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A new cell primo-culture method for freshwater benthic diatom communities

T. Debenest · J. Silvestre · M. Coste · F. Delmas · E. Pinelli

Abstract A new cell primo-culture method was developed for the benthic diatom community isolated from biofilm sampled in rivers. The approach comprised three steps: (1) scraping biofilm from river pebbles, (2) diatom isolation from biofilm, and (3) diatom community culture. With a view to designing a method able to stimulate the growth of diatoms, to limit the development of other microorganisms, and to maintain in culture a community similar to the original natural one, different factors were tested in step 3: cell culture medium (Chu No 10 vs Freshwater “WC” medium modified), cell culture vessel, and time of culture. The results showed that using Chu No 10 medium in an Erlenmeyer flask for cell culture was the optimal method, producing enough biomass for ecotoxicological tests as well as minimising development of other microorganisms. After 96 h of culture, communities differed from the original communities sampled in the two rivers studied. Species tolerant of eutrophic or saprobic conditions were favoured during culture. This method of diatom community culture affords the opportunity to assess, in vitro, the effects of different chemicals or effluents (water samples and industrial effluents) on diatom communities, as well as on diatom cells, from a wide range of perspectives.

Keywords Diatom community · Culture · Biofilm

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Introduction

Diatoms are important primary producers in aquatic ecosystems, and represent an important source of food for aquatic grazing organisms (Round et al. 1990). These micro-algae are crucial for the development of fish larvae and other young animals in upper trophic levels (Stevenson and Pan 1999). Perturbations in diatom communities lead indirectly to disruption of the productivity of the whole aquatic ecosystem (Berard et al. 2003). Man-made disturbances, such as fertiliser or pesticide contamination, can alter the balance of benthic diatom communities (Stevenson and Pan 1999). In this context, the study of freshwater benthic diatom community variations is particularly relevant both to the understanding of the whole aquatic ecosystem succession and to the practical determination of good ecological status recommended by the European Water framework Directive (2000) (McCormick and Cairns 1994). Up to now, most research has focussed on the behaviour of mono-specific cultures exposed to chemicals (Kasai 1999; Nelson et al. 1999; Peterson et al. 1997; Tang et al. 1997). However, it is difficult to relate the responses of unialgal cultures to the reactions of algae communities in rivers. Under natural conditions, species interactions and differential sensitivity of taxa modulate the response of a diatom community to a disturbance (DeNoyelles et al. 1982). Moreover, transfer of a species to culture induces genetic selection of individuals and alters their sensitivity to chemicals (Peterson et al. 1997). For these reasons, classic mono-specific tests do not predict the effects of perturbations at the community level, especially under natural conditions (Badr et al. 1997).

To overcome these limitations, numerous studies have been carried out with benthic diatoms in natural biofilms fixed on artificial substrata submerged in rivers and

transferred to the laboratory (Berard et al. 2003; Dorigo and Leboulanger 2001; Guasch et al. 1997, 1998; Guasch and Sabater 1998; Gustavson and Mohlenberg 2003; Navarro et al. 2002). River biofilm is quite a complex matrix composed of organic matter (OM), extra-cellular polymeric substances (EPS), and numerous organisms, including bacteria, algae and fungi. Thus, the in vivo observation of benthic diatom cells in natural biofilm samples is quite difficult. For these reasons, benthic diatom studies have focussed mainly on species composition of the community. The identification of species is based on the shapes and ornamentations of the siliceous diatom valves (Lenoir and Coste 1996; Prygiel and Coste 1996; Whitton and Kelly 1995). This descriptive ecological approach has been shown to be limited in its ability to discriminate between nutrient enrichment effects and other environmental stress impacts (Peres et al. 1996; Sabater 2000). Thus, many functional tests, based e.g. on classical pigment analysis or chlorophyll *a* fluorescence assessment, have been developed to measure the sensitivity of natural periphytic algae to a stress in vivo (Dorigo and Leboulanger 2001; Schreiber et al. 2002). However, this approach is performed either on mono-specific cultures or on whole algae communities without distinction between diatoms and the other algae fixed in the biofilm.

In this context, development of a culture method for diatom communities would represent a significant improvement, allowing ecotoxicological experiments on freshwater benthic diatoms at the community level, as well as the individual cell level. Therefore, experiments were carried out aimed at designing a novel method able to isolate from the biofilm a freshwater benthic diatom community similar to the original community collected in the river, and to maintain it in culture. Two culture media were compared for their ability to favour diatom growth and to limit the development of other microorganisms (other algae, bacteria). The influence of the inoculum (the diatoms being either free or fixed on a substrate), the nature of the vessel used for the culture in liquid medium, as well as the effect of culture time were studied. The method was tested on two different diatom communities sampled in two rivers located in the southwest of France.

Materials and methods

Biofilms were scraped off numerous pebbles in the flow of the river Garonne in south-western France. The biofilm solution was transferred as quickly as possible to the laboratory and enriched with nutrient solution for diatoms. The mix was placed in a clear plastic box under controlled conditions [temperature 24±0.5°C/ 18±0.5°C day/night cycles; photoperiod 16 h under daylight fluorescent lamps providing 400 μmol photons.m^{-2}.s^{-1} (Philips 600 W, Netherlands); relative humidity 70%] in a phytotron. Microscope slides, previously washed with distilled water, were submerged in this solution and held vertically by pieces of dense polystyrene. The plastic box was closed. After 3 days, the slides were removed from the plastic box. This duration of submersion was chosen in order to collect a maximum of benthic diatoms as well as to limit the presence of other microorganisms (especially other algae) on the slides. The inoculum obtained after scraping the slides was used to test different methods of culture in terms of medium and vessel used. In order to select the optimal duration of culture, this inoculum was also compared with another inoculum from the river Ariege.

Selection of medium for cell culture

Two nutrient media (Table 1) for the culture of benthic diatoms were studied: Chu No. 10 medium (Nichols 1973) as modified by Hughes and Lund, and a nutrient solution developed in our laboratory similar to Freshwater "WC" medium (Guillard and Lorenzen 1972). These media, without vitamins, were autoclaved at 120°C for 20 min. Vitamins were added in the same proportions to both media: 0.1 mg.L^{-1} vitamin B1, 0.5 μg.L^{-1} biotin and 0.5 μg.L^{-1} B12. Vitamin solutions and the oligo-element solution were filtered (0.2 μm) and frozen before use. Major element solutions and oligo-element solutions can be stored at 4°C. Media for diatom culture were prepared immediately prior to use. Benthic diatoms collected as described above were transferred to Erlenmeyer flasks for cell culture and shaken slowly (70 rpm).

Selection of vessel for cell culture

Some slides used for the isolation of benthic diatoms were not scraped, and were transferred into a smaller plastic box (180×135×90 mm). Samples of scraped cell inoculum were introduced into an Erlenmeyer flask or a centrifuge tube. At the start of culture, two aliquots (12 mL and 0.8 mL) of cell inoculum were inserted into 150 mL (cell culture Erlenmeyer flask) and 10 mL (centrifuge tube) of fresh medium, respectively, in order to obtain the same cell density. The plastic box, the Erlenmeyer flask and the centrifuge tube were slowly shaken. The rest of the inoculum was used to study the ecological succession of the community.

Cell density assessment

The growth of the diatom community was followed with a Malassez counting chamber, by assessing viable cell density based on the cell score with chloroplasts. Each sample was

Table 1 Major nutrients and micronutrients for Freshwater "WC" medium and Chu No.10 modified medium

	CAS number	Freshwater "WC" medium (Guillard and Lorenzen 1972)	Chu No. 10 modified medium (Nichols 1973)
Major nutrients (in mM)			
$Ca(NO_3)_2$	13477-34-4		0.042
K_2HPO_4	7758-11-4		0.228
Na_2CO_3	497-19-8		0.188
$MgSO_4$	10034-99-8	0.150	0.100
$Na_2SiO_3·5H_2O$	10213-79-3	0.100	0.202
$NaNO_3$	7631-99-4	1.000	
KH_2PO_4	7778-77-0	0.064	
$NaHCO_3$	144-55-8	0.150	
$CaCl_2·2H_2O$	10035-04-8	0.250	
Micronutrients (in µM)			
EDTA-Fe	15708-41-5	268.6	268.6
$MnSO_4·H_2O$	10034-96-5	8.900	1.000
H_3BO_3	10043-35-3	24.100	5.000
$ZnSO_4·7H_2O$	7446-20-0	1.700	0.100
$CuSO_4·5H_2O$	7758-99-8	3.900	0.050
$(NH_4)_6Mo_7O_{24}·4H_2O$	12054-85-2		0.007
$Na_2WO_4·2H_2O$	10213-10-2		0.010
KBr	7758-02-3		0.100
KI	7681-11-0		0.050
$Cd(NO_3)_2$	10022-68-1		0.050
$Co(NO_3)_2·6H_2O$	10026-22-9		0.050
$(NH_4)_2Ni(SO_4)_2·6H_2O$	7785-20-8		0.050
$Cr(N0_3)_3·9H_2O$	7789-02-8		0.010
NH_4VO_3	7803-55-6		0.010
$AlK(SO_4)_2·12H_2O$	7784-24-9		0.050
$Na_2MoO_4·2H_2O$	10102-40-6	0.1	
$CoCl_2·6H_2O$	7791-13-1	0.042	

counted three or four times. In the case of the plastic box, a part of each slide was scraped on both sides. For the Erlenmeyer flask and the centrifuge tube, diatoms were scraped off the surface of the vessel with a scraper. For all vessels, the surface scraped and the volume of the sample were noted to correct for variations due to the scraping.

Culture duration experiment

Inocula from two different rivers located in southwest France (Garonne and Ariege) were cultured in modified Chu No. 10 medium. The aim was to determine the optimal culture time to obtain both sufficient diatom biomass and a community as similar as possible to the original natural community. After 72 h of growth, benthic diatoms were treated for 24 h with 10 $mg.L^{-1}$ amoxicillin T trihydrate. According to previous results on the kinetics of nutrient uptake by a diatom community in Erlenmeyer flasks (nitrate, phosphate and silicium) (data not shown), the medium was renewed every 72 h and at the end of antibiotic treatment (72, 96, 168, 240 h). Samples were taken at each step of the culture: during the transfer to the Erlenmeyer flask, at the beginning and at the end of antibiotic treatment, and 10 days after the transfer. All samples were fixed with formaldehyde. According to the French standard for the "Determination of the Diatom Biological Index (IBD)" (AFNOR NF T90-354) samples were treated with hydrogen peroxide to digest cell organic content, and subsequently centrifuged (2,571 *g* for 10 min) four times and washed in order to eliminate the hydrogen peroxide. For all samples treated, an aliquot of 200 µL was dried on a cover slip. The diatom frustules fixed on the cover slip were mounted on a microscope slide with Naphrax. Slides were scanned with a light microscope (Leica DMRD Microsystems, Wetzlar, Germany) at a magnification of 1,000 X, and about 400 frustules were identified as recommended by French standard NF T90-354. From the species composition data obtained after each slide scan, the Shannon index $[-\sum_{i=1}^{S} p_i \ln p_i]$ where S is the number of species, $p_i=n_i/N$ (n_i the number of individuals in each species, and N the total number of individuals) was calculated to assess variation in species diversity. This diversity index accounts for abundance as well as evenness of the species present in the community.

Data analysis

A descriptive analysis of the data was used to calculate the mean and the standard deviation. The results of cell density for the two media tested were analysed statistically with one-way ANOVA, Kruskal-Wallis test and Dunn's test (Sigmastat, SPSS, Cary, NC). For each sample, the growth rate (μ) was calculated according to:

$\mu = (D_{tb}/D_{ta})*24/t.$

where D_{ta} and D_{tb} represent cell densities at the start (t_a) and at the end (t_b) of the growth period considered, and t the duration of this period ($t = t_b - t_a$) in hours. The growth rate is expressed per day. The maximum growth rate (μ_{max}) observed for each culture was calculated. Pearson correlations (Sigmastat) were also calculated to study the succession of species composition during the culture compared with the initial natural community in the river. Histogram charts were used to study the variations in abundance for the main species.

Results

Effects of cell medium on diatom growth

Cell density was assessed during culture to study the growth of diatom biomass. The results showed that the length of the lag time after inoculation was 66 h (Fig. 1). Maximum growth rate was achieved immediately after the lag time, i.e. between 66 h and 90 h of culture; thereafter, the growth rate gradually decreased. . The maximum growth rate of the diatom community was higher in Freshwater "WC" medium (μ_{max}=3.41 day^{-1}) than in Chu No. 10 medium (μ_{max}=1.88 day^{-1}). For the two media studied, the lowest growth rates (μ=1.24 day^{-1} for Freshwater "WC" and μ=1.26 day^{-1} for Chu No 10) were observed between 117 and 138 h. Maximal diatom biomass was obtained at 138 h (107 cells.mm^{-3} for Freshwater "WC" and 79 cells.mm^{-3} for Chu No. 10). At 90, 117 and

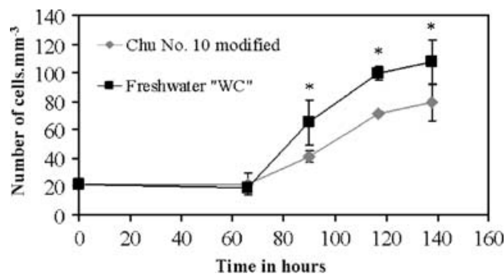

Fig. 1 Growth curves of diatom communities in two different media (Chu No. 10 modified and Freshwater "WC") from cell density assessment. * Cell density values are statistically different between the two media; ANOVA $P<0.05$

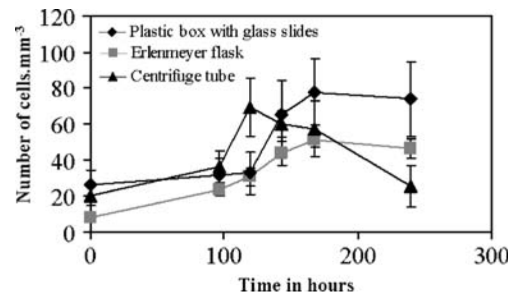

Fig. 2 Growth curves of diatom communities for three different culture vessels (plastic box with glass slides, Erlenmeyer flasks, centrifuge tubes) from cell density assessment. * Cell density values are statistically different between the two media; ANOVA $P<0.05$

138 h, the cell density was significantly higher for the community in the Freshwater "WC" medium (ANOVA, $P<0.05$). Microscope observations showed that the latter medium favoured the growth of other algae such as *Scenedesmus* sp. or *Chlamydomonas* sp.

Diatom growth in different vessel types

In centrifuge tubes, the maximum growth rate (μ_{max}= 1.9 day^{-1}) was achieved between 96 and 119 h (Fig. 2). The highest cell density (69.9 cells.mm^{-3}) reached at 119 h with this vessel is close to that obtained with glass slides (77.8 cells.mm^{-3}) and in Erlenmeyer flasks (51.1 cells.mm^{-3}) at 167 h. After 119 h, diatom growth in centrifuge tubes ceased and cell density decreased. For the other vessel types, μ_{max} was higher for glass slides (2 day^{-1}) than in Erlenmeyer flasks (1.42 day^{-1}). In these two vessels, stationary phase was achieved at 167 h. Statistical analysis showed, at each time point, no significant differences between the different vessels (ANOVA or Kruskal Wallis $P>0.05$).

Succession of benthic diatom communities isolated from the biofilms of two different rivers

As shown in Fig. 3, the correlation between the natural community and the isolated community (t=0 h) was positive and significant (0.982* for the River Garonne community and 0.759* for the River Ariege community, *$P<0.05$). The isolation of diatom cells on glass slides did not affect the species composition of the communities or the diversity assessed by the Shannon index.

For diatoms isolated from the River Garonne, the cultured community stayed relatively similar to the natural one up to 96 h, the correlations being positive and significant. Between 72 and 96 h, the diversity decreased from 3.6 to 3.24; thereafter the succession of the community in culture was more pronounced.

In the case of diatoms isolated from the River Ariege, the community changed at 72 h. The correlation (0.279)

with the natural community was not significant. During culture, a continuous decrease in diversity was also observed.

In parallel, the study of the density values showed that the cell density reached values close to 80 cells.mm^{-3} for the Garonne culture and 40 cells.mm^{-3} for the Ariege culture.

In order to study more precisely the ecological succession during culture, the relative abundance of the main species was determined (Fig. 4). The same global succession pattern was observed in the two cultures. Dominant species reported in original natural communities [*Encyonema minutum* (Hilse in Rabh.) D.G. Mann, *Nitzschia fonticola* Grunow in Cleve et Möller, *Nitzschia dissipata* (Kützing) Grunow, *Diatoma vulgaris* Bory and *Navicula tripunctata* (O.F.Müller) Bory] were replaced during culture by *Nitzschia palea* (Kützing) W.Smith or *Gomphonema parvulum* (Kützing) Kützing.

Among the dominant species in the natural communities, the decline of *N. dissipata*, *E. minutum* and *D. vulgaris* during culture was not related to the isolation step. Their abundance remained almost stable (except *N. dissipata* in the culture from the River Ariege biofilm) or increased during this step. The culture conditions also affected the development of different Gomphonema species scored in the Ariege culture [*Gomphonema minutum* (Ag.) Agardh and *Gomphoneis minuta* (Stone) Kociolek & Stoermer] and in the Garonne culture [*Gomphonema olivaceum* (Hornemann) Brébisson]. The abundances of these species decreased. Likewise, other species, such as *Navicula tripunctata* and *Achnanthidium biasolettianum* (Grunow in Cl. & Grun.) Lange-Bertalot, exhibited a decrease in abundance in both cases.

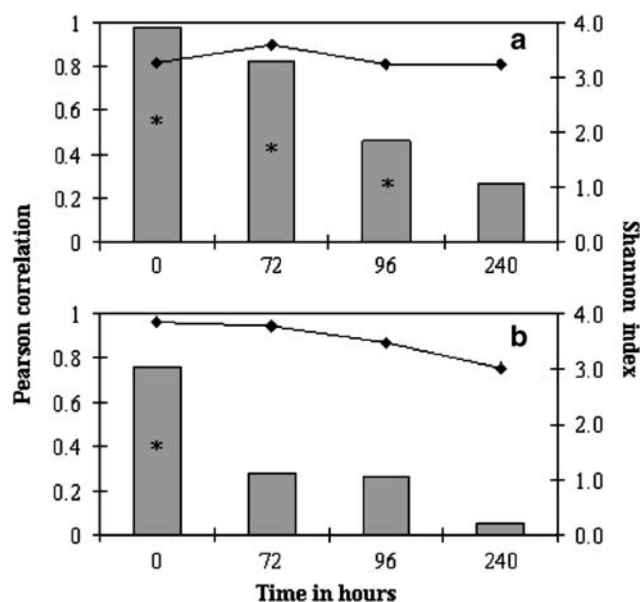

Fig. 3 Diversity curves and histograms of Pearson correlations between the cultured diatom community at different dates and the natural community sampled in (**a**) the River Garonne and (**b**) the River Ariege. *Significant correlation $P<0.05$

A few species were more adapted to culture conditions, e.g. *N. palea*, which showed the largest development in the two cultures. It increased in abundance faster in the community isolated from the River Ariege sample. Likewise, another species, *G. parvulum*, grew very quickly during the last hours of culture and then stabilised. However, this species was abundant only in the culture isolated from the River Garonne. In the case of *N. fonticola*, growth stopped at 72 h and afterwards decreased. However, the decrease was limited and, for both cultures, the final relative abundance of *N. fonticola* was stable at around 200 inviduals per 1,000 cells.

Two species, *Mayamaea atomus* (Hustedt) Lange-Bertalot and *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, developed suddenly in relatively high abundance after 96 h for each culture. *Mayamaea atomus* was more abundant in the community isolated from the River Ariege and *F. saprophila* in the community isolated from the River Garonne.

Discussion

The data obtained in these experiments allow discussion of the influence of the selected culture method on the growth and succession of diatom communities.

The first experiment was carried out to select an efficient medium for the growth of benthic diatoms that limits the development of bacteria and other algae. The comparison of modified Freshwater "WC" medium (Guillard and Lorenzen 1972) with Chu No. 10 medium (Nichols 1973) indicated that Freshwater "WC" medium was rapidly colonised by cyanobacteria and other algae (green algae). Such contamination may be related to the absence of inhibitor elements in the micronutrient solution, such as vanadium for cyanobacteria (Nalewajko et al. 1995). Although the diatom biomass growth rate was higher in Freshwater "WC" medium, the contamination of the culture by other microorganisms is a negative factor for further ecotoxicological experiments. These microorganisms produce EPS that can agglomerate diatoms and other algae and thus disturb diatom cellular observations under the microscope. In contrast, Chu No. 10 allowed sufficient production of diatom biomass and was very selective for other microorganisms and algae. For these reasons, Chu No. 10 was used in subsequent experiments.

The growth rates obtained with this medium ($1.2<\mu<1.9$ day^{-1}) were close to those reported for unialgal cultures in this medium at the same temperature (Butterwick et al. 2005; Hayakawa et al. 1994). Growth rate data for diatom cultures are limited to unialgal cultures (Round et al. 1990). Casotti et al. (2005) mentioned that culture of

Thalassiosira weissflogii (Grunow) reached the stationary phase of growth within 72 h whereas, in this study, this phase was reached at 117 h. This difference may be related to the use of another medium by Cassoti and coworkers. The presence of diatom species growing at different speeds in the community culture could also explain this difference.

In the second experiment, different vessels for diatom cell culture were tested. The results show that polypropylene centrifuge tubes are not appropriate for the culture of diatom communities. Cell density decreased after 119 h, probably due to poor oxygenation. On glass slides, diatom cell culture exhibited a higher maximum growth rate. However, the communities of diatoms differed between slides after only 2 days of culture (data not shown). In addition, the variability in cell density was very high during the growth period. For the Erlenmeyer flasks, the maximum growth rate of the community was lower than that on glass slides. Nevertheless, it is possible to reproduce diatom development with this vessel. Diatom community growth remained more homogenous for the duration of the culture (239 h) compared to communities on glass slides. The results illustrated that the Erlenmeyer flask is the most

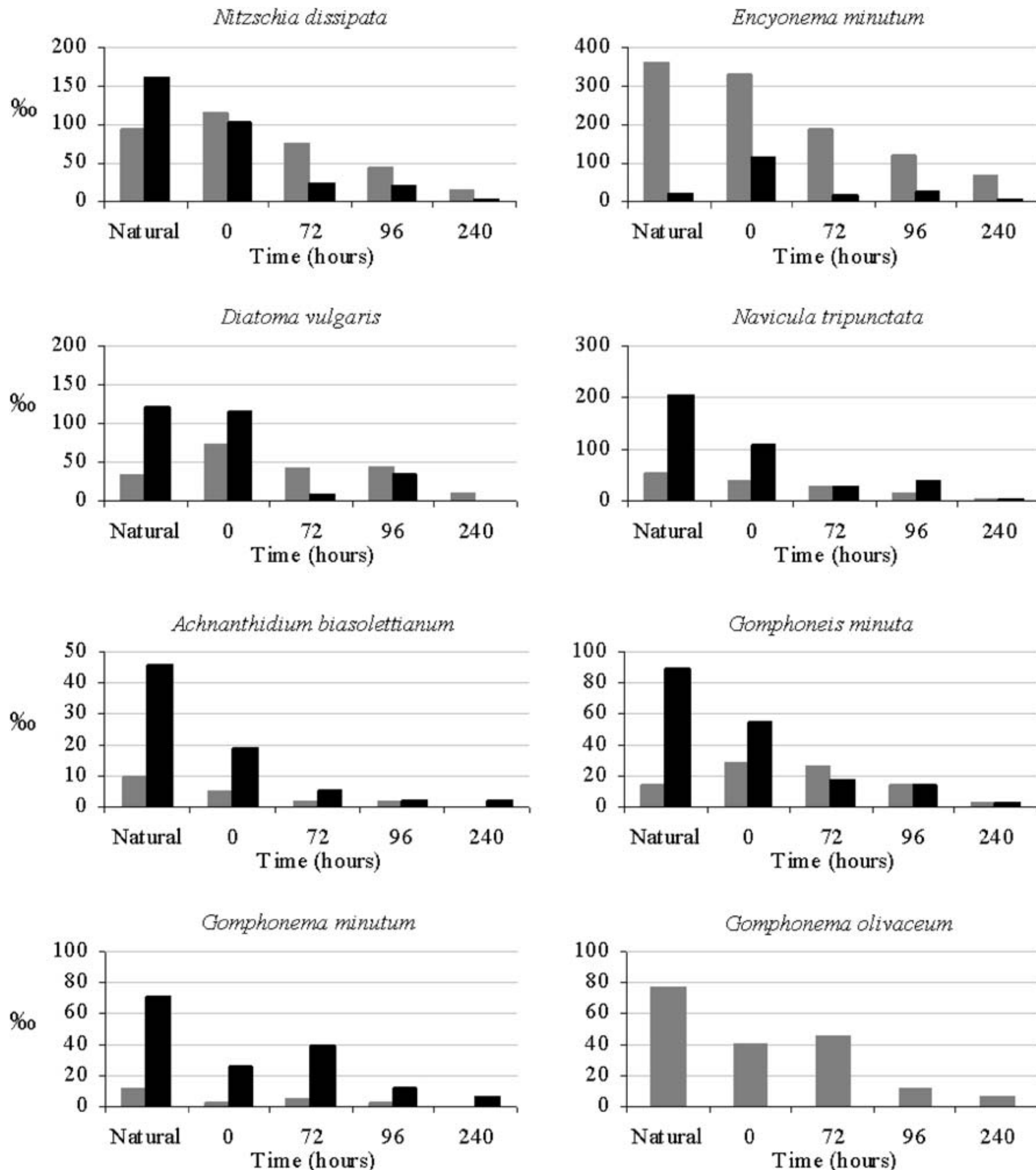

Fig. 4 Histograms of species relative abundance (‰) in natural communities, in communities isolated on glass slides (t=0 h) and in communities during culture (t=72, 96, 240 h) for two inocula. *Gray* Garonne, *black* Ariege

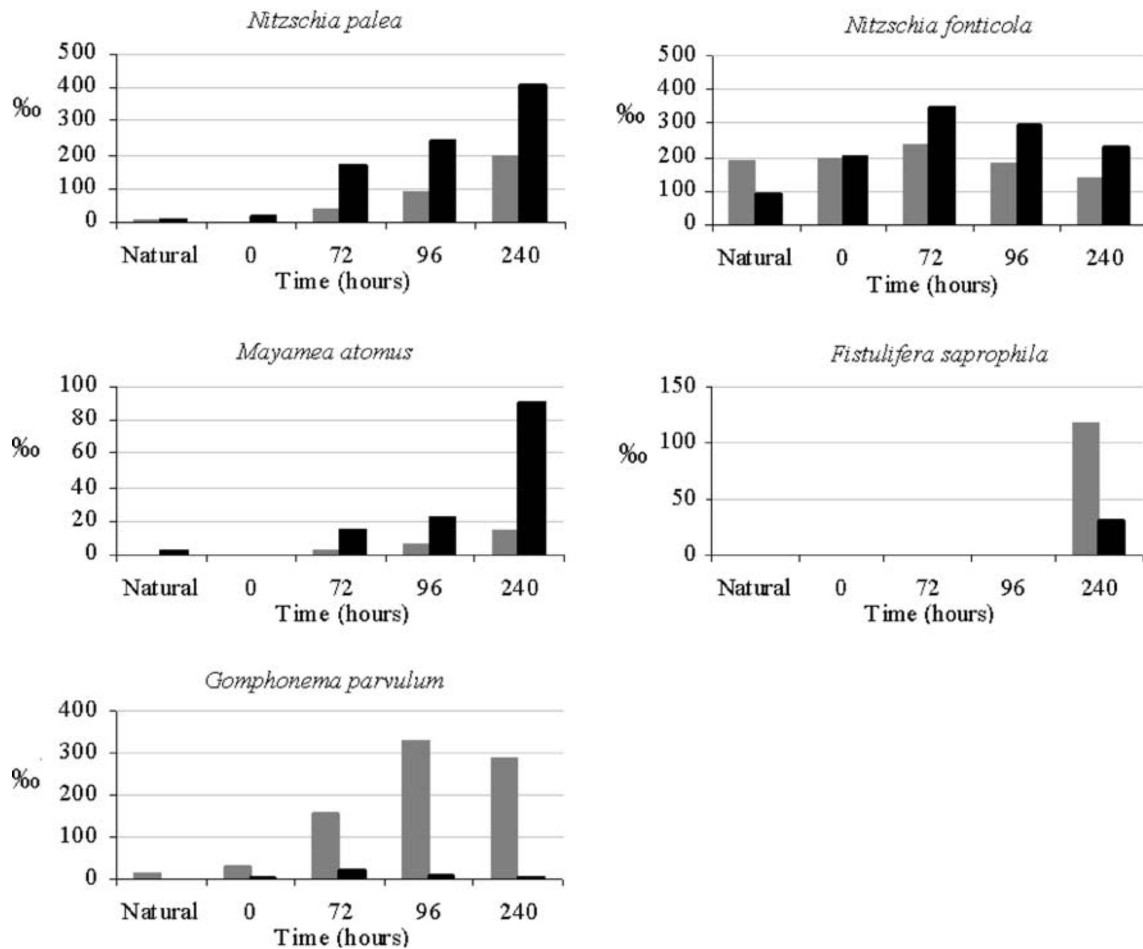

Fig. 4 (continued)

appropriate vessel to allow homogenous development of the cells and to maintain diatom community in primo-culture, although glass slides can provide heterogeneous biological material for further applications in ecotoxicological experiments.

In the third experiment, the optimum time of culture was studied in Erlenmeyer flasks. The purpose of this experiment was to select the optimum duration to (1) obtain a community close to the original natural community, and (2) to produce enough biomass for experiments on diatoms. The results showed that the isolation step (described in Material and methods) immediately prior to primo-culture had no significant effect on community composition. The communities isolated were composed of species that are very sensitive to organic pollution (*Nitzschia fonticola*, *Nitzschia dissipata*, *Navicula tripunctata*, *Gomphonema olivaceum*, *Diatoma vulgaris*, *Gomphonema minutum*, *Gomphoneis minuta* and *Encyonema minutum*) according to Krammer et al. (1986). After 72 h of cell culture, the abundance of some species was significantly reduced. Some species, such as *Gomphonema olivaceum*, are more adapted to oligotrophic than to eutrophic culture conditions (Guasch et al. 1998; Rott et al. 1998). Other species, e.g. *E. minutum* and *Nitzschia dissipata*, were still observed in moderate proportions due to their high abundance in the community isolated from the river Garonne. At the same time of culture, *Gomphonema parvulum* and *Nitzschia palea* appeared in high abundance in cultures stemming from the communities of the Garonne and Ariege rivers. These two species are recognised largely for their tolerance of high nutrient concentrations and poor oxygenated conditions (Guasch et al. 1998; Lange-Bertalot 1979; Rott et al. 1998; Van Dam et al. 1994). After 96 h of culture, the community structure remained unchanged. No significant differences in community composition of diatoms isolated from the Garonne River were observed following antibiotic treatment. The antibiotic treatment is not absolutely required. Nevertheless, it makes it possible to control the unwanted development of other algae and bacteria. In the Ariege culture, the low correlation obtained at the same date between community in culture and original natural community could be related to the dominance of oligotrophic species in the natural community. Therefore, in eutrophic culture conditions, the succession was more marked.

After 240 h of culture, diatom communities reflect highly eutrophic to saprobic conditions. At this time, two species not enumerated previously, *Mayamaea atomus* and *Fistulifera saprophila*, appeared in high proportions (9% and 12%, respectively). These two species are particularly tolerant to eutrophic and poorly oxygenated conditions (Krammer et al. 1986; Rott et al. 1998). In contrast, *Nitzschia dissipata*, *Navicula tripunctata*, *Gomphonema olivaceum*, *Diatoma vulgaris*, *Gomphonema minutum*, *Gomphoneis minuta* and *Encyonema minutum* had almost disappeared. As mentioned previously, these species are known to be sensitive to organic pollution and to poorly oxygenated conditions. *Nitzschia fonticola* was always observed with relatively stable abundance in all cell cultures. This species is known for its tolerance to a wide range of trophic conditions (Coste 1982). The occurrence of this species under eutrophic conditions is confirmed by its moderate sensitivity value (3.5) according to Coste (1982) in the Polluo-Sensitivity Index.

In conclusion, the optimal duration of our primo-culture method would be 72 or 96 h. After this, the communities obtained in culture were too significantly different and no longer reflected the natural communities. In this ecological succession, species tolerant of eutrophic to saprobic conditions and poorly oxygenated conditions were most favoured. Sensitive species may not be able to modulate the development of tolerant species with interspecific signal (Casotti et al. 2005). Moreover, oxygenation by flask agitation appeared inadequate and favoured growth of species tolerant of poorly oxygenated conditions. Air bubbling may be a potential improvement to maintain species that favour oxygenated conditions.

To sum up, the culture of a diatom community provides the opportunity to test the effects of a wide range of parameters including contaminants such as pesticides, or the harmfulness of different effluents, at the community level as well as at the cellular level. With this aim, further investigations will be necessary to increase the time of culture as well as to obtain a diverse diatom community relatively similar to the original natural community.

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