the biodiversity induces a reduction of biomass productivity (Ptacnik et al. 2008, Striebel
26 et al. 2009). This could possibly apply here, especially to the NR and 0.22 μm microcosms. In
27 these enclosed environments, specific individuals could be grazed or destroyed, and the lack
28 of cell addition through renewal process could induce a reduction in the community diversity.
29 The dramatic changes in population ratios observed here may be related to such a reduction in
30 diversity. A subsequent reduction in productivity could occur, and the populations would then
31 be reduced.
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33 From these experiments, we showed that microcosms without renewal, or the use of cell-free
34 sea water for renewal, did not fulfil our objective of a phytoplankton community, whose
35 composition would be close to the natural surrounding environment: The cell ratio was not
36 representative of natural surrounding waters.
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38 When 25-, 50- or 200- μm filtered waters were added, the distribution of populations after 13
39 days exhibited an intermediate pattern when compared to surface waters over the period.
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Furthermore, PCA on pigment fingerprints showed these microcosms as intermediate compared with surface water samples. These results demonstrate the positive effect of a partial renewal to maintain the phytoplankton community similar to the natural surrounding one.

Such results are also consistent with our previous work (Stachowski-Haberkorn et al. 2009), which demonstrated similar diversity indices, a similar number of species, and the same three major groups in communities in 200- μm filtered microcosms and the surrounding surface waters.

Present results also showed a relationship between the reduction of the filtration cut-off (200, 50, and 25 μm) of the renewal water, and the increase of the total cell populations. We hypothesize that such a reduction in filtration cut-off may induce a reduction in the microzooplankton content (smaller than 200 μm): this could induce a reduction of the grazing pressure, and thus an increase of the phytoplankton populations in these microcosms. Such high grazing pressure of microzooplankton on the <20 μm phytoplankton fraction has already been reported (Gifford et al. 1995). Similar cell density increases were also observed in experiments where the grazing pressure was reduced by dilution (Landry et al. 1993).

Therefore, in order to maintain the top-down control of phytoplankton populations by grazers in microcosms at levels close to those of the surrounding waters, we must avoid reducing the grazer population when performing water renewal. This can be done by using water supply subjected to only a large filtration cut-off, thus allowing the phytoplankton community in microcosms to be similar to the natural surrounding waters. As total cell count is still higher in microcosms than in natural surrounding waters, even in 200- μm filtered microcosms, a higher filtration cut-off should be tested that could possibly mimic the natural conditions better.

These experiments suggest that the positive role of renewal in maintaining a close-to-natural phytoplankton community is mainly due to the inoculation of fresh grazers and cells.

Thus, we can hypothesize that, in microcosms where renewal is omitted, microzooplankton is rapidly destroyed: because these organisms cannot operate their daily vertical migration, they could be affected by light conditions during the day.

As this could then induce a reduction of the grazing pressure on larger eukaryotes, these possibly mixotrophic cells could increase their competitive and grazing pressure on smaller Prasinophytes and *Synechococcus*.

4.3 Herbicide impact assessment

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2 When bentazon or dimethenamid were added at $1 \mu\text{g.L}^{-1}$, the concentrations detected at the
3 end of the experiment were close to the expected values for both herbicides. The detected
4 concentrations for $10 \mu\text{g.L}^{-1}$ herbicide treatments were lower than expected. For the $100 \mu\text{g.L}^{-1}$
5 treatments, they were less than half the expected value for the $100 \mu\text{g.L}^{-1}$. This could be due
6 to the possible adsorption/desorption during the dilution process, on bottle walls and/or on the
7 organic matter present in seawater. Such losses are consistent with previous data obtained in
8 the same kind of experiments (Stachowski-Haberkorn et al. 2009) and were also reported by
9 Damiens et al. (2004). Pesticide concentrations encountered in estuarine areas are usually
10 below $1 \mu\text{g.L}^{-1}$ (Lehotay et al. 1998; Oros et al. 2003; Steen et al. 2001). However, for
11 example, a glyphosate concentration of $1.2 \mu\text{g.L}^{-1}$ was detected in Marennes-Oléron bay over
12 an 11-day period (Burgeot et al. 2007), and analyses performed by IDHESA Laboratory
13 (Brest, France) reported maximal concentrations of $0.41 \mu\text{g.L}^{-1}$ for glyphosate and $0.64 \mu\text{g.L}^{-1}$
14 for its degradation product, AMPA, in the Bay of Brest (Gaël Durand, pers. comm.). We
15 can therefore consider the lowest concentration tested here likely to be representative of peak
16 pollution in coastal areas.
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4.3.1 Bentazon

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31 Flow cytometry analysis showed a significant reduction in the larger eukaryote counts for the
32 lowest tested concentration of $1 \mu\text{g.L}^{-1}$. This impact is confirmed by a modification of the
33 pigment fingerprints, demonstrated using both PCA and pigment ratio comparison. The same
34 effects on larger eukaryotes and pigment ratios were observed for $10 \mu\text{g.L}^{-1}$ treatments. At
35 $100 \mu\text{g.L}^{-1}$ exposure, however, a different impact was observed on cell counts and on pigment
36 ratio, and on the PCA graph (Fig. 5).
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44 Such results show that the modification of the phytoplankton community structure differs
45 depending on the pesticide concentration. We may hypothesize that, the larger eukaryote
46 population was impacted at 1 and $10 \mu\text{g.L}^{-1}$ herbicide concentrations, but that increased
47 concentration ($100 \mu\text{g.L}^{-1}$) could impact the grazers of Prasinophytes and larger eukaryotes,
48 thus offsetting the direct effect on larger eukaryotes and inducing the increase in
49 Prasinophytes cell counts.
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4.3.2 Dimethenamid

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When exposed to 1 $\mu\text{g.L}^{-1}$ dimethenamid for 12 days, only Chl *a* exhibited a significant increase. The 10 $\mu\text{g.L}^{-1}$ and 100 $\mu\text{g.L}^{-1}$ doses induced increases in both Chl *a* content and larger eukaryote counts, with significant dose-correlation. These positive dose-correlations, suggest that grazers could be the impacted population, inducing an increase of the photosynthetic community by reduction of the grazing rate. However, compared to bentazon, whose impact was stronger, even though it was added over a shorter 7-day period, these results suggest that the phytoplankton community could be less sensitive to dimethenamid.

5 Conclusion

Microcosms attached to immersed structures allow enclosed phytoplankton communities to be exposed to natural light and temperature conditions. A recurrent partial renewal, by addition of natural, cell-containing water obtained through high cut-off filtration, was required to maintain the characteristics of the phytoplankton community close to those of the surrounding environment. Further work is needed to confirm the putative role of grazers in such microcosms, and to test the effects of higher filtration cut-off, and other renewal frequencies. Both bentazon and dimethenamid induced modifications of the phytoplankton community starting at 1 $\mu\text{g.L}^{-1}$, the lowest tested concentration. This suggests that these pesticides could impact phytoplankton communities in polluted coastal areas.

The populations directly or indirectly impacted differ depending on the dose and herbicide molecule tested. This illustrates the diversity of targets concerned by phytoplankton toxicity, and thus the advantage of using microcosms, rather than monospecific cultures, for environmental toxicity assessments. Further experiments are still needed to investigate the mechanisms involved on zooplankton and/or phytoplankton herbicide toxicity.

The equipment presented here allows a large set of these microcosms to be run efficiently and simultaneously, for periods up to two weeks. This makes it an efficient tool for impact assessments of multiple chemical compounds on phytoplankton communities in “close-to-natural” conditions, over ecologically realistic periods.

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3 analysis, and Louis Quiniou and Hansy Haberkorn for advice on data treatment.
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Figure 1: Partial view of the microcosm support frame, shown hoisted out of the water as for partial microcosm renewal.

Figure 2: Chlorophyll *a* in surrounding surface water during the experiment and in microcosms after 13 days. Renewal water was prepared from surface water filtered through 0.2, 25, 50, or 200 μm mesh. Vertically-aligned crosses represent replicates. Shared letters show where there is no significant difference (Mann-Whitney test).

Figure 3: PCA of pigment fingerprint data from microcosms and surface water samples. **d0, d4, d13**: replicate samples from surface water on day 0, day 4, and day 13, respectively. **0.22, 25, 50, 200**: samples from microcosm replicates subjected to 10% water renewal every other day with water filtered through 0.2, 25, 50, or 200 μm filters, respectively. **NR**: microcosm replicates with no water renewal.

Figure 4: Flow cytometry analysis of samples from surface water during the experiment and from microcosms after 13 days. Bars and dots represent the mean of 4 or 5 microcosm analyses, error bars represent standard errors.

A: Cell count distribution of populations

B: Total cell count.

Figure 5: PCA of pigment fingerprint data sets (17 pigments) from microcosms exposed to bentazon for 7 days. Each dot represents a treatment replicate.

0: no bentazon; **1**: 1 $\mu\text{g/L}$ bentazon; **10**: 10 $\mu\text{g/L}$ bentazon; **100**: 100 $\mu\text{g/L}$ bentazon.

Figure 6: Pigment ratio from HPLC analyses, in microcosms exposed to bentazon for 7 days. Only pigments in which differences were detected are shown (17 pigments analysed).

Bars represent the average of 4 or 5 microcosm analyses, error bars represent standard errors.

*: significant difference ($p < 0.05$; Mann-Whitney) compared with 0 $\mu\text{g/L}$.

Figure 7: Cell count distribution from flow cytometry analysis in microcosms exposed to bentazon for 7 days. Bars represent the mean of 4 or 5 microcosm analyses, error bars represent standard errors.

*: significant difference ($p < 0.05$; Mann-Whitney) compared with 0 $\mu\text{g/L}$.

Figure 8: Chlorophyll *a* in microcosms exposed to dimethenamid for 12 days. Vertically aligned dots represent replicates.

Figure 9: Cell count distribution from flow cytometry analysis in microcosms exposed to dimethenamid for 12 days. Bars represent the mean of 4 or 5 microcosm analyses, error bars represent standard errors.

*: significant difference ($p < 0.05$; Mann-Whitney) compared with 0 $\mu\text{g/L}$.

Figure 10: PCA of pigment fingerprint data sets (17 pigments) from microcosms exposed to dimethenamid, for 12 days. Each dot represents a treatment replicate.

0: no dimethenamid; **1**: 1 $\mu\text{g/L}$ dimethenamid; **10**: 10 $\mu\text{g/L}$ dimethenamid; **100**: 100 $\mu\text{g/L}$ dimethenamid.



Figure 1

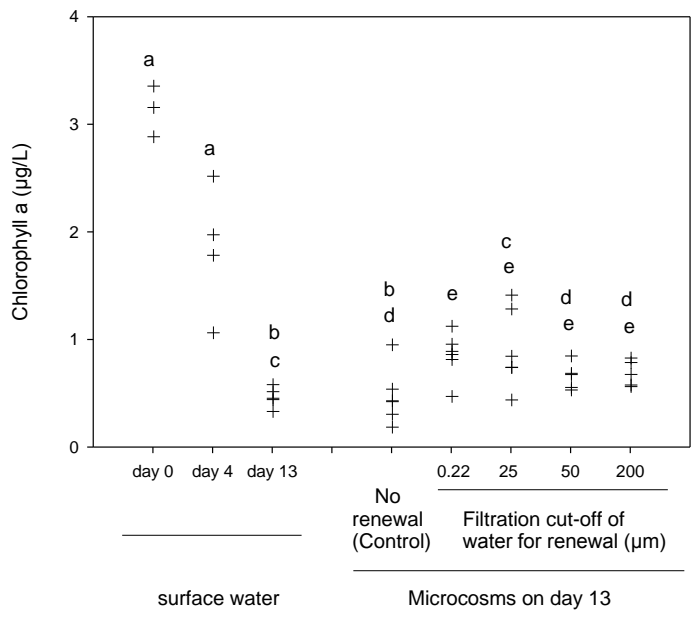


Figure 2

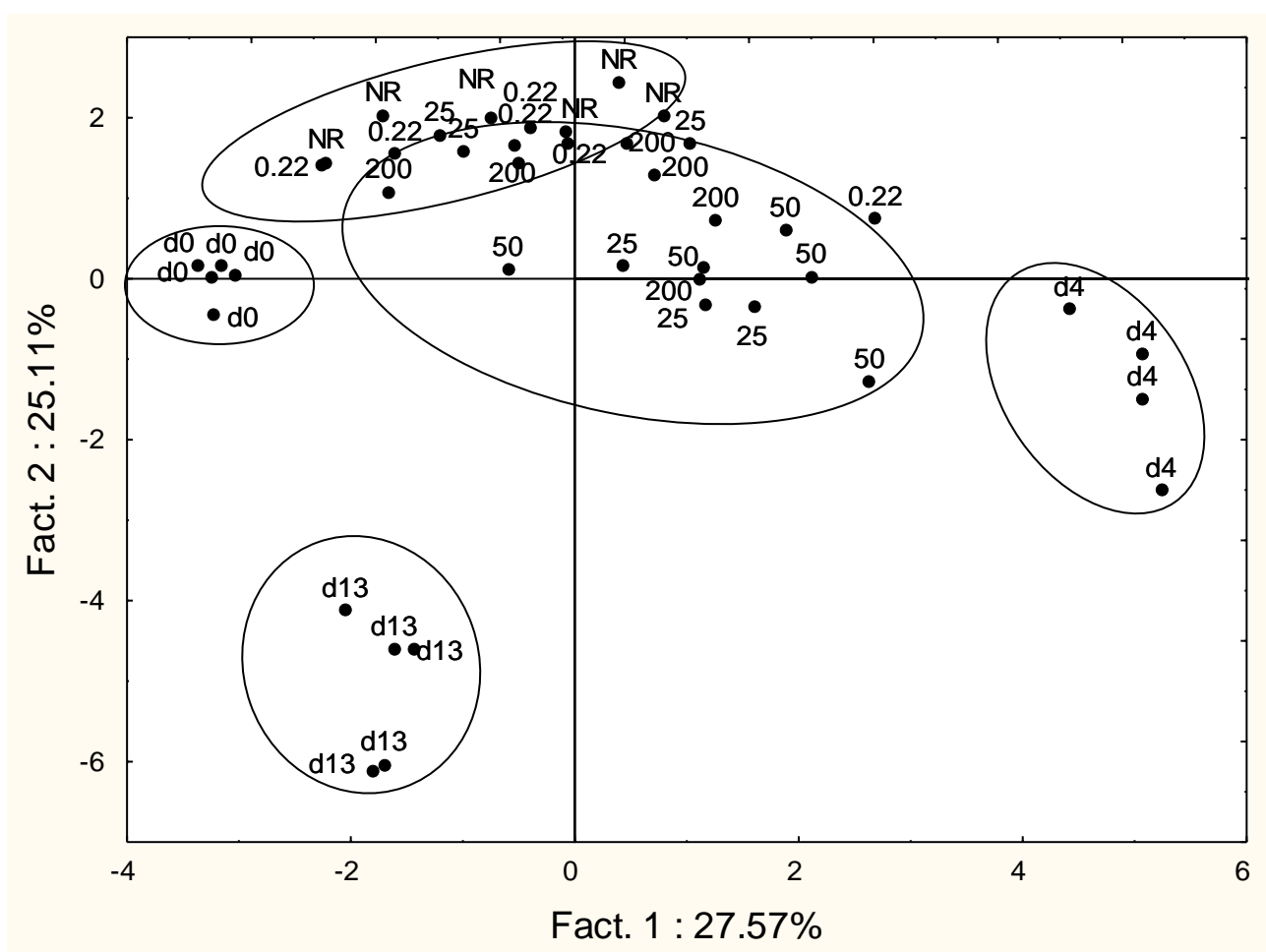


Figure 3

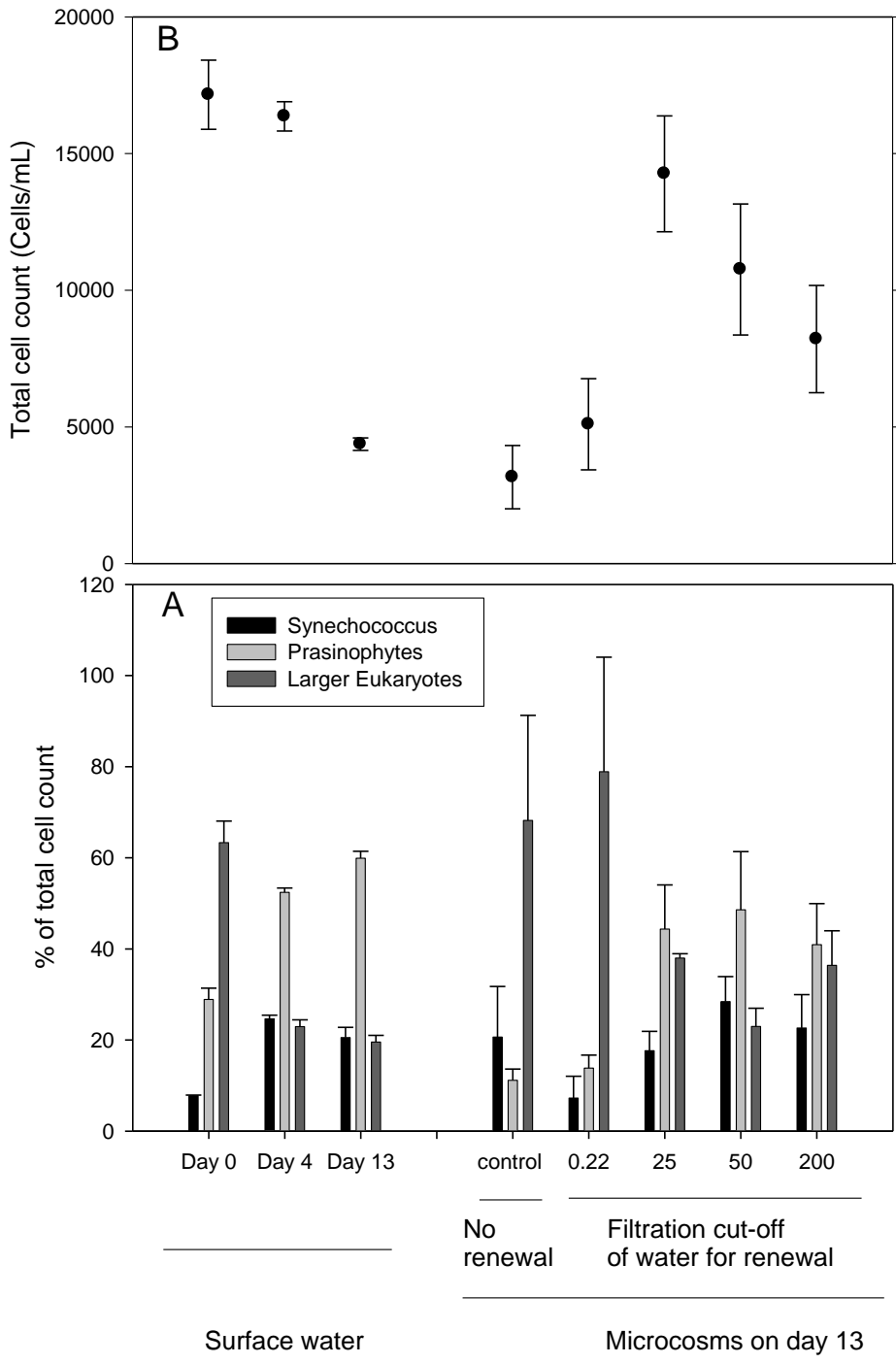


Figure 4

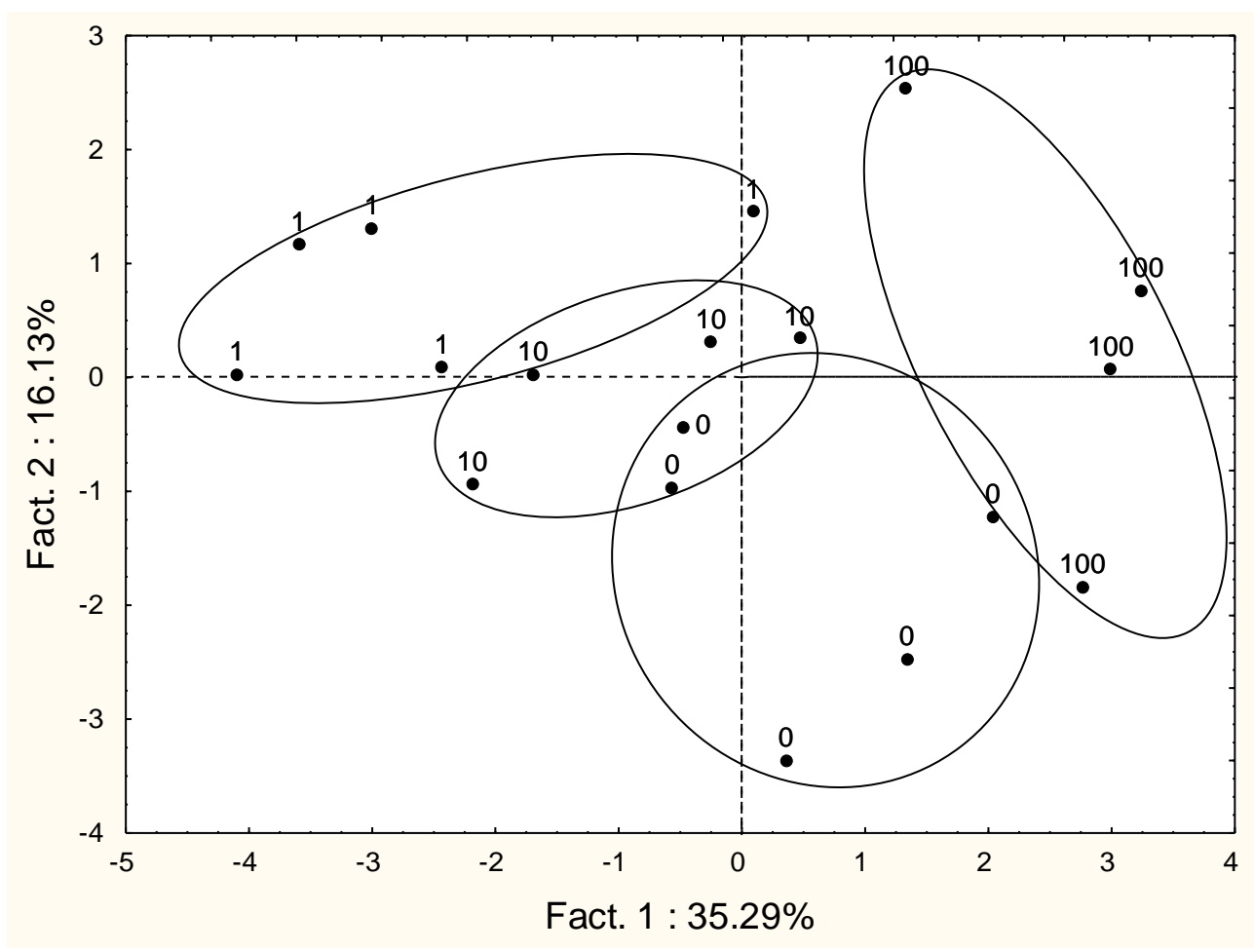


Figure 5

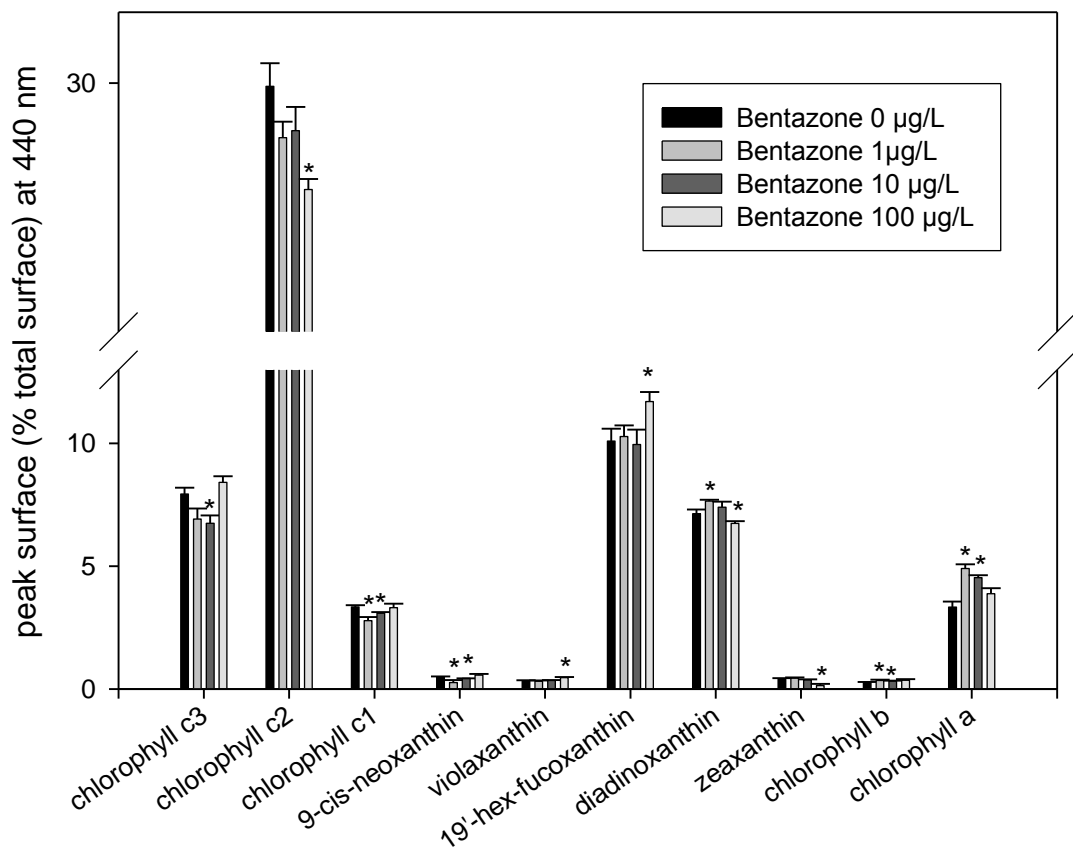


Figure 6

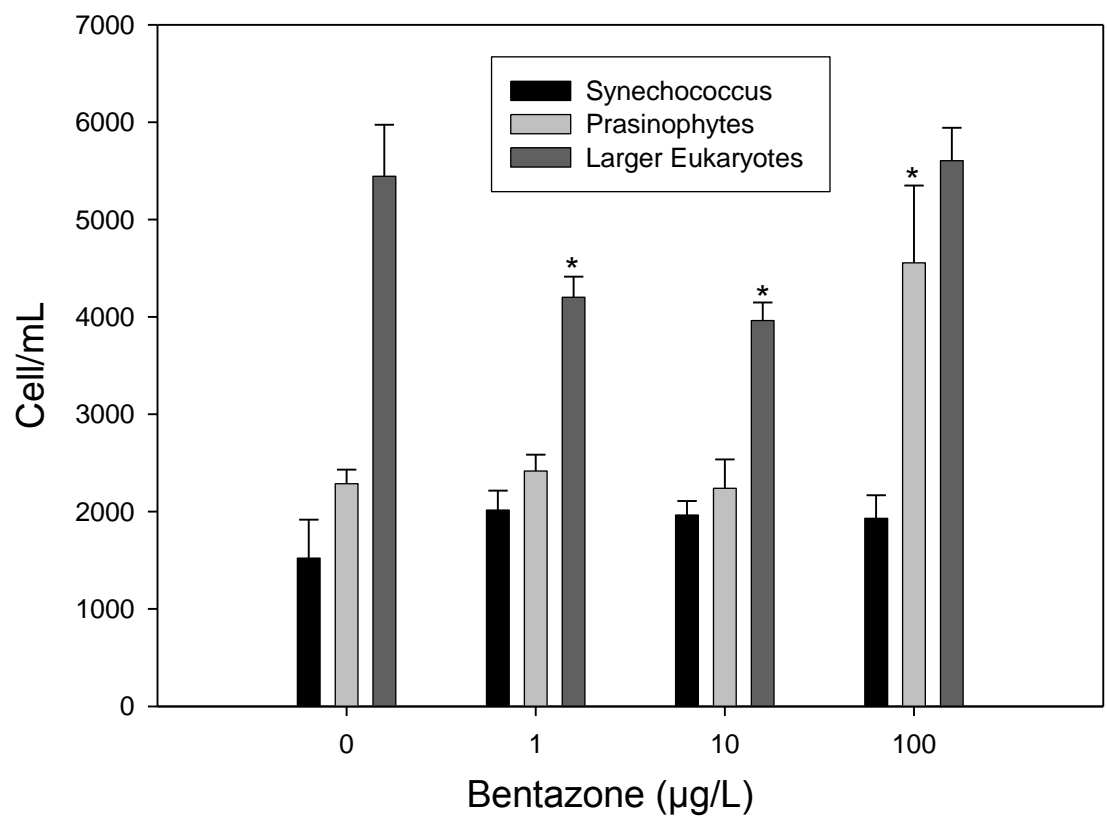


Figure 7

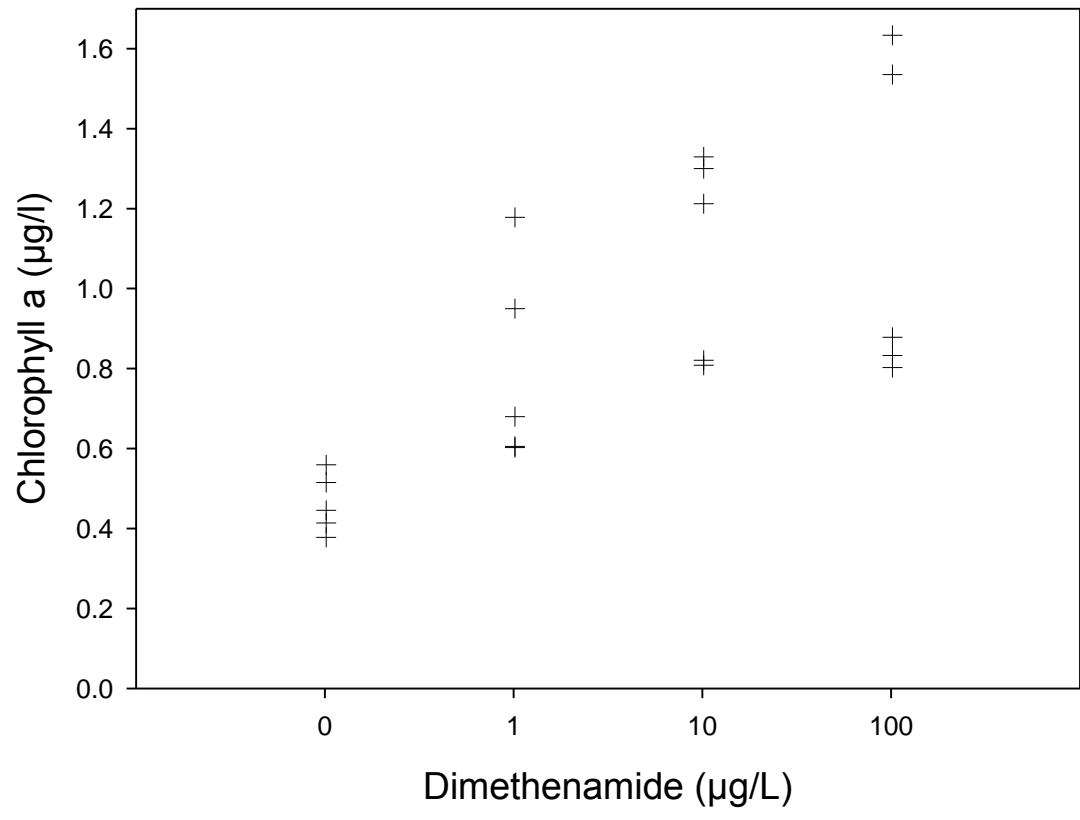


Figure 8

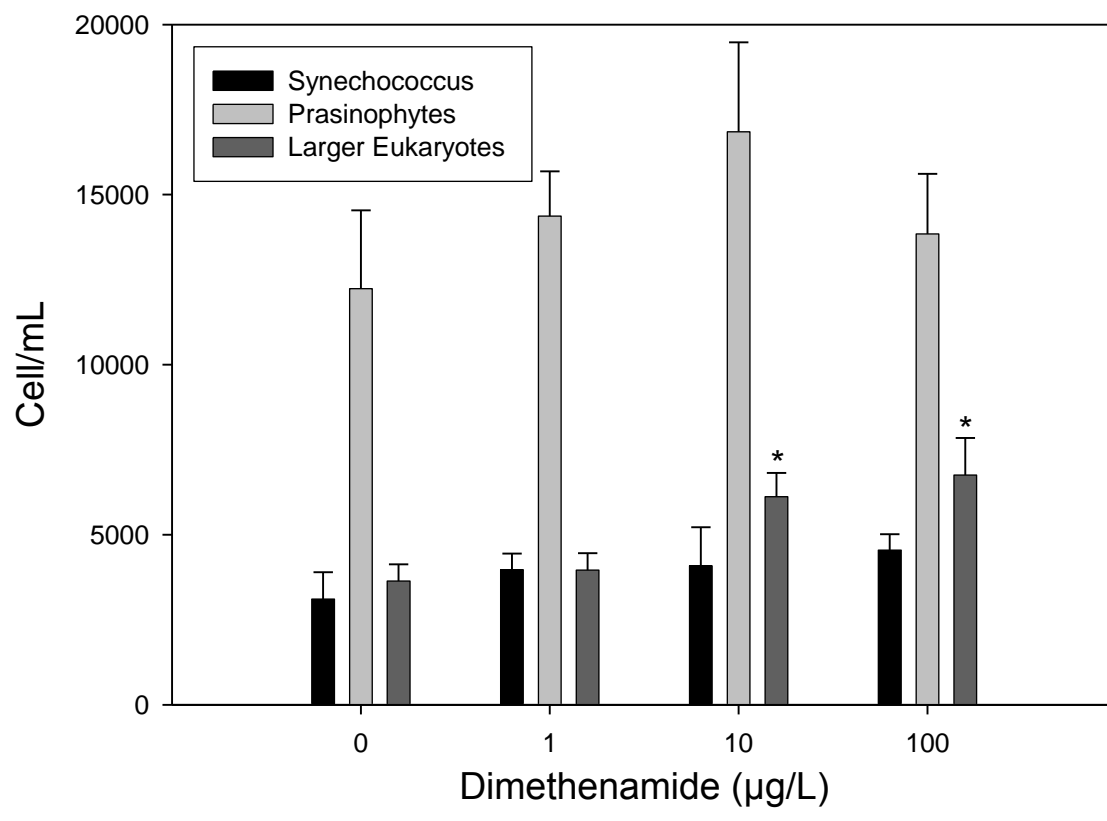


Figure 9

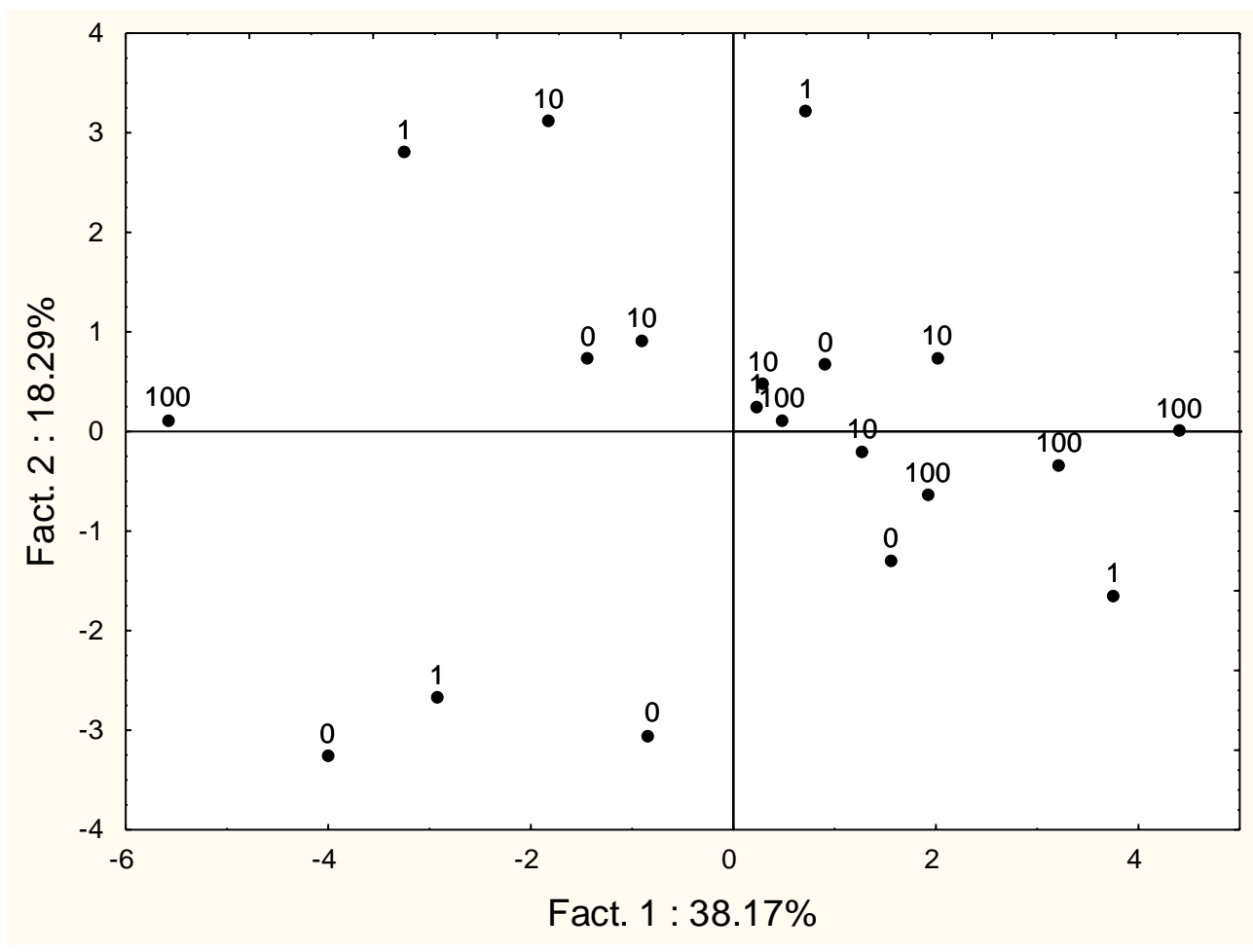


Figure 10