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Evidence of a new toxin in the red-tide dinoflagellate *Prorocentrum minimum*

D.Grzebyk[1,3], A.Denardou[2], B.Berland[1,4] and Y.F.Pouchus[2]

[1]*Centre d'Océanographie de Marseille, CNRS UMR 6540 Dimar, Station Marine d'Endoume, rue de la Batterie des Lions, F-13007 Marseille and* [2]*Faculté de Pharmacie, UMR 11 Toxinologie Marine, SMAB - ISOMer, 1 rue G. Veil, BP 1024, F-44035 Nantes Cedex 01, France*

[3]*Present address: Laboratory of Marine Microbiology, Department of Applied Bioscience, Graduate School of Agriculture, Kyoto University, Kyoto 606-01, Japan*

[4]*To whom reprint requests should be addressed*

Abstract. Several clones of *Prorocentrum minimum* were isolated from various French Mediterranean and English Channel sites. Mouse tests performed using methanolic extracts from cultures revealed a neurotoxic activity in four clones. The water-soluble toxin detected worked rapidly, killing mice within a few minutes at high doses. Toxin production in cultures was observed during the phase of decline and appeared to be weak or nil during the growth phase. Toxin production was stimulated by associated bacteria in the culture, but a clone rendered axenic remained able to produce toxins. The potential risks of human poisoning from consumption of shellfish harvested during or after toxic blooms of *P.minimum* are discussed.

Introduction

The bloom-forming dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller (also known as *Exuviaella mariae-lebouriae* Parke and Ballantine and *P.mariae-lebouriae*) has rarely been associated with toxic effects. Firstly, in 1942, 114 people living around a coastal lagoon (Lake Hamana) in Japan died after consuming oysters and clams (*Venerupis semidecussata*); a toxin, venerupin, was isolated in shellfish (Akiba and Hattori, 1949). The source of toxin was attributed to *E.mariae-lebouriae* after finding a relationship between the toxicity of shellfish and an abundance of these dinoflagellates in the seawater (Nakazima, 1965a,b,c, 1968). Venerupin shellfish poisoning (VSP) has peculiar symptoms, including liver damage (Akiba and Hattori, 1949).

In the Obidos Lagoon in Portugal, *P.minimum* (initially identified as *Exuviaella baltica* and later as *Prorocentrum balticum*) was considered to be responsible for several episodes of human poisoning subsequent to the consumption of shellfish. The symptoms were considered to be characteristic of paralytic shellfish poisoning (PSP) (Silva, 1963, 1980; Silva and Sousa, 1981). In Norway, a case of poisoning after consumption of mussels was attributed to *P.minimum* which had bloomed several weeks before in the mussel harvesting area; the symptoms (especially nausea and late gastrointestinal disorders) were similar in their nature and development to those of VSP, but much less dramatic (Tangen, 1980, 1983). Occasionally, the presence of toxins has been investigated at the time of a *P.minimum* bloom. However, Kimor *et al.* (1985) failed to find any toxicity in

Baltic Sea dinoflagellates, and mouse tests revealed only very minor symptoms of toxicity in Black Sea mussels (Moncheva, 1991).

Red tides of *P.minimum* or *E.baltica* (actually *P.minimum*) have also been implicated in various effects on marine animals. Blooms were considered highly toxic since fish and other marine animals died or were forced to flee (Silva, 1980; Rabbani *et al.*, 1990; Tseng *et al.*, 1993). However, in one case deaths were observed 3 days after a reduction in dissolved oxygen (Rabbani *et al.*, 1990) and in both of these cases no study was undertaken to verify the toxicity.

Although many observations cast doubt on the toxicity of *P.minimum*, the possibility needs to be reconsidered with respect to public health, especially since this species often proliferates in shellfish farming areas and its blooms are increasing worldwide (Smayda, 1990). Moreover, a number of years ago, toxicity was detected in mussels during a large bloom of *P.minimum* and *P.micans* in the Sète region on the French Mediterranean coast. The symptoms observed with mouse tests indicated a rapid neurological effect, but it could not be clearly demonstrated that the dinoflagellates were the source of this toxicity (Belin, REPHY network, IFREMER, Nantes, France, personal communication).

The present study reports on the toxicity of extracts of several *P.minimum* clones newly isolated for this topic and on conditions of toxin production in *P.minimum* cultures.

Method

Prorocentrum minimum cultures

Eight *P.minimum* clones isolated from natural environments along French coasts were cultured for toxicity studies. Table I indicates their place of origin and the date of their isolation. The cultures were performed in continuous light at 22°C, generally in batch cultures, but also in semi-continuous mode, using the medium described by Antia and Cheng (1970) without silicates. The semi-continuous cultures were inoculated with two clones, PmB and PmS1, in order to produce toxins in greater quantity and in relatively stable conditions (logarithmic growth phase). Volumes of medium (2–4 l) were inoculated with around one-fifth of the final culture volume using 3- or 4-day old mother cultures in exponential growth. Several series of cultures were inoculated. The cultures were harvested by

Table I. Date and place of isolation of the clones of *P.minimum* isolated in order to test toxicity

Clone	Date of isolation	Zone	Place	Type
PmH	September 1989	English Channel	Le Havre	Harbour
PmS1	June 1989	Mediterranean Sea	Sète	Mussel farming
PmS2	June 1989	Mediterranean Sea	Sète	Mussel farming
PmS3	June 1989	Mediterranean Sea	Sète	Mussel farming
PmB	February 1990	Mediterranean Sea	Berre Lagoon	Marine lagoon
PmC1	March 1990	Mediterranean Sea	Gulf of Fos	Mussel farming
PmC2	March 1991	Mediterranean Sea	Gulf of Fos	Mussel farming
PmC3	March 1991	Mediterranean Sea	Gulf of Fos	Mussel farming

filtration on glass fiber filters (Whatman GF/A), 10–11 days after seeding, providing a sufficiently long period for achievement of growth.

Growth was measured by microscopic enumeration using a Neubauer hemocytometer. At least 400 algal cells were counted per sample, to a precision of ±10% (Lund *et al.*, 1958).

In order to study the influence of culture age on toxicity, cultures of PmS1 and PmB were half-harvested using 8.0 μm cellulose ester filters twice during their development: the first half-culture was harvested during (PmS1) or at the end (PmB) of the growth phase, the second half-culture was harvested after 3–4 days in the senescent phase (see Figure 1). After completing 8.0 μm filtration of PmS1 and PmB senescent cultures, liquid filtrates were refiltered on 0.22 μm cellulose ester filters in order to harvest bacteria. Toxicity was tested in these <8.0 μm fractions to ascertain whether toxicity could be related to associated bacteria in the cultures.

As a complementary approach in studying the influence of associated bacteria in toxin production in *P.minimum* cultures, axenic cultures were also prepared. The first time, axenization was performed using a mixture (1:1 by weight) of two antibiotics, framycetin and amoxillin, according to a protocol similar to that of Berland *et al.* (1972). A series of culture tubes containing a concentration ranging from 500 to 20 μg ml^{-1} of each antibiotic was used (i.e. total antibiotic concentration of 1000–40 μg ml^{-1}). Exposure times to antibiotics ranged from half a day to 7 days. Tests for bacterial contamination were performed in an algal liquid medium enriched with organic substrates (1 l contained 0.5 g peptone, 0.5 g yeast extract, 50 mg glucose, 25 mg glycine) and PO_4^{3-} (250 μmol l^{-1}). Tubes (5 ml) of the bacterial medium were inoculated with 1 ml of the algal culture to be tested

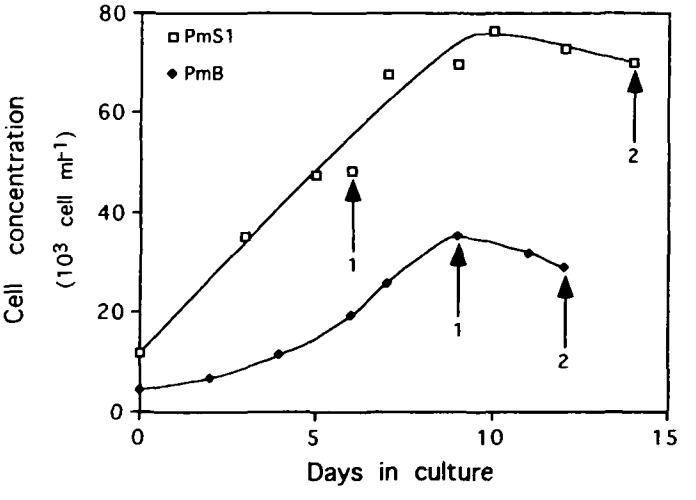

Fig. 1. Growth of two cultures of PmS1 and PmB, with two different times (1 and 2) of cell harvesting in order to test variation of cell toxicity with age of culture.

and were kept in the dark for at least a month at room temperature. Algal cultures were considered axenic if no bacterial or fungal growth appeared in three successive subcultures in a medium without antibiotics. The PmS1 subclone chosen (PmS1ax) was the one rendered axenic after the shortest exposure time at the lowest successful total antibiotic concentration of 600 μg ml^{-1}. During experiments, *Prorocentrum* cultures were systematically checked for axeny by cultural tests and direct microscope observations. The framycetin–amoxillin treatment did not render PmB axenic, bacterial flora proved resistant. However, a subclone (PmB342) was chosen to be tested for toxicity; although not bacteria free, it had a reduced bacterial contamination after antibiotic treatment.

A second antibiotic treatment, a mixture of 200 μg ml^{-1} cefotaxime and 100 μg ml^{-1} vancomycin, considered to provide a wide range of antibiotic effectiveness (Kooistra *et al.*, 1991), was applied but without success. After this treatment, two non-axenic subclones were tested for toxicity: PmB112 obtained after a 24 h exposure to the treatment and PmB131 obtained after a 1 week exposure.

Extractions

The different types of extracts studied were prepared according to the protocol shown in Figure 2. Methanolic extracts were obtained from cultures filtered on glass fiber (Whatman GF/A) or cellulose ester (Millipore) filters used for differential filtrations (8 and 0.22 μm porosities). With glass fiber filters, which retain more water, the first extraction was performed with absolute methanol, followed by two others with 80% aqueous methanol. With cellulose ester filters, the three extractions were completed with 80% methanol. Filters in aqueous methanol were broken up in glass tubes using a glass stick. The methanolic extracts obtained were evaporated to dryness.

To study the degree of hydrophilia of the toxic substance, the extract was dissolved in pure water and extracted with diethyl ether. The two fractions obtained were evaporated to dryness for testing purposes.

Acute toxicity test with mice

The dry extracts were redissolved in a 1% Tween 60 solution and injected intraperitoneally into Swiss male mice weighing ~20 g. Acute toxicity was characterized by the observed symptoms and the survival time of the mice. The quantities injected were expressed as the equivalent number of algal cells injected per gram of mouse (cells g^{-1} mouse).

High-pressure liquid chromatography analyses

To look for the presence of identified dinoflagellate toxins in different extracts toxic to mouse, high-pressure liquid chromatography (HPLC) analyses were carried out. Analyses of diarrhetic shellfish poisoning (DSP) toxins were performed according to Lee *et al.* (1987). Analyses of paralytic shellfish poisoning (PSP) PSP toxins were performed according to Oshima *et al.* (1984) and Oshima (1989).

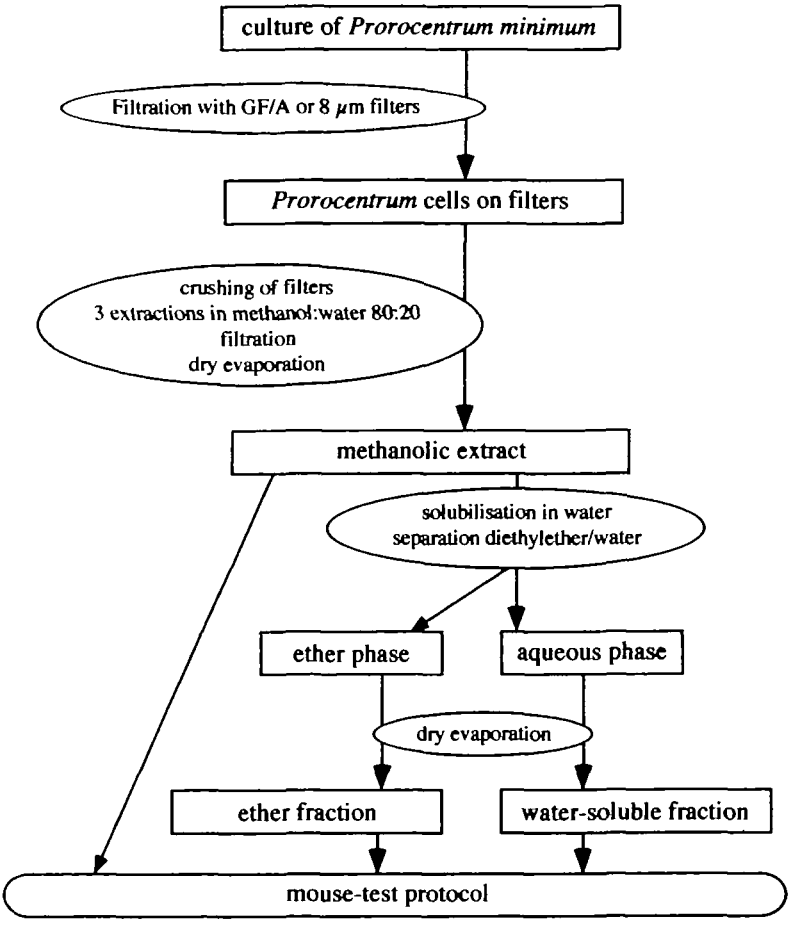

Fig. 2. Extraction method of *P.minimum* toxin from cultures, and diethyl ether–water partitioning for the study of the degree of hydrophilia.

Hepatotoxicity test with hepatocyte cultures

Hepatocyte cultures are widely used to detect hepatotoxic effects of substances and have been used in studies of algal toxins (Aune, 1989). We carried out a hepatotoxicity test with hepatocyte cultures because hepatotoxic effects have been described during the previous VSP intoxications or in experimental studies with *P.minimum* extracts (Akiba and Hattori, 1949; Okaichi and Imatomi, 1979). Toxic extracts (after mouse test) from the different *P.minimum* cultures were placed in contact with cellular suspensions of rat hepatocytes prepared according to the methods of Tore and Kjetil (1986) and Guquen-Guillouzo and Gripon (1988). The toxic extracts were tested at concentrations ranging from 0.001 to 1 mg ml^{-1} of hepatocyte culture. Each concentration was tested on four different hepatocyte cultures during a 20 h incubation period.

Results

Screening for toxic clones

Four clones failed to produce the slightest symptom of toxicity, whereas the other four proved to be extremely toxic (Table II). The symptoms in intoxicated mice were similar for the four toxic clones. Within 1–8 min after the injection of lethal doses of the toxin extract, the mice experienced convulsions, with contraction of the hind paws sometimes accompanied by muscle contractions in the trunk and neck. Death occurred within 3–20 min, depending on the quantity of toxin injected. In a few cases, death was delayed. These mice experienced several convulsions and then became rapidly and deeply asthenic, with occasional spasms in the hind paws or the paralyzed hindquarters. Death occurred later, within 3–15 h after injection.

For sublethal doses, mice experienced several convulsions a few minutes after injection, then became rapidly asthenic and entered into a coma phase. They recovered progressively within 5–24 h. This symptom suggests neurotoxicity.

No DSP or PSP toxins were detected in toxic extracts using HPLC analyses carried out with extract of PmB (sublethal toxicity at 97.4 mg injected in mice), PmS1 (sublethal toxicity at 100.0 mg injected in mice) and PmS1ax (sublethal toxicity at 105.0 mg injected in mice).

The level of toxicity was related to the relative quantity of injected toxic extract (Table III). Injections of lower quantities lengthened survival time, and levels of sublethal toxicity were readily reached.

Conditions of toxin production: influence of growth stage or of culture age on toxicity

Semi-continuous cultures were inoculated with two toxic clones: PmB and PmS1. In these cultures, the growth was always in exponential or linear phase. With PmB, a mouse test was performed using an extract obtained from the sampling of several semi-continuous cultures (around one-tenth of the total culture volume harvested). The injected quantities represented a number of cells estimated at 2.6–3.0 $\times$ 10^6 cells g^{-1} mouse. This extract induced delayed death 15 h after

Table II. Results of toxicity screening with mouse tests on the clones of *P.minimum* with, for the toxic clones, the minimum lethal dose injected in mice (10^6 cells g^{-1} mouse). The name of mouse tests plotted in Figure 3 is given in parentheses

Clone	Cells injected in mice ($\times$ 10^6 g^{-1})	Toxicity
PmH	3.7	No toxic symptom
PmC1	1.2	No toxic symptom
PmC2	0.9	No toxic symptom
PmC3	0.35	No toxic symptom
PmS1	0.85	Death at 8 min (PmS1.b)
PmS2	1.19	Death at 6 min (PmS2)
PmS3	1.38	Death at 4 min 30 (PmS3ce)
PmB	0.35	Death at 10 min (PmB.a2˜)

Table III. Relationship between quantity of extract (volume of extract or number of cells of *P.minimum*) injected into mouse and toxicity, with time of survival. The name of mouse tests plotted in Figure 3 is given in parentheses

Clone	Extract	Cells injected in mice ($\times 10^6$ g^{-1})	Toxicity
PmB	ce	0.71	Death at 8 min (PmB.a2′)
		0.35	Death at 10 min (PmB.a2″)
		0.14	Sublethal toxicity
PmS1	ce	1.70	Death at 5 min (PmS1.a′)
		0.20	Death at 6 min (PmS1.a″)
PmS2	ce	1.19	Death at 6 min (PmS2)
		0.69	Sublethal toxicity
PmS3	ce	1.38	Death at 4 min 30 (PmS3ce)
		0.76	Sublethal toxicity
	wse	2.17	Death at 3 min (PmS3wse)
		0.54	Sublethal toxicity

ce, crude extract; wse, water-soluble extract.

injection, whereas a similar quantity of cells had led to rapid death during the previous screening tests. With PmS1, mouse tests performed with similar quantities of injected cells produced only sublethal intoxications.

After checking up this lower toxicity in these semi-continuous cultures never containing senescent cells, we studied the influence of the growth stage on toxicity in batch culture mode. Figure 1 shows the growth curves of PmS1 and PmB performed for this study at both times of harvesting. For the two clones, toxicity in *P.minimum* cells after 3–4 days in the phase of decline was much higher than that in cells harvested during or at the end of the growth phase (Table IV).

Assumed origin of the toxin

Toxicity in particles <8 μm. This test was performed to determine whether toxic particles remained in the liquid after filtration of algal cells, resulting mainly from bacteria but also from algal fragments after senescent cell lysis. The extract of liquid filtrate (~0.75 l previously containing 52×10^6 cells) of the PmS1 senescent culture pre-filtered on 8 μm (proved highly toxic; Table IV) produced a sublethal toxicity with the characteristic immediate symptoms followed by a period of asthenia lasting several hours (>3 h) before complete recovery 8 h after injection. The extract of ~1.0 l filtrate (previously containing 28×10^6 cells) from the senescent PmB toxic culture (Table IV) pre-filtered on 8 μm caused no particular symptoms after injection.

Toxicity in algal cells after antibiotic treatments. The toxicities of four different PmS1ax cultures were tested. No characteristic symptoms of toxicity were noticed in three cases: two 3 week cultures and a 7 week culture. However, with the fourth culture, a quantity equivalent to 1.8 l of culture (not cell count) produced the characteristic symptoms (convulsions 3 min after injection) and induced death within 6 min.

Table IV. Variation in toxicity (using mouse tests), in relation to the growth stage, in the two *P.minimum* toxic clones PmS1 and PmB. See Figure 2 for growth curves. The name of mouse tests plotted in Figure 3 is given in parentheses

Clone	Sample	Growth stage 10^3 cell ml^{-1} in culture	Cells injected in mice ($\times 10^6$ g^{-1} mouse)	Toxicity
PmS1	Sample 1	Growth phase 48.5	1.5	Sublethal toxicity (asthenia)
(PmS1.a')	Sample 2	Phase of decline for 4 days 70.0	1.2	Death at 6 min
PmB (PmB.a1)	Sample 1	End of growth phase 35.2	1.5	Death at 17 min
(PmB.a2')	Sample 2	Phase of decline for 3 days 28.8	0.71	Death at 8 min

Two non-axenic (but with reduced bacterial flora) subclones, PmB342 and PmB112, were tested twice. No toxic symptoms were apparent with a 3 week culture. With the 7 week cultures, mouse death occurred very quickly: in 3.5–4 min with 1.74×10^6 cells g^{-1} mouse for PmB342 and with 2.58×10^6 cells g^{-1} mouse for PmB112. The last non-axenic subclone PmB131 was tested with a 7 week culture, and death occurred only after several hours (>3 h) with 2×10^6 cells g^{-1} mouse.

Toxin polarity

The affinity of the toxic fraction of crude methanolic extracts was determined for a 6 week PmB culture and a 5 week PmS3 culture. In both cases, toxicity was found in the water-soluble fraction and showed the same characteristic symptoms as with crude extracts. With this fraction, death occurred in 3 min for PmS3 with 2.17×10^6 cells g^{-1} mouse (1.33 l of culture) and in 7.5 min for PmB (1.25 l of culture, no cell count). In both cases, injection of the ether-soluble fraction produced no symptoms.

Comparison of clonal toxicities

Our results for acute toxicity with all clones and subclones indicate an inverse relationship between the number of *P.minimum*-equivalent cells injected per gram of mouse and survival time before death (Figure 3). Most of the tests reported here were performed with cultures in senescence when the toxin concentration per cell was maximal. This relationship suggests that these maximal concentrations were comparable in the four clones. Only two results differed from this tendency. The test referred to as PmB.a1 corresponded to the half-culture harvested at the beginning of the stationary phase (Figure 1). The other half-culture harvested 3 days later in the phase of decline yielded the PmB.a2 tests. For the PmB.b test, the culture may not yet have entered into the phase of decline for a sufficiently long time (the growth curve was not checked before harvesting the culture).

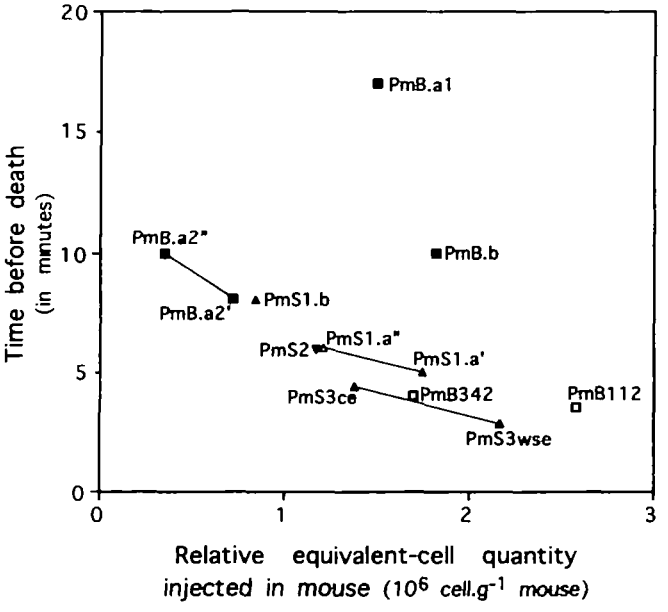

Fig. 3. Relationship between *P.minimum*-equivalent cell quantities injected in mice and the time of survival of the mice, for test showing high toxicity (fast mortality). Plotted here are the results given in this study for the different clones (PmB, PmS1, PmS2, PmS3) and subclones (PmB342 and PmB112 obtained after antibiotic treatments) except for the test named PmB.b. All plotted tests were carried out with crude extracts, except PmS3wse (water-soluble extract).

Hepatotoxicity test with hepatocyte cultures

Two toxic extracts of different PmB cultures were tested. Quantities of extracts injected in 20 g mice to check the toxicity level, 97.4 and 72.8 mg, respectively, provoked sublethal toxicity symptoms before recovering within 24 h. No hepatotoxic effects were observed with the different extract concentrations incubated with hepatocyte suspensions, even with the maximal concentration of 1 mg of extract per milliliter of hepatocyte culture.

Discussion

Toxicity and potential risks of human poisoning

The existence of toxic as well as non-toxic clones of *P.minimum* accounts for the different observations concerning the toxicity or non-toxicity of blooms of this species. The four clones which proved toxic in this study came from two French Mediterranean sites ~150 km apart: the Berre Lagoon and coastal waters near Sète. Toxin production appeared as a common characteristic of the three clones isolated in the same bloom from the Sète area.

The new form of *P.minimum* toxicity produced neurotoxic symptoms which appeared rapidly in the mice, killing them within a few minutes when a sufficient dose was injected. However, this violent effect changed markedly when the doses

were reduced, becoming sublethal. This factor could account for underestimations of the toxicity of *P.minimum* blooms in the past. The symptoms differed from those of PSP and their rapid effect distinguished them from those of DSP, while the main toxins associated with these toxicities were not detected in HPLC analyses. In addition, the rapidity of death in tested mice (within a few minutes) and the nature of the symptoms are not compatible with hepatotoxic effects, unlike the observations described in the experimental work of Okaichi and Imatomi (1979) or those of the VSP poisonings observed in the Lake Hamana event (Akiba and Hattori, 1949). Moreover, the inactivity of the PmB toxic extracts *in vitro* on hepatocytes with high concentrations confirms the absence of hepatotoxic substances. However, the water-soluble nature of PmB and PmS3 toxins is in agreement with previous results obtained in *P.minimum* culture (Okaichi and Imatomi, 1979; Silva and Sousa, 1981) or with *Venerupis* shellfish (Akiba and Hattori, 1949). This confirms the difference with DSP toxins such as okadaic acid which have intermediate polarity. Thus, extraction of the toxin would appear to be better with methanol than acetone. This could account for the low toxicity observed by Moncheva (1991) with acetone extracts, although there is no evidence that the toxin involved in Moncheva's results was the same as the one described here.

Now we have no data to say whether this new toxin could be transmitted in marine food chains (mainly through shellfish) and then if the toxin remains active after ingestion, how it could affect human consumers. Nevertheless, the potential risk of toxicity has to be taken into account and we have to consider whether the toxicity levels measured here experimentally in different clones of *P.minimum* are consistent with observations made in shellfish during *P.minimum* blooms. During an *Exuviaella cordata* bloom (probably *P.minimum* according to Marasovic *et al.*, 1990), with cell concentrations of 10.9×10^6 to 418×10^6 cells l^{-1}, the intestinal algal content of mussels reached $1–2 \times 10^6$ cells with up to 89% of *E.cordata* (Moncheva, 1991). In these bloom conditions, toxicity in shellfish might be detectable when using the mouse test to monitor shellfish safety. Akiba and Hattori (1949) showed that after transplantation from a VSP-safe area into a VSP-contaminated area, clams became toxic in 10 days. In addition, some experimental work studied the ingestion and assimilation of toxic dinoflagellates and the subsequent accumulation of their toxins by shellfish. These data, obtained especially with the *Alexandrium* genus, indicate the rapid accumulation of water-soluble paralytic toxins in shellfish tissues, reaching within a few days the critical level requiring the discontinuation of harvesting (Lassus *et al.*, 1989, 1992; Bricelj *et al.*, 1990, 1991). However, the problem of the ingestion and real assimilation of dinoflagellates, and thus of the bioaccumulation of toxins, is raised from experimental studies showing that cells filtered by shellfish can be rejected directly in pseudofeces or can be found undigested in feces. This was shown to be the case with *P.minimum* (Cucci *et al.*, 1985; Shumway *et al.*, 1985; Shumway and Cucci, 1987).

Conditions controlling toxin production

The toxicity of *P.minimum* appears to be characteristic of the cell itself since the axenic PmS1ax clone remained toxic, producing symptoms identical to those

induced by the unpurified strain. The relationship between the number of injected cells and the period of lethality (Figure 3) indicates intracellular production and storage of the toxin, and that the maximal concentration in cell (in the phase of decline) seems constant. The slight toxicity in particles measuring 0.2–8 μm could not have been due to bacteria present in the medium and producing the toxin, but to algal fragments from cell lysates. Unlike Kodama *et al.* (1988, 1989, 1990) in their work concerning *Alexandrium tamarense*, toxin does not appear to be produced by intracellular bacteria. We did not observe bacteria in the cytoplasm or the nucleus in the two toxic clones (PmB and PmS1) in almost 100 sections studied under a transmission electron microscope (unpublished results).

The cell density is not an adequate parameter to estimate potential toxin quantity in *P.minimum* because toxin production was not the same at different stages of the culture. The results obtained with PmB and PmS1 show that toxin production was low or non-existent during the growth phase. Toxin production occurred at the end of the growth phase or during the declining phase when the medium is limited in nutritive elements. During this phase, cells settle in the culture vessel, some of them are lysed or can form temporary cysts (Grzebyk and Berland, 1996). These old cultures are viscous, indicating excretion of polysaccharide compounds. Moreover, other *P.minimum* clones are already known to be able to produce two other types of secondary metabolites massively secreted in culture 1–3 days after the beginning of the stationary phase: the siderophore prorocentrin (Trick *et al.*, 1983a,b) and the norcarotenoid β-diketone with antibiotic activity (Andersen *et al.*, 1980; Trick *et al.*, 1981, 1984).

The two antibiotic treatments applied to the PmB clone were partly effective and contributed to modifying the expression of toxicity, implying a slow or decreasing toxin production. In PmB342 and PmB112, toxicity was found in very old cultures, and later than with the unpurified PmB clone. With PmB131, the lengthening of culture time did not lead to an increased toxicity in cells. The presence of associated bacteria in the culture could play a direct role in stimulating toxin production in dinoflagellates. Through the antibiotic treatments performed on PmB, a progressive selection of bacterial strains was achieved. A modification of toxin production (intervals before expression, yield level) apparently resulted from this selective process, although the effect on algal cell production was stimulated more by certain bacterial strains than by the total bacterial population. We also hypothesized that these peculiar bacterial strains grow mainly at the end of the *P.minimum* growth phase using organics from algal exudation or cell lysates as complex substrates (Bell *et al.*, 1974; Fukami *et al.*, 1983; Painchaud and Therriault, 1989; Amon and Benner, 1994, 1996; Nakano, 1996). This might explain the decrease in toxicity observed in semi-continuous cultures frequently diluted: in addition to the fact that the senescent phase was never reached, the use of young cultures to inoculate new media could have diluted this peculiar bacterial population (unable to develop in young algal cultures).

The association of *P.minimum* with bacteria seems similar to those observed with the dinoflagellate *Ostreopsis lenticularis* and the diatom *Pseudonitzschia pungens* f. *multiseries*. With *O.lenticularis*, toxin production increases in old

cultures at the beginning of the phase of decline, related to the increased number of associated bacteria, particularly in the genus *Pseudomonas*, bound to the algal cells (Tosteson *et al.*, 1986, 1989; Gonzalez *et al.*, 1995). With *P.pungens* f. *multiseries*, the production of neurotoxic domoic acid is maximal in the stationary phase; non-axenic strains, like the axenic strains after the reintroduction of bacterial strains, also produce greater quantities of toxins per cell than axenic cultures (Douglas and Bates, 1992; Douglas *et al.*, 1993; Bates *et al.*, 1995).

In conclusion, with respect to the divergent observations about variable toxicity of *P.minimum* blooms, our results suggest the additional, complex conditions needed for the development of toxicity in *P.minimum* blooms: (i) clones able to produce toxins; (ii) particular associated bacterial strains which stimulate toxin production in senescent *P.minimum* cells; (iii) stable conditions in the environment, long enough in duration for the dinoflagellate bloom to become senescent, allowing the development of the bacteria stimulating toxin production.

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