Impact of Roundup on the marine microbial community, as shown by an in situ microcosm experiment

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A B S T R A C T

The effects of the herbicide Roundup® (glyphosate) on natural marine microbial communities were assessed in a 7-day field experiment using microcosms. Bottles were maintained underwater at 6 m depth, and 10% of their water content was changed every other day.

The comparison of control microcosms and surrounding surface water showed that the microcosm system tested here can be considered as representative of the natural surrounding environment. A temporal temperature gradient gel electrophoresis (TTGE) was run on 16S and 18S rDNA-amplified extracts from the whole microbial community. Cluster analysis of the 16S gel showed differences between control and treatment fingerprints for Roundup at 1/9.262 gL⁻¹ (ANOSIM, p = 0.055; R = 0.53), and 10/9.262 gL⁻¹ (ANOSIM, p = 0.086; R = 0.40). Flow cytometry analysis revealed a significant increase in the prasinophyte-like population when Roundup concentration was increased to 10/9.262 gL⁻¹.

This study demonstrates that a disturbance was caused to the marine microbial community exposed to 1/9.262 gL⁻¹ Roundup concentration, a value typical of those reported in coastal waters during a run-off event.

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1. Introduction

The massive use of pesticides induces acute or chronic contamination of aquatic biota via spray drift, leaching or runoff from urban and agricultural areas. Herbicides in estuarine areas have been reported at concentrations ranging from several 10s to 100s of ng L⁻¹ (Lehotay et al., 1998; Chesworth et al., 2004; Steen et al., 2001; Oros et al., 2003). These contaminants can negatively impact non-target organisms, including microbial eukaryotes and prokaryotes. In microbial aquatic communities, phytoplankton are potentially vulnerable to herbicides, due to their physiological homologies with terrestrial plants (DeLorenzo et al., 2001; Dorigo et al., 2004). Furthermore, impacts of pesticides on this microbial community could seriously damage 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., 1999a).

Numerous studies have been published about pesticide toxicity assessment on phytoplankton, using single-species tests (Sabater and Carrasco, 2001; Rioboo et al., 2002; Sabater et al., 2002; Weiner et al., 2004; Gatidou and Thomaidis, 2007; Santin-Montanya et al., 2007). But Béard et al. (1999) demonstrated that single-species tests may fail to predict indirect or system responses to toxicants, such as changes in population competition or succession. According to these authors, studies focusing on the whole natural community provide more reliable predictions about herbicide safety in aquatic environments.

Toxicity effects on natural communities can be assessed in several ways: by sampling at different natural sites and/or times to look for pollution-related characteristics (Kostanjsek 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) and, finally, by running studies using in situ microcosms. Recently, de la Broise and Palenik (2007) 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. Such in situ micro- or mesocosm experiments provide the best aspects of both laboratory
and open field systems, as this experimental setup allows both the testing of multiple replicates and ecological realism (van den Brink et al., 2002). Microcosms setup in natural environments allows a wider species spectrum to be tested, including populations that are hard to maintain, and can thus provide a wider survey of prokaryote and eukaryote susceptibility to toxicants (Yasuno et al., 1993). Seguin et al. (2001) and Leboulanger et al. (2001) pointed out the need for reliable toxicity data using such microcosm systems.

Genetic fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) or temperature gradient gel electrophoresis (TGGE) (Yoshino et al., 1991) can provide an overview of whole community composition, including unculturable strains, which can account for 90% of microorganisms (Ward et al., 1990). Both DGGE and TGGE methods have been useful as tools to describe the diversity of the whole prokaryote or eukaryote microbial communities in aquatic environments (Murray et al., 1996; van Hannen et al., 1998; Bernard et al., 2000; Diez et al., 2001; Schäfer et al., 2001; Marie et al., 2006).

More recently, these methods were successfully applied to detect disturbances in microbial communities exposed to pollution (Petersen et al., 2004; Kostanjsek et al., 2005; Pesce et al., 2006, 2008).

Besides genetic fingerprinting techniques, flow cytometry can also provide information about natural microbial community populations, and their abundances (Hofstraat et al., 1994; Jonker et al., 1995; Marie et al., 1999; Rutten et al., 2005). Toxicity assessment using flow cytometry was reported in studies involving either phytoplankton cultures (Cid et al., 1995, 1996; Lage et al., 2001; Stauber and Adams, 2005; Yu et al., 2007) or natural photosynthetic communities (de la Broise and Palenik, 2007).

Glyphosate is the most commonly used herbicide in the world, for both agricultural and non-agricultural weed control (Woodburn, 2000). The use of this non-selective and broad-spectrum herbicide increased dramatically after the introduction of genetically modified glyphosate-resistant crops in 1997 (Giesy et al., 2000). The main formulation of glyphosate is Roundup, where glyphosate is present as an isopropylamine (IPA) salt and its efficiency is enhanced by addition of the surfactant polyoxyethylene amine (POEA) (Tsui and Chu, 2003).

As receivers of terrestrial pollution, coastal areas can suffer glyphosate contamination over episodes of several days: for instance, glyphosate was detected over a 11-day period at a peak value of 12.2 µg L⁻¹ in the Marennes–Oléron bay (Atlantic coast, France) in late spring 2004 (Burgeot et al., 2007). However, little is known about its impact on natural aquatic microbial communities. To our knowledge only one study has revealed significant disturbance in the sediment bacterial community from a lake, exposed to environmentally relevant glyphosate concentrations (Widenfalk et al., 2008).

To examine the effects of Roundup on marine microbial communities, the objectives of this study were

1. To compare microbial communities in microcosms and in surrounding water in order to evaluate the relevance of in situ microcosms.
2. To expose microbial communities to Roundup at environmentally relevant concentrations, in microcosms in situ.
3. To assess the pesticide impacts using flow cytometry and TTGE for analysis of prokaryote and eukaryote natural communities.

2. Materials and methods

In order to assess the effects of Roundup on eukaryote and prokaryote marine microbial communities, a set of microcosms containing natural surrounding filtered seawater was placed in situ and exposed to Roundup. The genetic fingerprints of microcosm communities were obtained from the whole community DNA amplified extracts, using TTGE analysis. Chlorophyll a (chl a) measurements were made from biomass collected on filters, and microcosm subsamples were also analysed using flow cytometry, and species determination by microscopy.

2.1. Pesticide

The pesticide tested in this study is the commercial formulation of the herbicide glyphosate (Roundup®, Monsanto, Saint-Louis, MO, USA). Concentration values represent those of the pure active substance. The pesticide stock solution (10 mg L⁻¹ glyphosate) was prepared in 0.22-µm filtered seawater, previously autoclaved for 20 min at 121 °C. After agitation for 48 h using a magnetic stirrer, the solution was kept at −24 °C.

Freshly thawed stock solution was added directly into the microcosm bottles just before immersion. Pesticide concentrations were analyzed on the first day of the experiment, on one additional microcosm for each treatment, and on the last day of experiment on pooled water samples from all the replicate bottles of each treatment. Samples were frozen until analysis.

Pesticide analyses were performed by Idhesa Laboratory (Brest, France). The determination of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) residues was performed using liquid chromatography with fluorescence detection after liquid–liquid extraction and derivatization with 9-fluorenylmethylchloroformate (FMOC–Cl). Detection limit was 0.05 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 July 27 to August 3. This bay is submitted to tide amplitude of 3–5 m, and maximum tidal currents are up to 0.5 m s⁻¹ at the experimental site. Light at 6 m depth was measured twice during the experiment (spherical quantum sensor LI-193 SA from LICOR corporation), and showed about 10-fold intensity reduction as compared to surface light, indicating low turbidity waters. Microcosms consisted of hermatically 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 phytoplankton communities, and enclosed with about 300 mL of air (de la Broise and Palenik, 2007).

Fifteen microcosms were prepared: five replicates each of the three treatments: control and two different concentrations of Roundup (1 and 10 µg L⁻¹).

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, regardless of the tide (Fig. 1). The replicate microcosm bottles of each treatment were distributed alternately all around the frame.

On days 2, 4 and 6, the frame was hauled out of the water and 200 mL of the 2 L seawater content of each bottle was collected in a tank for proper disposal, and replaced with the same volume of fresh 200-µm filtered surrounding seawater and the frame was reimmersed. No analysis was carried out on these 200 mL samples. Bottles were finally collected on day 7 for analyses.

On the first and last days of experiment, five samples of surrounding surface seawater (2 L) were also collected for analysis.

One litre of water from each microcosm and one from the surrounding seawater sample were filtered through 0.22-µm poly-sulfone filters. These filters were stored at −80 °C until DNA
ent geographic locations, as prasinophytes (D. Marie, unpublished identified by flow cytometric sorting in marine samples from differ-
eukaryotes was considered as part of the overall picoeukaryotic dis-
olution (SIGMA, chlorophyll \(a\) concentration, measured at 440 nm, was calibrated against a chl
four single solvents were used in the present study. Chl
whereas Zapata used two eluents prepared from mixed solvents,
cence (580 nm) scatter signals and their natural red (>630 nm) and orange fluores-
2.4. Flow cytometry

Half of each frozen 0.22-\(\mu\)m polysulfone filter was extracted in the dark, in 1 mL of 95% methanol as follows: the 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-\(\mu\)m PVDF syringe filter to remove debris. Aliquots (200 \(\mu\)L) 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 mm \(\times\) 4.6 mm, 3.5 \(\mu\)m particle size, 100 \(\AA\) pore size). The mobile phase was a gradient mixture of four solvents: methanol (100%), acetonitrile (100%), acetic acid (100%) and aqueous pyridine solution (0.25 M), as described in Zapata et al. (2000). The elu-
ent gradient program was adapted from Zapata et al. (2000), but whereas Zapata used two eluents prepared from mixed solvents, four single solvents were used in the present study. Chl \(a\) concent-
ration, measured at 440 nm, was calibrated against a chl \(a\) standard solution (SIGMA, chlorophyll \(a\) from spinach for HPLC Ref. 10865).

2.4. Flow cytometry

Samples were run using an FACSort flow cytometer equipped with a 488-nm argon laser and standard filter setup (Becton Dickin-
on, San Jose, CA). Two populations of photosynthetic organisms were discriminated in microcsm samples, on the basis of their scatter signals and their natural red (>630 nm) and orange fluores-
cence (580 ± 20 nm): Synechococcus and pico-eukaryotes (<10 \(\mu\)m). Usually, picoplankton is considered to range from 0.2 \(\mu\)m to 2 \(\mu\)m, and nano/plankton from 2 \(\mu\)m to 20 \(\mu\)m. But here, using flow cytometry, the term “pico-eukaryotes” is used for cells <10 \(\mu\)m.

For more precise analysis, a clear sub-population of small eukaryotes was considered as part of the overall picoeukaryotic dis-
bution (Fig. 2). Cells belonging to this sub-population have been identified by flow cytometric sorting in marine samples from different geographic locations, as prasinophytes (D. Marie, unpublished data) and for this reason, we call them prasinophyte-like in the fol-
lowing text. Data were analysed using the WinMDI v2.9 software (J. trotter, http://facs.Scripps.edu/).

2.5. Species determination

Determination and quantification of micro- and nano-
phytoplankton cells were carried out at species level as follows: sub-samples of 50 mL were settled into Utermöhl settling cham-
bers (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).

The Shannon–Wiener diversity index \(H\) was calculated using the formula:

\[
H = \sum p_i \log_2(p_i)
\]

where \(p_i\) is the proportion of the total count arising from the \(i\)th species.

2.6. DNA extraction and purification

Half of each polysulfone filter (0.22 \(\mu\)m pore size) was cut into small pieces and put in 5-mL polypropylene tubes. Sterile glass beads (0.5 g, 0.1 mm diameter, SIGMA, Ref. G8993) and 500 \(\mu\)L of TNE buffer (10 mM Tris–Cit, 100 mM NaCl, 10 mM EDTA, pH 8) were added. Tubes were vortexed for 1 min in order to disrupt the cells.

2.6.1. Nucleic acid extraction

Lysozyme was added (final concentration, 2 mg mL\(^{-1}\)) and tubes were then kept at 37°C for 45 min. Proteinase K (final concentration, 0.5 mg mL\(^{-1}\)) and sodium-dodecyl-sulfate (SDS, final concentra-
tion, 1%) were then added, and tubes incubated at 37°C for 30 min. The lysates were purified twice by extraction with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), and the residual phenol was removed by two extractions with an equal volume of chloroform–isoamyl alcohol (24:1). Two volumes of isopropanol and sodium acetate (10% of the total volume, 3 M, pH 5.2) were added to the aqueous phase. After precipitation (1 h at room temperature), the tubes were centrifuged for 15 min at 13,000 rpm. The supernatant was discarded and 1.5 mL of 70% ethanol were added. Tubes were centrifuged for 10 min at 13,000 rpm, and the super-
natant discarded. The tubes were then left for a few minutes at room temperature to ensure ethanol evaporation, and nucleic acids were resuspended into 300 \(\mu\)L of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8).

2.6.2. RNA digestion

Pre-incubated RNase (10 min at 80°C) was added (final concentration, 20 \(\mu\)g mL\(^{-1}\)) and tubes were incubated for 30 min at 37°C.

2.6.3. DNA purification

This procedure was carried out as for the nucleic acid extraction described above, except that proteinase K and SDS final concentrations were 50 \(\mu\)g mL\(^{-1}\) and 0.1%, respectively. DNA pellets were suspended in 0.1× TE buffer.

2.7. PCR

Two PCRs were run for each DNA extract.
For the first PCR, eukaryotic 18S ribosomal DNA (rDNA) universal primers were used. The primers were Euk1A and Euk516r-GC (Diez et al., 2001), which amplify a fragment of approximately 560 bp. The PCR program (Diez et al., 2001) included an initial denaturation step 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.

For the second PCR set, prokaryotic 16S rDNA universal primers were used. The primers were 341F-GC (Muyzer et al., 1993) and 907RC (Schäfer et al., 2001). The PCR program (Massana and Jürgens, 2003) included an initial denaturation step at 94 °C for 5 min, followed by 10 touchdown cycles including denaturation at 94 °C for 1 min, annealing for 1 min (starting at 65 °C and decreasing by 1 °C per cycle), and extension at 72 °C for 3 min. The next 20 cycles were composed of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min. A final extension step was performed for 5 min at 72 °C.

For both PCRs, approximately 15 ng of extracted DNA (estimated using spectrophotometry) were used as a template: the reaction mixture (50 µL) contained sterile nuclelease-free water, each primer at a final concentration of 0.3 µM, bovine serum albumin (final concentration 0.2 µg µL−1) and Promega PCR Master Mix (in which the low T product, and 63–68 °C for 16S PCR products. 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, Natic, MA).

For practical reasons, only four replicates for control and three replicates for each pesticide treatment were processed for TTGE analysis.

2.9. Statistical analysis

The chl a 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 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 pair wise 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 using the one-way ANOSIM function in the Past v1.77 software (Hammer et al., 2001).

3. Results

3.1. Comparison between control and surrounding water

The chl a concentration in the surrounding water (Fig. 3) ranged from 0.93 ± 0.07 µg L−1 (mean ± S.E.) on day 0 to 1.71 ± 0.097 µg L−1 on day 7, showing almost a twofold increase. In controls on day 7, the chl a concentration had increased up to 1.31 ± 0.16 µg L−1, but this was not significantly lower than the level in surrounding water on the same day (p = 0.11).
Table 1
Number of species and Shannon–Wiener index calculated from microscopic analyses of samples from the surrounding water (SW) and the microcosm experiment

<table>
<thead>
<tr>
<th></th>
<th>SW Day 0</th>
<th>SW Day 7</th>
<th>Control</th>
<th>Roundup 1 (µg L⁻¹)</th>
<th>Roundup 10 (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of species</td>
<td>31</td>
<td>30</td>
<td>32</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>H’</td>
<td>2.12</td>
<td>2.29</td>
<td>1.99</td>
<td>1.59</td>
<td>1.87</td>
</tr>
</tbody>
</table>

Five pooled samples were used for each analysis.

Similarly, the total cellular concentration obtained using flow cytometry analysis (Fig. 4) ranged from 5667 ± 306 cell mL⁻¹ on day 0 to 11 268 ± 131 cell mL⁻¹ on day 7, showing a twofold increase. The value obtained for control microcosms on day 7 (9 943 ± 480 cell mL⁻¹) was not significantly different from that of surrounding seawater on the same day.

The Synechococcus cellular concentration in the surrounding water (Fig. 4) did not increase significantly between day 0 and day 7 (3152 ± 180 and 3467 ± 138 cell mL⁻¹, respectively). This population was significantly lower in the control microcosms on day 7 (1454 ± 131 cell mL⁻¹, Mann–Whitney, p < 0.05) compared to the surrounding water.

The total pico-eukaryote cellular concentration in the surrounding water (Fig. 4) increased from 2515 ± 164 cell mL⁻¹ on day 0, to 7801 ± 200 cell mL⁻¹ on day 7 and values were not significantly different between control microcosms (8489 ± 562 cell mL⁻¹) and surrounding water on day 7 (p = 0.27).

On the first day of experiment, 31 species were identified in the algal community of the surrounding water using microscope. On the last day of experiment, 30 were found. A similar value (32 species) was observed in the control on the last day. The Shannon–Wiener diversity indexes (H’) were also close in surrounding water on the first and last days, and in control microcosms on the last day (Table 1).

Nine main groups composed the surrounding water and control communities on the first and last days of experiment (Fig. 5). Relative abundance of diatoms increased from 31% in the surrounding water on the first day to 84% on the last day, while it accounted for 63% in the control. The diatom group was mostly represented by the genus Chaetoceros (25% of the total abundance on the first day, 53% on the last day and 50% in the control), and the species Leptocylindrus danicus (2.5% of the total abundance on the first day, 21% on the last day and 10% in the control). Dinoflagellate relative abundances were not higher than 12% in the surrounding water on the first day (11% Gymnodinium spp.), but they dropped to 0.8% by the last day, and accounted for 1.2% in the control. The most abundant group, undetermined flagellates, represented 57% of the total abundance in surrounding water on the first day, 15% on the last day and 36% in the control.

From these data, it appears that the abundances of these four main groups observed in the control match the range of abundances observed in the surrounding water between the first and the last day of the experiment.
### Table 2
Glyphosate (µg L⁻¹) detected in microcosms and surrounding water on the first and last days of experiment

<table>
<thead>
<tr>
<th>Day</th>
<th>Surrounding seawater</th>
<th>Control</th>
<th>Roundup 1 (µg L⁻¹)</th>
<th>Roundup 10 (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>0.11</td>
<td>3.56</td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>0.13</td>
<td>3.08</td>
</tr>
</tbody>
</table>

Detection limit: 0.05 µg L⁻¹.

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3.2. Microbial community exposure to Roundup

3.2.1. Pesticide exposure

The control microcosms and the surrounding seawater did not contain detectable glyphosate during the experiment (Table 2). The concentrations detected for Roundup-treated microcosms on the first day of experiment ranged from 11% of the nominal amount added for the 1 µg L⁻¹ treatment, to 36% for the 10 µg L⁻¹ treatment. At the end of the experiment, levels ranged from 13% to 31% for the 1 and 10 µg L⁻¹ treatments, respectively. The metabolite AMPA was not detected in any of the samples.

3.2.2. Chlorophyll a in microcosms

The chl a concentration (1.31 ± 0.16 µg L⁻¹ in control microcosms, Fig. 6) was not affected by the Roundup exposure, either at 1 or 10 µg L⁻¹ (Mann–Whitney, p = 0.99 and 0.71, respectively).

3.2.3. Roundup effects on prokaryote communities

Image analysis of the TTGE gel from the 16S PCR products (Fig. 7a) allowed the detection of 12–16 major bands for the control microcosms, 14–17 for microcosms treated with 1 µg L⁻¹ of Roundup, and 10–16 for the 10 µg L⁻¹ exposure. A total of 23 different bands was detected on the whole gel.

The dendrogram generated from this banding pattern (Fig. 7b) exhibited a clear separation of the control microcosms on one hand, and the herbicide-treated microcosms on the other. Only one sample fingerprint (a 10 µg L⁻¹ Roundup replicate) was situated outside of these two clusters. The ANOSIM from the cluster analysis between the control group and the Roundup treatments showed p-values of 0.055 and 0.086 for the Roundup at 1 and 10 µg L⁻¹, respectively. The R-values obtained were 0.53 for Roundup 1 µg L⁻¹ and 0.40 for Roundup 10 µg L⁻¹. There was no evidence of a difference in the effect of 1 and 10 µg L⁻¹ treatments on these communities, as fingerprints from both treatments were grouped together.

When the control group was tested against both treatments together, a p-value of 0.021 (R = 0.44) was observed.

The prokaryotic Synechococcus counts in microcosms, obtained from flow cytometry analysis (Fig. 8) did not show significant differences between the controls and either Roundup treatment.

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Fig. 6. Chl a concentrations (µg L⁻¹) in microcosms on the last day of experiment.

Fig. 7. (a) TTGE profiles from 16S PCR products. C = control; R1 = Roundup 1 µg L⁻¹; R10 = Roundup 10 µg L⁻¹; a–d = replicates. (b) UPGMA cluster analysis of Dice similarity matrix calculated from 16S-TTGE banding patterns. C = control; R1 = Roundup 1 µg L⁻¹; R10 = Roundup 10 µg L⁻¹; a–d = replicates.

Fig. 8. Cellular concentrations of the photosynthetic populations in microcosms on the last day of experiment, discriminated using flow cytometry.
Roundup does not show any significant effect on *Synechococcus* relative fluorescence values (data not shown).

### 3.2.4. Roundup effects on eukaryote communities

Image analysis of the TTGE gel from the 18S PCR products (Fig. 9a) allowed the detection of 9–14 major bands for the control microcosms, 7–12 for microcosms treated with 1 μg L⁻¹ of Roundup and 13–17 for the 10 μg L⁻¹ exposure. A total of 22 different bands were detected on the whole gel.

The cluster analysis (Fig. 9b) revealed no clear separation of the patterns, even for the highest pesticide concentration, and the ANOSIM results did not indicate significant differences ($p > 0.10$, data not shown).

Flow cytometry analysis revealed no significant difference in total pico-eukaryote counts (Fig. 8) or fluorescence (data not shown) between the control microcosms (8489 ± 562 cell mL⁻¹, mean ± S.E.) and either 1 μg L⁻¹ (7638 ± 522 cell mL⁻¹) or 10 μg L⁻¹ (8675 ± 635 cell mL⁻¹) Roundup treatment.

However, though the prasinophyte-like cell count was not significantly enhanced (Mann–Whitney, $p = 0.06$) for the 1 μg L⁻¹ treatment, it was significantly higher at 10 μg L⁻¹ Roundup, with a cellular concentration increase of 43% (Fig. 10).

The microscopic observation of samples allowed the identification of 32 species for the control, but only 24 and 26 for the Roundup treatments at 1 and 10 μg L⁻¹, respectively. The associated Shannon–Wiener diversity indexes also dropped in treatments compared with the control (Table 1). Although a statistical treatment cannot be applied to such data, the quantification of the main groups (Fig. 11) suggests, in treated microcosms during the experiment, a higher decrease of *L. danicus* and a lower increase of *Chaetoceros* sp. cells than in control microcosms and in surrounding waters (Fig. 5). Undetermined flagellates were maintained at values much higher in treated microcosms than in control or in surrounding waters.
4. Discussion

4.1. Are microcosm contents representative of the mixed layer?

For practical reasons, seawater for renewal was collected in the 50-cm surface layer. The mixed layer of ocean surface waters is estimated to fluctuate from several meters depth in very calm conditions, to 100 meters or more in strong wind and wave conditions (Bendtsen et al., 2006; Nilsen and Falck, 2006). Therefore, at the beginning of experiment, the water content of such microcosms can be considered as representative of coastal surface waters. The in situ microcosms used in the present study were exposed to natural underwater temperature and light conditions. Following previous experiments, it was decided to renew 10% of the bottle content every other day in order to mimic the change of the surrounding water, and an air overlay was also included to simulate gas exchanges occurring in free seawater (de la Broise and Palenik, 2007).

The chl-a measurements in controls on day 7 exhibited values that were not significantly different from the surrounding water on the same day. Total abundances and pico-eukaryotes were greatly increased during the experiment, both in surrounding water and in controls. Only 27% of the microcosm water content was changed overall during this period. However, control and surrounding water values were quite close on day 7. Only Synechococcus counts were clearly lower in controls on day 7 compared with surrounding seawater. However, the abundance of this cyanobacterium is known to fluctuate rapidly over a wide range in coastal waters. Li et al. (2006) reported daily variations of Synechococcus biomass in the Yellow Sea, ranging from 2 to 8 mgC m⁻². Uysal and Koksalan (2006) reported a fourfold increase of Synechococcus counts over a 6-day period in a survey on eastern Mediterranean Sea coastal waters. The authors found that the observed fluctuations were characteristic of shallow coastal waters, with rapidly changing environmental conditions. Therefore, based on its reported natural variations, the present result showing a Synechococcus concentration reduced by half in controls compared with surrounding water does not seem surprising.

The number of species and diversity index showed similar values for control and surrounding water at the end of experiment. Furthermore, the three major groups identified were also the same. These data suggest that the environmental conditions that drive short-term fluctuations were realistic enough in microcosms for the maintenance of a phytoplankton community representative of natural water in the area.

4.2. Pesticide exposure

As medium renewal was 10% every other day, the pesticide loss in microcosms after 7 days should be approximately 27% of the nominal amount added. Data from glyphosate analyses on the first and last days of experiment showed concentrations much lower than those expected. This could be due to an underestimation of the glyphosate present in the samples, or to its possible adsorption to the bottle walls and/or on the organic matter or multivalent cations present in seawater. Indeed, Freuze et al. (2007) demonstrated that the FMOC method applied in this study could underestimate both glyphosate and AMPA in the presence of multivalent cations. They also showed that only free forms of AMPA and glyphosate were able to react with the FMOC reagent. Overall, the authors indicate that the efficiency of the method depends on the water sample type and content (cations, organic matter etc.).

No detectable concentrations of AMPA were observed in treatments on day 7. This could be explained by AMPA concentrations being below the detection limit, by the same adsorption mechanism described for glyphosate or as a result of slow glyphosate degradation kinetics, under such conditions.

To our knowledge, very few reports on glyphosate concentrations in coastal waters are available. This lack of data may be linked to the high polarity of the molecule, close in structure to many naturally occurring amino acids, that makes its detection difficult in water samples (Skark et al., 1998; Rubio et al., 2003). This difficulty is probably made more pronounced by the low concentrations occurring in coastal waters. A peak value of 1.2 μg L⁻¹ over a 11-day period was detected in the Marennes–Oléron bay (salinity 33‰) (Atlantic coast, France) in late spring 2004 (Burgeot et al., 2007). Analyses performed by Idhesa Laboratory (Brest, France) reported maximal concentrations in the Bay of Brest (2002–2004), of 0.41 μg L⁻¹ for glyphosate and 0.64 μg L⁻¹ for AMPA, in salinity 27‰ or higher (Gaël Durand, personal communication). While the highest concentration tested in the present experiment (10 μg L⁻¹ added, 3.6 μg L⁻¹ detected on day 0) can be found in rivers or may represent an outstanding pollution peak in estuarine areas, the lowest concentration tested (1 μg L⁻¹ added, 0.11 μg L⁻¹ detected on day 0) is representative of coastal pollution.

4.3. Roundup impact on prokaryote communities

Comparison of TTGE fingerprints from control and Roundup-treated communities revealed significant differences (p-values in the range 0.05–0.1). The K-values were 0.53 and 0.40, for Roundup 1 and 10 μg L⁻¹, respectively, indicating that the two treated groups separate from the control group in the high dimensional space defined by the similarity matrix (Clarke, 1993). The p-value was reduced to 0.021 when both treatments were tested together against the control. This increased discrimination when treatments were grouped is probably due to the fact that such a non-parametric analysis is not very powerful, especially with small samples. These results clearly show a change in the prokaryote community composition, even with 1 μg L⁻¹ (0.11 μg L⁻¹ detected on day 0). Other studies have been reported that used TTGE from 16S PCR products to monitor pesticide toxicity on natural riverine microbial communities, using either pooled DNA extracts from replicates before PCR (Pesce et al., 2006), or no replication (Pesce et al., 2008). In our study, three or four replicates per treatment were extracted, amplified and separated. This allowed the statistical tool ANOSIM to be applied to test for significant differences between groups of fingerprints. As far as we know this is the first time that the ANOSIM has been applied to an ecotoxicological study using TTGE gel cluster analysis, and its relevance was clearly illustrated.

In spite of this significant difference observed in Roundup treatments compared to controls, no difference was observed in the number of bands detected: the prokaryote diversity might have been disturbed qualitatively, rather than quantitatively. The changes in the TTGE patterns cannot be precisely interpreted, since no sequencing of discriminating bands or other measurement on the bacterial compartment were done. Flow cytometry data show that Synechococcus was apparently not affected by Roundup. As Synechococcus is the major autotrophic prokaryote in mesotrophic and coastal areas (Olson et al., 1988; Partensky et al., 1996), we can then hypothesize that the impacted organisms were either heterotrophic prokaryotes or other minor autotrophs that could not be discriminated using flow cytometry.

Various hypotheses could explain this result: the Roundup could affect some prokaryote populations and thus allow the increase of their competitors, or some populations could even be stimulated (el Fantroussi et al., 1999). The changes could also indicate modified grazing pressure due to the possible pesticide impact on heterotrophic eukaryotes. TTGE changes could even indicate...
modified nutrient sources for prokaryotes, due to Roundup disturbance on other organisms of the microbial loop. Working on bacterial community composition of Lake Erken (Sweden) sediments, Widenfalk et al. (2008) recently reported significant changes in microbial communities of sediments exposed to 150 µg·L⁻¹ (dry weight) of glyphosate, using the terminal restriction fragment length polymorphism (T-RFLP) analysis. Unfortunately, such data are scarce in the literature, and should be complemented with further studies.

4.4. Roundup impact on eukaryote communities

The whole eukaryote community, including autotroph and heterotroph planktonic cells, as well as grazers (<200 µm), was targeted in the 18S-TTGE analytical procedure. The cluster analysis of the 18S community fingerprints did not indicate any impact of Roundup on eukaryotes. However, the trophic diversity of organisms targeted, together with the possible bias introduced by the heterogeneity of 18S rRNA gene and its amplification (preferential amplification of some sequences, gene copy number per cell, van Hannen et al., 1998), can complicate the detection of potential effects. Based on these aspects, we can hypothesize that a slight effect on a reduced number of eukaryotic components would probably not be detected using this method. Flow cytometry analyses revealed a significant increase in the cellular concentration of the prasinophyte-like population. This indicates that the growth of these organisms had been stimulated, which may have occurred directly, or indirectly by an effect on a potential competitor. This effect could also be explained by a possible impact on grazers, inducing a modified grazing pressure, or changes in nutrient sources. It should be noted that the p-value of the test for prasinophyte-like concentration at 1 µg·L⁻¹ of Roundup was 0.06. This suggests that the effect of Roundup on prasinophyte-like concentration starts as low as 1 µg·L⁻¹.

In this case flow cytometry analyses seem more sensitive than TTGE for detecting the impact of Roundup on the eukaryote community.

Flow cytometry analyses are run on approximately 250–300 µL of sample and the diameter of the flow cell is below 80 µm. So only small cells (<10 µm) at relatively high concentrations can be discriminated, which usually correspond to the smallest part of the nanophytoplankton (2–20 µm) and picophytoplankton (0.2–2 µm). The larger nanophytoplankton cells and the microphytoplankton cells (20–200 µm) are not detected using flow cytometry because of their size and/or their low concentration in natural samples. This is the reason why qualitative and quantitative analyses of species, using microscopy, are required: they provide additional information and give a wider view of the eukaryote communities.

For practical reasons, the microscopic determination of species was run only on one pooled sample for each condition, and therefore no statistical treatments could be applied. However, data suggest that less species might be present in the treated microcosms compared with the control, and that the distribution of some species could be modified.

5. Conclusion

In situ microcosms were demonstrated to be a realistic model for toxicity studies on coastal waters. Our results show that the herbicide Roundup can affect natural coastal microbial communities after a 7-day exposure at 1 µg·L⁻¹ (0.13 µg·L⁻¹ detected) for prokaryotes and at 10 µg·L⁻¹ (3.1 µg·L⁻¹ detected) for some pico-eukaryotes. This effect was detected for glyphosate concentrations typical of those already observed in polluted coastal areas. It leads us to conclude that glyphosate pollution during run-off events can modify microbial communities in some coastal areas. Effects could be attributed to impacts on competitors for nutrients, leading to community reorganisation and to colonization of empty ecological niches. But these effects could also be attributed to changes in nutrient sources or to modified grazing pressure by the heterotrophic eukaryotes. This study provides information about the high sensitivity of planktonic microorganisms to glyphosate exposure in environmentally relevant conditions. As AMPA is also found in coastal waters, its impact on microbial communities along with the impact of other pesticides should also be assessed.

Further experiments would be needed in order to identify the organisms impacted and their contribution to the global equilibrium of the microbial system. It will also be necessary to clarify whether such effects could occur during other periods of the year.

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