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Herbicide effects on freshwater benthic diatoms: Induction of nucleus alterations and silica cell wall abnormalities

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ABSTRACT

Benthic diatoms are well known bio-indicators of river pollution by nutrients (nitrogen and phosphorus). Biological indexes, based on diatom sensitivity for non-toxic pollution, have been developed to assess the water quality. Nevertheless, they are not reliable tools to detect pollution by pesticides. Many authors have suggested that toxic agents, like pesticides, induce abnormalities of the diatom cell wall (frustule). High abnormal frustule abundances have been reported in natural diatom communities sampled in streams contaminated by pesticides. However, no direct link was found between the abundances of abnormal frustules in these communities and the pesticide concentrations in stream water. In the present study, a freshwater benthic diatom community, isolated from natural biofilm and cultured under controlled conditions, was treated with a known genotoxic herbicide, maleic hydrazide (MH). Cells were exposed to three concentrations of MH (5×10^{-6}, 10^{-6}, 10^{-7} M) for 6 h followed by a 24 h-recovery time. After MH treatments, nucleus alterations were observed: abnormal nucleus location, micronucleus, multinuclear cell or disruption of the nuclear membrane. A dose-dependent increase of nuclear alterations was observed. The difference between the control (9.65 nuclear alterations per 1000 cells observed (9.65‰), S.D. = 4.23) and the highest concentrations (29.40‰, S.D. = 8.49 for 10^{-6} M and 35.96‰, S.D. = 3.71 for 5×10^{-6} M) was statistically significant (Tukey test, $P < 0.05$). Diatoms also exhibited frustules with deformed morphology and abnormal ornamentation. Significantly increased abundances of abnormal frustules were observed for the highest concentrations (10^{-6} and 5×10^{-6} M; Tukey test, $P < 0.05$). These two parameters tended to increase together (Pearson correlation = 0.702, $P < 0.05$). The results suggest that the induction of abnormal frustules could be associated with the genotoxic effects of MH. The alterations observed could be related to the effects of MH on the synthesis of the proteins involved in frustule formation or in the regulation of the cytoskeleton of the diatom cells.

1. Introduction

Freshwater diatoms are fundamental primary producers for the aquatic ecosystems (Round et al., 1990). They are a source of food for numerous other organisms of the upper trophic levels. Alterations of these micro-organisms may disrupt the balance of the whole ecosystem (Stevenson and Pan, 1999) and water quality biomonitoring programs, based on studies of benthic diatoms, have been developed in Europe and North America to monitor river health (Whitton and Kelly, 1995; Potapova and Charles, 2007). Several monitoring tools like the diatom biological index (IBD) have been developed to assess the water quality of rivers based on the evolution of diatom community structure (Lenoir and Coste, 1995; Prygiel and Coste, 1996; Potapova and Charles, 2007). The IBD, standardized in 2000 (NF T90-354), is now widely used in Europe. This tool was built from a data base of 649 taxa classified according to their sensitivity to seven standard physico-chemical parameters: pH, conductivity, percent saturation of oxygen, BOD_5 and nutrient concentration in NH_4^+, NO_3^-, and PO_4^{3-}. A mathematical model integrates the sensitivities of the different species observed in a natural community to calculate a water quality score. Nevertheless, its efficiency to detect water pollution by pesticides is questioned (Dorigo et al., 2004). Indeed, many parameters like light exposure and nutrient concentrations may interfere in the response of diatoms to pesticides (Guasch et al., 1997, 1998). Thus, the potential impact of pesticides on algal communities was not demonstrated in agricultural watersheds where streams are contaminated by high levels of fertilisers (Berard et al., 1998).

Among photosynthetic micro-organisms, diatoms present the particularity of having a cell wall (frustule) composed of siliceous

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valves forming a shell around the cell. The morphology and the ornamentation of the frustule are specific for each species. Sometimes, abnormal morphology and disturbed ornamentation of the frustule have been observed in nature. Silica deficiency and high cell density have been proposed as causative agents of such abnormalities as have toxic compounds, like herbicides or heavy metals (Thomas et al., 1980; McFarland et al., 1997; Dickman, 1998; Rijstenbil, 2001; Gomez and Licursi, 2003; Cattaneo et al., 2004; Schmitt-Jansen and Altenburger, 2005). A field survey (eight sites) showed an increase of the abundance of abnormal frustules in diatom communities sampled in two streams highly contaminated by fertilisers and pesticides (unpublished data). In the unpolluted site of this survey, the mean value of abnormal frustule abundance was about 1.9/1000 (±0.6), whereas in the most contaminated watershed, the highest mean value was close to 14/1000 (14.2/1000 ± 7.5). However, the relationship between the presence of abnormal frustules and pesticide exposure is extremely difficult to establish in natural streams. This is due (i) to the heterogeneity of the chemicals present in water and (ii) to the difficulty to monitor the real pesticide concentrations to which organisms are exposed.

The cellular mechanisms involved in the formation of frustule abnormalities remain unknown. Abnormal frustules have been observed in diatoms exposed to colchicine (CAS No. 64-86-8) or to isoproturon (CAS No. 34123-59-6) (Coombs et al., 1968; Schmitt-Jansen and Altenburger, 2005). Genotoxic effects (DNA dispersion and multinuclear cells) were reported after treatments by colchicine or a polyunsaturated aldehyde (2-trans,4-trans-decadienal CAS No. 25152-84-5) (Coombs et al., 1968; Casotti et al., 2005). Although colchicine induced abnormal frustules as well as multinuclear cells, no link was established between the induction of frustule abnormalities and the genotoxicity.

Thus, a laboratory experiment was carried out to study the potential relationship between the induction of frustule abnormalities and the formation of nucleus alterations. The diatom cells were isolated from natural biofilm and maintained in culture (Debenest et al., 2008). Diatom culture was exposed to a well known genotoxic herbicide, maleic hydrazide (MH CAS No. 123-33-1). MH is used in France as a growth regulator for potatoes, onions and tobacco and as a herbicide in agriculture or in parks and gardens. This chemical was chosen for use as a positive control in the micronucleus tests (Cotelle et al., 1999). In higher plants, MH induces the formation of micronuclei, DNA strand breaks or bridges between strands (Cotelle et al., 1999; Grant, 1999; Kong and Ma, 1999; Marcano et al., 2004; El Hajjouji et al., 2007). In the present study, the potential genotoxicity of MH on diatoms was estimated by the nucleus alteration count. To evaluate the role of herbicides in the induction of frustule abnormalities, the number of abnormal fustules was counted.

2. Materials and methods

2.1. Diatom cultures

The benthic diatoms were cultured as described by Debenest et al. (2008). The biofilms were collected from pebbles taken in rivers located in the basin of the river Garonne in the Southwest of France. A specific diatom medium (Chu No. 10) was added to the biofilm samples which were placed in plastic boxes under controlled conditions in regulated room with day/night air temperatures of 24 ± 0.5/18 ± 0.5 °C and a relative humidity of 70/75%. Diatoms were exposed to 400 μmol m^{-2} s^{-1} of light provided by fluorescent lamps [Philips 600 W, Eindhoven, Netherlands] with a 16L:8D light-dark cycle. In order to isolate the benthic diatoms, microscope slides were submerged vertically in the biological samples mixed with the medium. After 3 days, the diatoms were scraped off the slides and grown in Erlenmeyer flasks filled with 150 ml of fresh medium.

2.2. Diatom toxic agent exposure

After a 2-week of culturing, 5 ml-subsamples were placed in 12 centrifuge tubes (15 ml) containing the same volume of fresh nutrient solution. The tubes were held horizontally in a small box and slowly shaken. Powdered maleic hydrazide 99% (MH, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in demineralised water to make 10^{-5} M stock solution. Aliquots of it were then added to the centrifuge tubes to obtain final concentrations of 10^{-7}, 10^{-6}, and 5×10^{-6} M. Three replicates were done for each concentration and for the control. The cells were exposed to MH for 6 h. At the end of exposure, to remove the MH, the cells were centrifuged (693 *g* for 10 min) and re-suspended in fresh medium. The diatom cells were kept for a 24 h-recovery period in the same conditions as the original culture. The duration of the MH exposure was chosen in comparison with bibliographic data (Cotelle et al., 1999; Casotti et al., 2005) and followed by a recovery time fixed in relation to the growth rate assessed previously during other cell cultures (Debenest et al., 2008).

2.3. Diatom species identification and abnormal frustules count

For the identification of the diatom species and to count the abnormal frustules, all samples were fixed in formaldehyde (CAS No. 50-00-0). Following the French standard for the determination of the "Diatom Biological Index (IBD)" (NF T90-354) samples were treated with hydrogen peroxide (CAS No. 7722-84-1) to digest the organic components. They were subsequently centrifuged and the siliceous diatom cell walls (frustules) re-suspended in water to eliminate the hydrogen peroxide. For all treated samples, an aliquot (about 200 μl) was dried on a glass coverslip. The frustules fixed on the glass cover were mounted on a microscope slide with Naphrax®, a resin with a high refractive index (1.73). The slides were scanned with a light microscope (Leica DMRD, Leica Microsystems GmbH, Wetzlar, Germany) at a magnification of 1000×. For each MH concentration and for the control, a total of at least 1000 frustules was identified to record the abundance of abnormal frustules.

2.4. Microscopic nucleus observation

For the laboratory experiment, the diatom nuclei were stained with 2% of Hoescht 33342 (CAS No. 23491-52-3, Sigma Chemical Co.) solution at 2 g/l. The slides were prepared for each replicate after the recovery time. Nucleus alterations were counted under 600× magnification with an epifluorescence microscope (BX41 Olympus America Inc., Center Valley, PA, USA) with a specific DAPI filter (U-MWU2, Ex. filter: 330–385λ, Em. filter: 420λ, dicromatic filter: 400λ). At least 1000 cells total for each treatment were counted to determine the mitotic index (number of cells in division/total number of cells counted × 100) and the frequency of nucleus alterations. To be able to observe these alterations, preliminary studies with a confocal microscope (DRMX A2 Leica Microsystems GmbH, Wetzlar, D) were performed to identify the specific and correct stain for DNA in diatoms. The stain Hoescht 33342 was selected rather than other fluorochromes such as propidium iodide (CAS No. 25535-16-4) which did not discern nucleus from chloroplasts.

2.5. Statistical analysis

A descriptive analysis of the data was used to calculate the mean value and the standard deviation of the abundance data. The val-

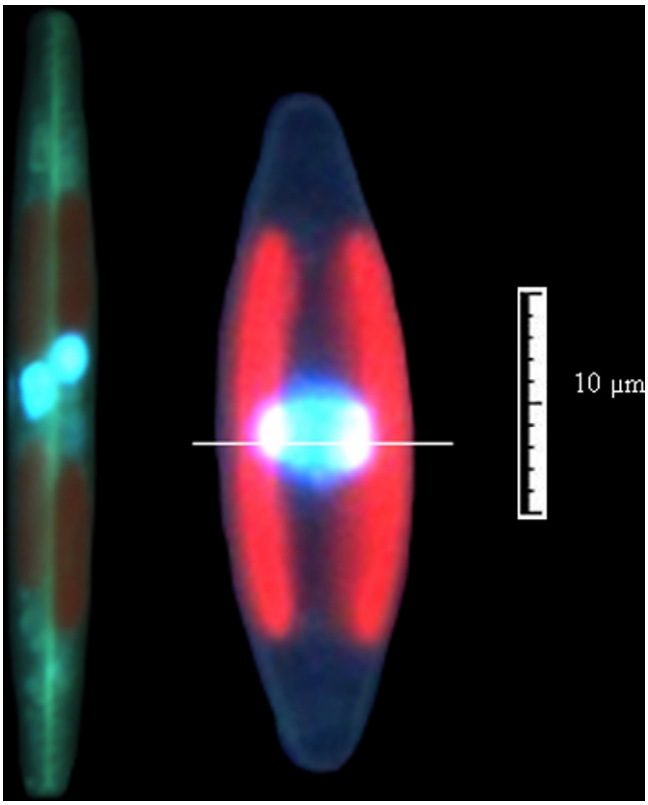

Fig. 1. Benthic diatoms in division with anaphase stage (left) and metaphase stage (right, transapical axis in white) observed under the epifluorescence microscope (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

ues of abundance for the abnormal frustules and for the nucleus alterations were statistically analyzed with ANOVA and a Tukey test (SigmaStat, Systat Software Inc., San Jose, CA, USA) to compare the treated groups with the control group. Pearson correlation (SigmaStat, Systat Software Inc.) was also used to test for any relationships between these two types of cell alterations.

3. Results

The cells in mitosis were counted in order to calculate the mitotic index and, thus, to assess the impact of the MH on the cell division frequency. Cells are considered in mitosis if two similar nuclei were observed in the transapical plane (white axis Fig. 1). The results of Table 1 show that no significant variations (ANOVA, $P > 0.05$) in mitosis frequency occurred between the control and the diatom communities treated with the three different concentrations of MH.

The highest abundances of nuclear alterations were obtained for diatom communities exposed to MH (Table 1). These alterations appeared in significantly (Tukey test, $P < 0.05$) higher proportions for the highest MH concentrations (10^{-6} and 5×10^{-6} M). Different kinds of nuclear alterations were identified: abnormal nucleus location (Fig. 2a), micronucleus induction (Fig. 2b) and fragmentation of the nucleus into multiple parts (Fig. 2c). Moreover, morphological changes of the nucleus (nuclear membrane breakage and DNA spreading out) were also observed (Fig. 2d). Nevertheless, this morphological change was not counted as a nucleus alteration.

In the case of the abnormal frustules, their abundance increased with the MH concentrations applied (Table 1). The abnormal frustules were significantly more abundant in the communities treated with the highest doses (10^{-6} and 5×10^{-6} M) than in the control communities. Two types of frustule alterations were identified: abnormal morphology (Fig. 3a) or disrupted ornamentation (Fig. 3b). In *Nitzschia* cells with abnormal ornamentation, the fibula was disturbed and the striae were not parallel. The abnormal cells of the *Ulnaria* diatom species showed an increase of the frustule deformations. For the first (a) and the second (b) cells, the striae were disturbed and the pseudo-raphe was not straight. The abnormalities of the last cell (c) were greater. The normal ornamentation was completely destroyed: no striae and pseudo-raphe were observed.

Pearson analysis showed a significant positive correlation between the abundance of nucleus alterations and the abundance of abnormal frustules (0.702, $P < 0.05$).

4. Discussion

Based on the methodology developed for the micronucleus tests in higher plants (Cotelle et al., 1999), the mitotic index was assessed to ensure that MH did not inhibit the cell division. The nuclear alterations are the results of mitotic anomalies (chromosome breaks or disturbance of the microtubular system) that are only recognisable during mitosis. Likewise, in diatoms, the frustule abnormalities may be also induced during the secretion of the new valves at the end of the mitosis (Duke and Reimann, 1977; Round et al., 1990; Van Den Hoek et al., 1995). In our experiment, the mitotic index showed no significant differences between the various MH treatments. These results indicate that MH exposure did not affect diatom cell division so it was possible to quantify and compare the different effects between treatments.

Four nucleus alteration types were identified after the exposure of diatoms to MH: abnormal nucleus location, micronucleus formation, nucleus fragmentation and nucleus membrane breakage. In the case of the nucleus location, many researchers have reported that the diatom nucleus is generally positioned centrally, either in the middle of the cell or to one side depending on how the cell is viewed (valvar or connective) (Edgar and Pickett-Heaps, 1984; Round et al., 1990; Pickett-Heaps, 1991; Van Den Hoek et al., 1995). Therefore, for benthic diatoms, it can be assumed that the nucleus is altered when it is clearly located in the apex (Fig. 2a). Indeed no evidence of this location is mentioned in the bibliography. An explanation could be that the toxic agent alters the cytoskeleton (microtubular system and other microfilaments), leading to a mod-

Table 1
The effect of maleic hydrazide (MH) concentrations on the mean abundance of cells with nuclear alterations, on the mean abundance of cells in division (mitotic index) and on the mean abnormal frustule abundance

Treatment (M)	Nucleus alteration abundance (per 1000)		Mitotic index (per 100)		Abnormal frustule abundance (per 1000)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	9.65	4.23	18.34	2.75	4.33	1.25
1×10^{-7}	23.04[a]	5.56	22.14[a]	2.37	12.00[a]	0.82
1×10^{-6}	29.40	8.49	23.74[a]	6.00	15.00	3.74
5×10^{-6}	35.96	3.71	22.51[a]	0.74	14.67	3.68

[a] S.D.: Standard deviation; no significant difference $P > 0.05$ with the control.

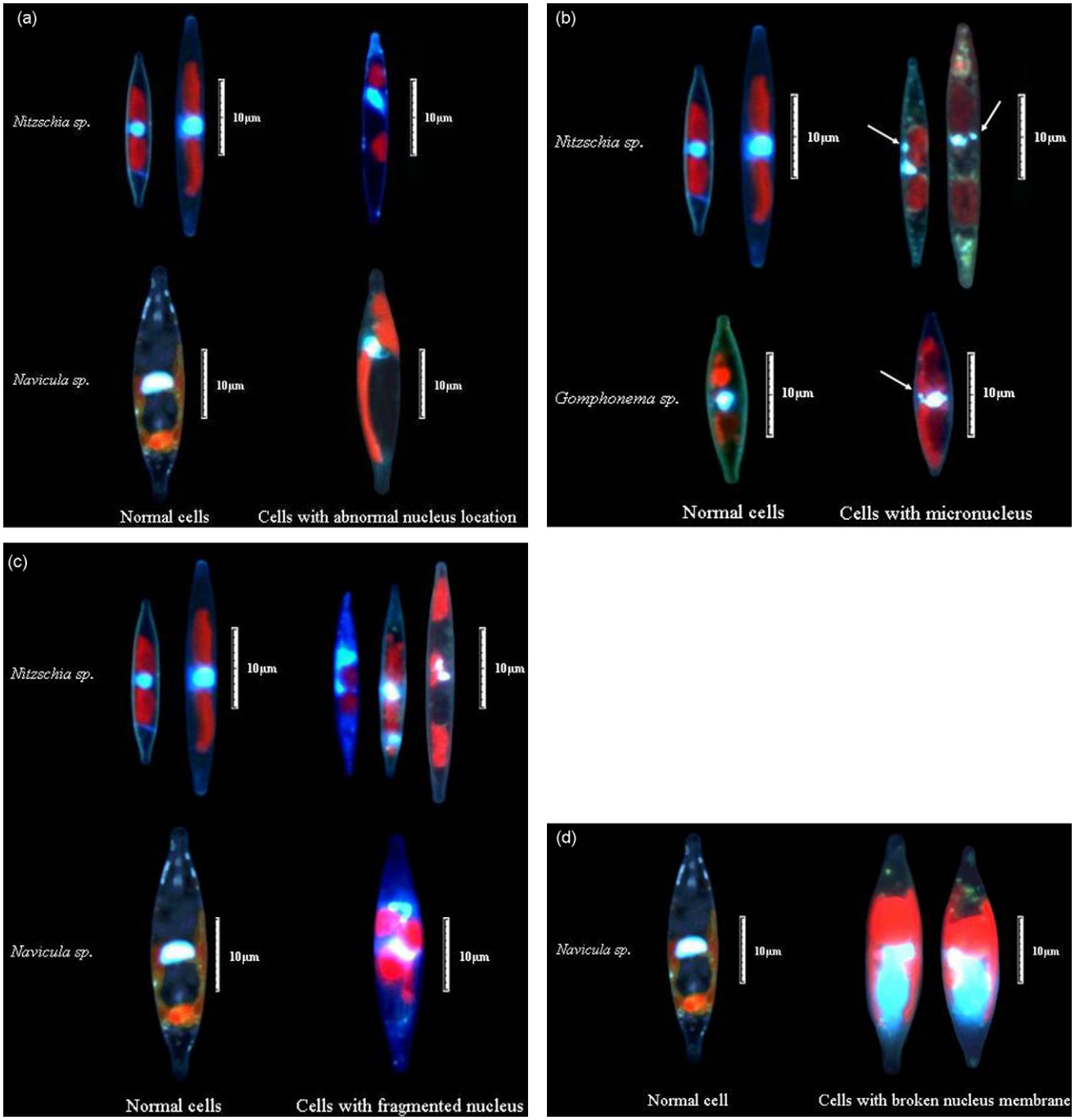

Fig. 2. (a) Cells of different diatom genera (Nitzschia and Navicula) with normal and abnormal nuclear location (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). (b) Cells of different diatom genera (Nitzschia and Gomphonema) without and with micronuclei (arrows) observed under the epifluorescence microscope (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). (c) Cells of different diatom genera (Nitzschia and Navicula) with normal and fragmented nucleus observed under the epifluorescence microscope (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). (d) Diatom cells (Navicula genera) with or without broken nucleus membrane observed under the epifluorescence microscope (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

ification of the nucleus position or to the disturbance of nucleus division during mitosis (Edgar and Pickett-Heaps, 1984; Spurck and Pickett-Heaps, 1994; Rank and Nielsen, 1997). In green algae treated with the pesticide isopropyl *N*-phenyl carbamate (IPC, CAS No. 122-42-9), Coss and Pickett-Heaps (1974) showed that abnormal microtubular spindles were formed after exposure. Micronucleus induction is a well known biomarker of the impact of genotoxic agents on the cells of higher plants like *Allium cepa* L., *Tradescantia* sp. and *Vicia faba* L. (Gichner et al., 1982; Grant et al., 1992; Cabrera and Rodriguez, 1999; Cotelle et al., 1999; Kong and Ma, 1999; Marcano et al., 2004). Micronuclei have never been shown to occur in benthic diatoms. The diatom nucleus fragmentation observed (Fig. 2c) in this experiment may be related to the nuclear alterations noted in *A. cepa* L. exposed to MH and in a green alga *Oedogonium cardiacum* treated with the herbicide IPC (Coss and Pickett-Heaps, 1974; Rank and Nielsen, 1997; Marcano et al., 2004). Micronucleus and nucleus fragmentation can be induced by DNA breaks or by a disturbed microtubular system, inducing abnormal

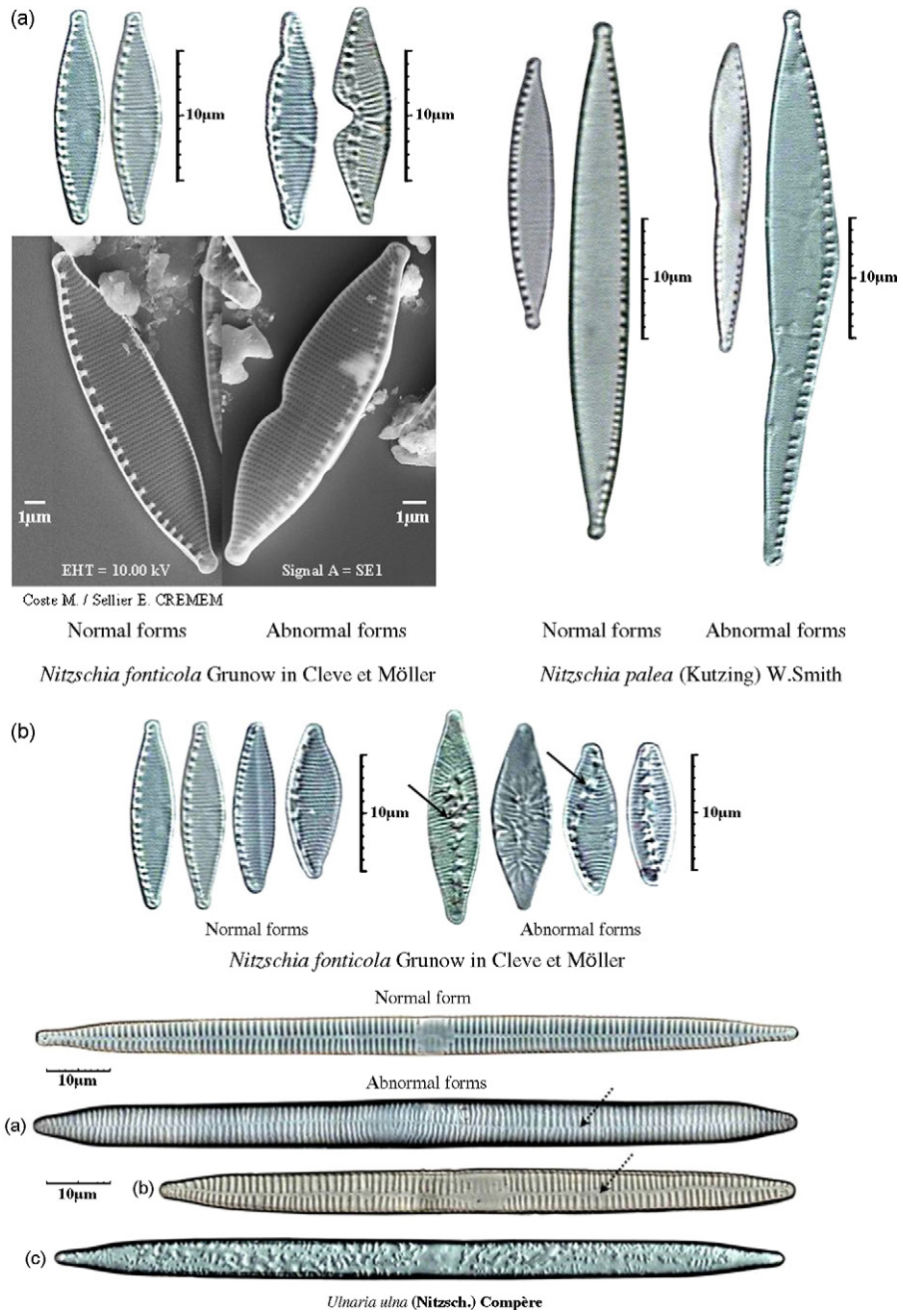

Fig. 3. (a) Light and scanning electron micrographs of abnormal frustule morphologies for two species, *Nitzschia fonticola* Grunow in Cleve et Möller and *Nitzschia palea* (Kutzing) W. Smith, exposed to MH. (b) Light micrograph of abnormal frustule ornamentations for two species, *Nitzschia fonticola* Grunow in Cleve et Möller (abnormal fibula ⟶) and *Ulnaria ulna* (Nitzsch.) Compère (abnormal pseudoraphe ⇢).

migration of the chromosomes during the cell division (Grant et al., 1992; El Hajjouji et al., 2007). The nuclear membrane breakage observed here is a less common effect of MH since it is not reported in studies carried out on higher plants. Coombs et al. (1968) noted abnormalities of the nuclear envelope without membrane breakage for diatoms treated with colchicine (Coombs et al., 1968). Casotti et al. (2005) observed that DNA was completely dispersed in marine diatoms exposed to a polyunsaturated aldehyde (2-trans,4-trans-decadienal) (Casotti et al., 2005). According to these authors, DNA spread could be related to a mechanism similar to apoptosis. For these reasons, nucleus membrane breakage was not considered as a nuclear aberration.

In the communities of diatoms exposed to MH, significantly higher abundances of abnormal frustules occurred (Fig. 3a and b). Abnormal shapes were reported in diatoms exposed to a herbicide (isoproturon) (Schmitt-Jansen and Altenburger, 2005) but the authors did not investigate the intra-cellular alterations. In our case, a positive and significant correlation was observed between the abundances of abnormal frustules and nuclear alterations. As previously suggested by Round et al. (1990), the forming valves could be in close interaction with the nucleus. Numerous studies have reported the development of abnormal frustules among communities exposed to toxic agents such as heavy metals or UVA radiation (Thomas et al., 1980; Fisher et al., 1981; Yang and Duthie, 1993;

Rijstenbil et al., 1994; McFarland et al., 1997; Rijstenbil, 2001; Gold et al., 2003; Gomez and Licursi, 2003; Cattaneo et al., 2004). These agents are known to promote genotoxic effects mediated by the induction of oxidative stress in algae (Palmer et al., 2002; Tripathi et al., 2006; Han et al., 2008). In diatoms, UV irradiation induces thymine dimers at the origin of chromosome bridges and nuclear alterations (Buma et al., 1995, 1996; Holzinger and Lutz, 2006).

Therefore, further intracellular effects might be associated with the induction of nucleus alterations and the development of frustule abnormalities. The formation of abnormal frustules could be related to the effects of MH on the synthesis of the proteins governing the process. Indeed a protein template is involved in the polymerisation of silicilic acid to produce opaline silica (Fisher et al., 1981; Round et al., 1990; McFarland et al., 1997). A disruption in the synthesis of this protein could lead to a lack of silica during diatom cell wall formation and, thus, to the secretion of abnormal frustules. A silica deficiency is suggested by many researchers as a potential explanation for the induction of diatom cell wall abnormalities (Thomas et al., 1980; McFarland et al., 1997). The toxic agent could also interfere with the diatom cytoskeleton (microtubular system and microfilaments). The microtubular system is known to play a central role in the division of the nucleus during the mitosis. It would also manage the migration of certain components for cell wall formation (Pickett-Heaps et al., 1979; Round et al., 1990; Pickett-Heaps, 1991; Lee and Li, 1992; Van Den Hoek et al., 1995). Edgar and Pickett-Heaps (1984) reported that the position of the nucleus and the integrity of the microtubules (MT) are important for the development of the valves. Therefore, disruption of the microtubules could lead to abnormal fragmentation of the nucleus during the cell division and a poor silica supply for the formation of new valves at the end of mitosis. In the case of a green algae exposed to IPC, two functional polar microtubule organisation centres and more than two daughter nuclei were observed at the same time (Coss and Pickett-Heaps, 1974). Likewise, for diatom cells treated with microtubule inhibitors, different authors have reported multinuclear cells and also abnormal frustules (Coombs et al., 1968; Duke and Reimann, 1977; Lee and Li, 1992). In our study, the observations of fragmented nucleus as well as of fragmented chloroplasts (Fig. 2c) support the idea that MH disturbs the diatom cytoskeleton.

This work has described the intracellular effects of a single herbicide on diatom cells. The nature of the cellular mechanisms involved in the formation of the two types of aberration studied needs to be investigated. In addition, other herbicides with different modes of action (inhibition of cell division, photosynthesis, microtubule organisation, . . .) must be tested to evaluate their impacts on cultured diatom cells.

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