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TOXIC PHYTOPLANKTON BLOOMS IN THE SEA

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Edited by

THEODORE J. SMAYDA
Graduate School of Oceanography
University of Rhode Island
Narragansett, RI 02882-1197
U.S.A.

and

YUZURU SHIMIZU
Department of Pharmacognosy
College of Pharmacy
University of Rhode Island
Kingston, RI 02881-0809
U.S.A

at 2800 lux and 25°C in a Percival (model WE-1012) environmental chamber.

A 2.5 L Fernbach flask containing 1 L of medium was used as an inoculum for an acclimation series of 10 L carboy cultures. Cells were acclimated to the larger carboy vessel by progressively increasing from 1 carboy to 2, 4 and finally 16 carboys. This was accomplished by removing a 0.5 L aliquot·carboy^{-1} from mid log phase cultures, averaging 15,000 cells·mL^{-1}, as inocula for the subsequent carboy cultures. Thus, starting culture densities were kept consistently around 700 cells·mL^{-1}. A culture was considered "acclimated" to its environment when three successive reproduction rates were similar [14, 15]. This process was necessary to assure uniformity in cellular fitness and potency among large-scale batch cultures.

Growth was monitored at 3 to 4 day intervals by aseptic removal of a 10 mL subsample. Subsamples were placed into 20 mm screw-cap test tubes, gently vortexed and the whole sample was read spectrophotometrically at 670 nm [16]. In addition, the sample was fixed with a 1% Lugol's iodine solution and cell counts (cells·mL^{-1}) were made with a 0.1 mL Palmer-Maloney counting chamber in triplicate. Growth curves and reproduction rates were calculated as described by Guillard [16].

Harvest and Extraction. Harvest times were normalized at 8 h into the light cycle to lessen cellular variation between harvests. An aliquot of each of the 16 carboys were removed on 3 to 4 day intervals, starting with day 0. Cells were harvested with a 20 µm plankton net and further concentrated by cold (5°C) centrifugation. The final rinse and centrifugation of the cell pellet was carried out in a pre-weighed vial with DDIW to remove excess salts. The DDIW was carefully decanted and the cell pellet immediately frozen at -20°C and lyophylized for 48 h.

Once dry, separate pre-weighed samples were extracted for pigment [17], toxin (see below), total lipid [17], protein [18] and carbohydrate assays [19]. Cell samples were disrupted using a Branson J-17A sonicator fitted with a 4 mm needle probe. Each sample was subjected to a series of three 1 min sonication bursts in 5 or 10 mL of an appropriate solvent (see below). After each burst, cell debris was pelleted by cold centrifugation and the supernatant carefully decanted. Fresh solvent was added before the next sonication. Extraction solvents (all HPLC grade) for each of the biochemical constituents were as follows: (1) lipids - 2:1 v/v chloroform methanol, (2) protein and carbohydrates - 1.0 N NaOH and (3) pigment and toxins - 100 % methanol. Sample dry weights were standardized to 50 mg for lipid, 20 mg for protein and carbohydrate, and 100 mg for pigment and toxin assays.

Toxins. All methanol extracts were filtered (0.22 µm) and brought to 30 mL total volume with fresh 100% methanol. Variation in potency was assessed by two assay procedures. First, a whole-animal mouse bioassay was employed to determine the LD_{50} [20] of FAT. Female Harlan Sprague Dawley ICR(BR) mice weighing 18-22 g were used. At least four geometrically increasing doses were administered to a minimum of two mice (i.p.), followed by a 2 h observation period. Each dose was prepared by drying a known weight of crude methanol extract under nitrogen and resuspending the sample in 0.5 mL of 0.15 M NaCl containing 1% Tween 60. Final values were expressed in mouse units (MU =LD_{50} dose for a 20 g mouse). Control mice were injected with the carrier solution only. Since FAT concentration and mouse toxicity far exceeded that of OA in crude extracts, a second analytical immunoassay (DSP Check, UBE Ind., Japan) was utilized to determine OA content of the same crude methanol extracts. A 1.0 mL aliquot of each of the methanol extracts was removed, taken to dryness under nitrogen and resuspended with 3.0 mL of 45% aqueous methanol. Subsequently, at least three 50 µl aliquots of the methanol extracts were subjected to a competitive enzyme-linked immunosorbent assay (ELISA) specific for the polyether OA [21]. Pure OA was provided with the test kit as a standard.

Control Experiments. Since the culture conditions employed in this experiment were not axenic, it was necessary to assess the possibility of bacterial toxin production. Subsamples from log phase cultures (10 mL) were utilized to inoculate three liquid bacterial media containing different organic loads. The three media included Difco's marine broth 2216 medium; 0.1% bacto peptone with 0.1% glucose; and 1% bacto peptone plus 0.1% glucose. Each medium was prepared aseptically in a 2.5 L Fernbach flask with 1 L natural seawater base and h/2 enrichments. All three cultures were completely covered with foil and incubated in the same environmental chamber. These cultures were harvested by cold centrifugation after a 30 day incubation period. Methanol extracts of 100 mg samples and mouse bioassays were conducted as above.

RESULTS AND DISCUSSION

Growth and Nutrient Uptake. Densities of *P. hoffmannianum* measured over the entire growth cycle ranged from 700 to 55,000 cells·mL^{-1} (Fig. 2). Growth during the log phase was 0.35 div·day^{-1}. pH in the culture medium increased significantly at the end of log phase (pH = 9.6) and leveled off for the duration of stationary phase (Fig. 2). Thus, it was possible that high pH became growth-inhibiting; however, some reports [22, 23] suggest the lack of available CO_2 in dense cultures is probably the most limiting factor.

Ammonium and phosphate levels were significantly reduced by early log phase (data not shown) but were not depleted or limiting, since logarithmic growth continued long after measurable amounts of these nutrients had been removed from the growth medium. NH_4^+ uptake was much more

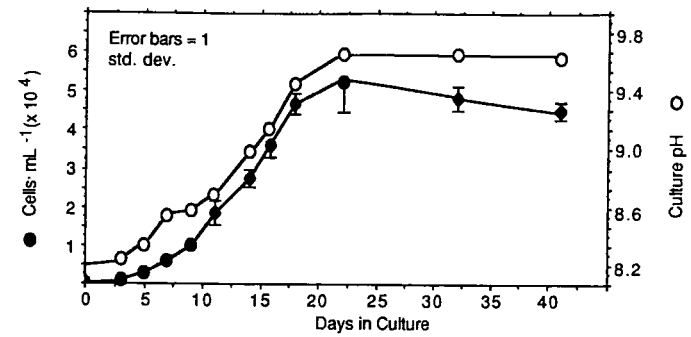

FIG. 2. Cell densities and pH of P. hoffmannianum cultures over one complete growth cycle.

rapid than both PO_4^{3-} and NO_3^- uptake. Nevertheless, a pulse of NH_4^+ was detected in late log phase (day 22), a phenomenon consistently found in *P. hoffmannianum* cultures, as well as other benthic dinoflagellate cultures in our lab (unpubl. data). Concurrent with the NH_4^+ pulse, cellular protein levels decreased (see Fig. 3A) during the time of maximal culture pH (Fig. 2). The pulse was probably the result of proteolysis or cell death; however, the additional NH_4^+ was rapidly assimilated by the remaining cells. PO_4^{3-} levels became undetectable after about 25 days for the duration of the experiment. If the NH_4^+ pulse was the result of cell lysis, then a concurrent pulse of PO_4^{3-} would be expected, but was not detected. This may be the result of a physical removal of the high PO_4^{3-} source from the culture medium, such as cellular membrane debris, during filtration prior to the final analysis. Phosphorus was probably the most limiting micronutrient, given the large surplus of NO_3^- still available in late log and stationary phases. Nitrate in the culture medium remained relatively unutilized until the late log phase, when it decreased from about 55 to 46 ppm NO_3^- (data not shown). The preferential assimilation of NH_4^+ over NO_3^- is highly variable among algae [see 24] but appears to be common among epiphytic/benthic dinoflagellates.

Chemical Composition. Total soluble proteins, lipids and reducible sugars (carbohydrates) were measured to assess the physiological condition of the cells over the complete growth cycle (Fig. 3). Cellular protein showed the greatest variability with two distinct peaks during the 41 day experiment. Protein levels increased from 1400 pg·cell^{-1} at day zero to ~2000 pg·cell^{-1} after 5 days in culture, but decreased to initial levels after 12 days. Thus, the initial accumulation of protein during lag phase was followed by a significant reduction during early log phase. This suggests that protein synthesis

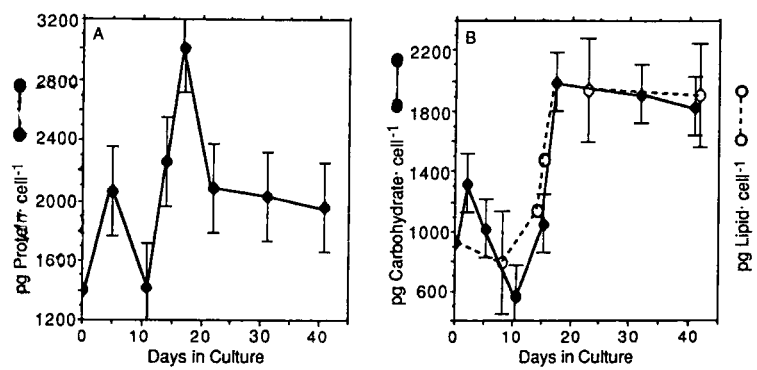

FIG. 3. Variation in total protein (A), carbohydrate and lipid (B) during culture development. Error bars were generated by the T-Method for comparison of means at a 95% confidence interval. Means whose intervals do not overlap are significantly different [25].

exceeded protein degradation during lag phase and the inverse occurred during early log phase. However, as the cultures progressed into mid log phase, protein levels increased significantly to a maximal level of 3000 pg·cell^{-1} at 16 days. This was followed by a significant reduction in protein levels (< 2100 pg·cell^{-1}) during late log and early stationary phase at 22 days. Anderson et al. [23] reported a similar convex-shaped pattern of protein synthesis in batch-cultures of the PSP dinoflagellate *Alexandrium fundyense*. However, they reported a range of cellular protein levels of 0 pg·cell^{-1} to about 7000 pg·cell^{-1} with maximal protein levels occurring in mid log phase.

The pattern of synthesis for the other primary metabolites closely resembled that of protein over the entire growth cycle. However, once maximal levels of 2000 pg carbohydrate·cell^{-1} and 1990 pg lipid·cell^{-1} were attained, no significant variation was observed for the remainder of late log and stationary phase (Fig. 3B). The cellular content of chlorophyll a and peridinin also closely paralleled the pattern of protein, with maximal levels of 65 and 40 pg·cell^{-1}, respectively, during mid log phase followed by a transient drop of both in early stationary phase (Fig. 4). Chlorophyll c_2 was more persistent, remaining at mid log phase levels (9 pg·cell^{-1}) until late stationary phase. The maintenance of mid log phase levels of chlorophyll c_2 in stationary phase may indicate a possible role for chlorophyll c_2 in the survival or protection of *P. hoffmannianum* cells under unfavorable conditions (i.e. nutrient stress, high pH, low dissolved CO_2 and O_2, or shading). Furthermore, during the latter stages of culture growth we also found maximal levels of carbohydrate and lipid (Fig. 3B), suggesting the beginning of encystment. Regardless, no cyst-like structures were detected, although many empty theca (ca. 7,000·mL^{-1}) were present in all cultures.

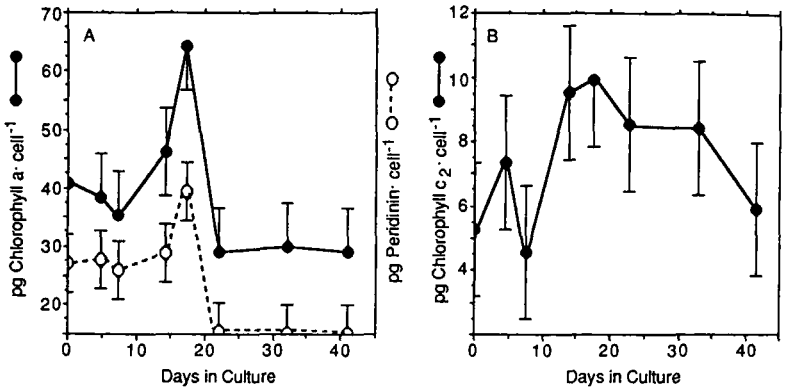

FIG. 4. Cellular content of (A) chlorophyll a, chlorophyll c_2 and (B) peridinin in *P. hoffmannianum* during one complete growth cycle. Error bars same as in Figure 3.

Toxins. FAT is a potent water soluble toxin named for its unique effects on mice. Mice injected with a median lethal dose (LD_{50}) died within 40 min or completely recovered [5]. In addition, Tindall & Miller [26] have reported an antagonistic effect of FAT on isolated guinea pig ileum preps, similar to maitotoxin from *G. toxicus* and suggested a molecular weight less than 1000 d. Unfortunately, the chemical structure of FAT is still unknown. OA is a polyether derivative of a C_{38} monocarboxylic fatty acid with a molecular formula $C_{44}H_{66}O_{13}$ [27]. OA is a lipid soluble toxin most commonly associated with gastrointestinal disorders (diarrhetic shellfish poisoning), rarely causing death. However, OA has been reported as a powerful inhibitor of type 1 and 2 protein phosphatases [28], as a potent non-phorbol ester-type tumor promoter on mouse skin [29], as well as many other unique biomedical activities [30].

Control Experiments. Crude methanol extracts of bacteria were non-toxic at doses 10x greater (> 2 mg·mouse^{-1}) than the dinoflagellate extracts for all test media. These data are supported by the study of Bomber et al. [31] in which crude extracts obtained from bacteria cultured from xenic cultures of *G. toxicus* failed to kill mice at doses well above 1 mg. Other studies have shown that axenic cultures of *P. concavum* and *G. toxicus* are capable of toxin production [31, 32]. Furthermore, toxic clones of *G. toxicus* have been examined with transmission electron microscopy and no endosymbiotic bacteria were detected [33]. Therefore, we conclude that toxin production in *P. hoffmannianum* was the result of normal dinoflagellate metabolism. Nevertheless, it has been shown that some PSP toxins are produced by marine bacteria [34 , 35], as well as a fresh water cyanobacterium [36].

Toxin Synthesis. The pattern of *de novo* synthesis of both FAT and OA were strikingly similar (Fig 5), despite their many chemical dissimilarities. The cellular content of both FAT and OA showed an initial decrease following inoculation (mid log phase cells) to the lowest measured content of 0.65 MU FAT·cell (x10^{-5}) and 3.46 pg OA·cell^{-1} at the beginning of log phase at 5 days. The initial decline in toxin content was followed by a steady increase to maximal levels of 2.85 MU FAT·cell (x10^{-5}) and ~20 pg OA·cell^{-1} during late log phase. Subsequently, FAT content showed a slight decline in late stationary phase, while OA levels remained constant throughout the stationary phase. This was probably the result of decreased toxin synthesis coupled with arrested cell growth. The slight decline in FAT content may be explained by a leakage of this water soluble toxin from senescing cells. Nevertheless, variation in total extractable FAT between mid log and stationary phase was similar to that of OA, ranging from 214 to 216 MU·100 mg dry cells^{-1} (data not shown). Similar patterns of toxin synthesis have been reported for the PSP dinoflagellate *Alexandrium fundyense* maintained in batch-culture [23]. However, most plots of toxin synthesis in that study were bell-shaped. These authors suggested that this pattern was the result of decreased toxin synthesis and/or the possibility of massive toxin leakage in late log to stationary phase.

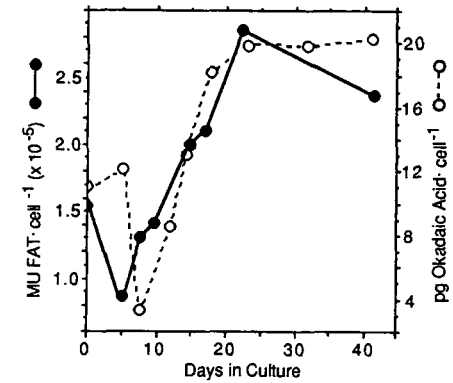

FIG. 5. Variation in FAT and OA cellular content during one complete growth cycle.

Our results (Fig. 5) indicate that toxin production in *P. hoffmannianum* follows a pattern similar to that of the other major cellular constituents (Fig. 3 & 4). From correlation analysis of all the biochemical parameters with potency over the growth cycle, the best relationship exists between toxin and lipid production (r = .935 for FAT vs. total lipid and r = .958 for OA vs. total lipid). This relationship is especially interesting since both toxins and lipids are probably competing for the same carboxylic acid (propionate acetate and butyrate) precursors [37, 38]. This is in contrast to a reported inverse relationship between the availability of arginine, a saxitoxin precursor, and toxin biosynthesis for *A. fundyense* [23]. These authors suggested a competition between the toxin and the pathways involved in cell division for the amino acid arginine.

Potency and nutrient uptake (NH_4^+ and PO_4^{3-}) in *P. hoffmannianum* had a strong negative correlation of r = -0.93 and -0.96, respectively, suggesting that the mechanisms of nutrient assimilation and toxin production are competing for some similar substrate or energy source. This is comparable to increased toxin content reported for *A. fundyense* in severely P-limited cultures [23]. However, the production of maitotoxin in *G. toxicus* was found to be significantly and positively correlated with growth rate [31] and NH_4^+ uptake rates [Bomber *pers. comm.*], suggesting a pleiotropic linkage between reproduction rate, NH_4^+ uptake and potency. These data indicate a strong relationship between potency and nutrient availability/uptake.

The ratio of FAT:OA for *P. hoffmannianum* in this study was 6:1 (OA data was normalized to mouse units, assuming 4 µg·mouse^{-1} = LD_{50} = MU for OA [27]). This ratio lies somewhere between that for water soluble toxins:ether soluble toxins in cultured *G. toxicus* 175 (~ 70:1), *G. toxicus* 350 (~ 35:1) [Tindall *unpubl. data*] and *Ostreopsis lenticularis* 872 (~ 2:1) [3]. Assuming maximal OA content (20 pg·cell^{-1}) and natural population cell densities (1.25 x 10^5 cells·gfw-macroalgal host^{-1}) reported by Carlson et al. [32], *P. hoffmannianum* can contribute as much as 2.5 µg OA·gfw-macroalgal host^{-1} in ciguatera endemic Caribbean waters. In addition to the OA contribution, we have uncovered a 6 fold production of the potent neurotoxin, FAT over that of OA in batch culture. Therefore, the contribution of FAT may have a more profound impact on ciguatera than OA. Nevertheless, the effect of both toxins coupled with the natural processes of biological magnification in the fish food chain in ciguatera endemic regions make *P. hoffmannianum* a viable etiological agent.

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DISTRIBUTION OF *Dinophysis* spp. AND *Alexandrium minutum* ALONG FRENCH COASTS SINCE 1984 AND THEIR DSP AND PSP TOXICITY LEVELS

CATHERINE BELIN
IFREMER, Rue de l'ile d'Yeu, BP 1049, Nantes cedex 01, France

ABSTRACT

This paper presents monitoring results for DSP-producing (*Dinophysis* spp.) and PSP-producing *(Alexandrium minutum)* species which are now a danger to consumers. Changes over the years in cell counts and DSP and PSP toxicity levels are given for different coastal areas. Three large regions are affected every year by the development of *Dinophysis* spp., and there has been an evident geographical spread of this dinoflagellate along French coasts since 1984. DSP toxicity levels are very variable, and some areas are apparently more exposed to high toxicity despite relatively low cell counts. *Alexandrium minutum is* at present confined to a small area off the northwestern coast of Brittany where PSP toxicity, first recorded in 1988, has never reached high levels.

INTRODUCTION

In 1983, approximately 3,000 DSP-type intoxications (Diarrhetic Shellfish Poison) were recorded in southern Brittany subsequent to consumption of mussels; the phytoplankton species found to be responsible belong to the genus *Dinophysis* Ehrenberg. In 1984, a monitoring network was set up along the entire French coastline (Atlantic and Mediterranean) in order to detect and monitor the occurrence of any toxic species [1]. In 1988, PSP (Paralytic Shellfish Poison) toxicity was recorded for the first time in France in mussels and oysters from the Abers region (ria coast) of northwestern Brittany; the implicated species was identified as *Alexandrium minutum* Halim [2].

METHODS

The French phytoplankton monitoring network consists of 110 sampling stations distributed along the entire French coastline. Thirty-seven stations (continuous monitoring) are sampled year round (twice a month in winter, once a week in summer). The remaining stations (warning stations) are sampled only in the event of growth of a toxic species. Observations and counts of phytoplankton species are performed on seawater samples (1 or 2 L) collected at the surface or sub-surface. Living and/or fixed (2.5 $ml.l^{-1}$ lugol + 2 $ml.l^{-1}$ formaldehyde) samples are examined under an inverted microscope using a 10 or 25 cm^3 water volume, according to the Utermöhl method [3]. The DSP toxicity assay consists of a mouse-test carried out on an extract of shellfish digestive gland according to a modified Yasumoto method [4], with a threshold equal to five hours [5], which corresponds to 2-4 µg of $OA.g^{-1}$ (Okadaic acid) of digestive gland. The PSP toxicity assay consists of a mouse-test carried out according to the AOAC method [6]. Positive results on toxicity bioassays lead to measures involving a ban on the marketing of incriminated shellfish; bans are lifted only after two weeks of successive negative assays.

RESULTS

Dinophysis spp.

Four dominant species have been found in France since 1983 during DSP occurrences: *Dinophysis* cf. *sacculus*, *D.* cf. *acuminata*, *Dinophysis sp.* and *D.* cf. *norvegica*, with the first three species related to the "*acuminata* complex" [7]; a smaller species identified as *D. skagi* has been found as well on a regular basis. Since these various species present significant morphological variations, their identification is not conducted systematically in the context of the monitoring network. Consequently, only the overall results on the *Dinophysis* genus, regardless of species, will be presented in this paper. Okadaic acid has a major responsibility in the overall DSP toxicity [8].

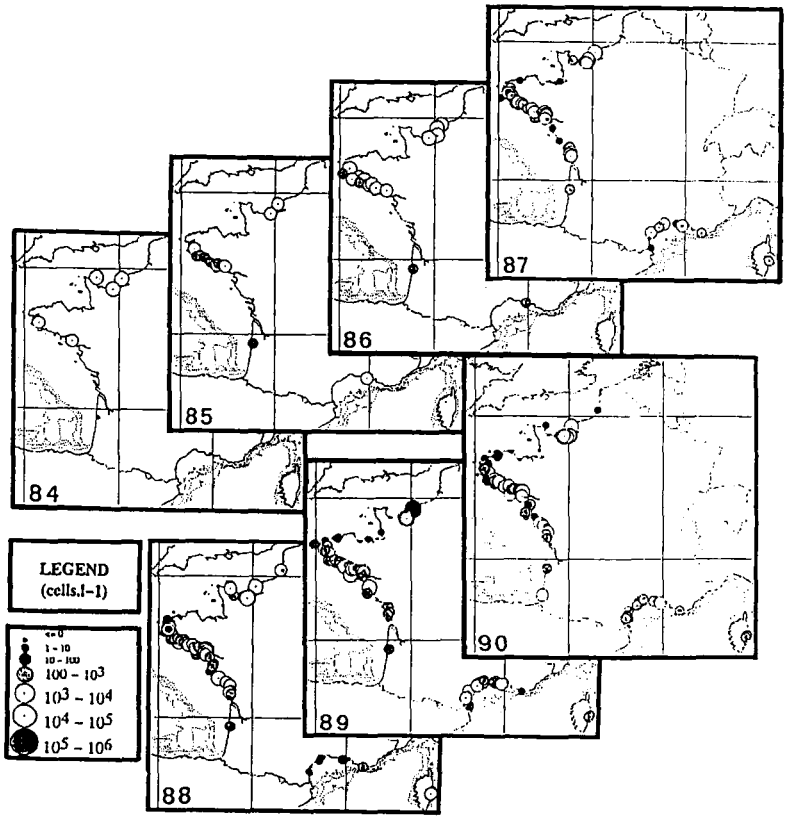


FIG. 1: Maximum cell counts of *Dinophysis* spp. along French coasts from 1984-1990 (from [14]).

Figure 1 shows that a significant portion of the French coastline is now suffering from regular occurrences of *Dinophysis*, most of them involving occurrences of DSP shellfish contamination of variable duration (Fig. 2). Maximum concentration periods, generally correlated to DSP toxicity occurrences, are primarily observed from the month of July along the Channel coastline (Normandy), and from May on the Atlantic (Brittany) and Mediterranean (Languedoc-Roussillon) coasts. The highest concentrations (>10^5 cells L^{-1}) are observed each year north of the Seine estuary. Local current and wind conditions may explain the preferential accumulation of *Dinophysis* cells in this area [9].

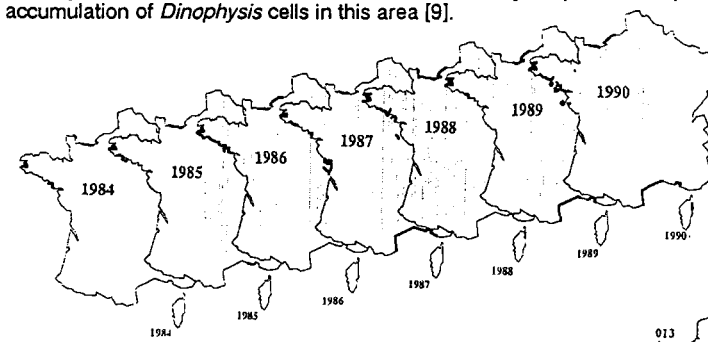


FIG. 2: Closed areas (for DSP toxicity) from 1984-1990 in France.

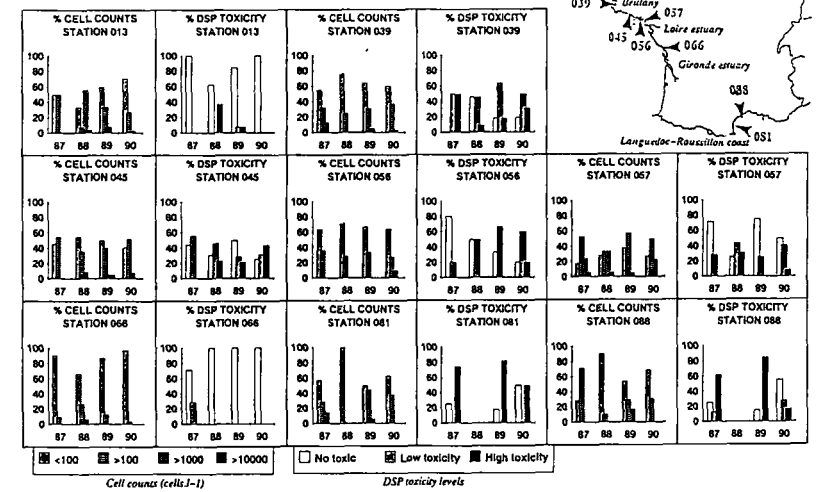


FIG. 3: *Dinophysis* spp. cell counts and DSP toxicity levels at eight sampling stations of the French coast from 1987-1990 (No toxicity = no mouse mortality, Low toxicity = between 5 to 24 hours survival time, High toxicity = mouse-test result below French health threshold of 5 hours survival time).

Figure 3 illustrates some examples representative of the existing disparity between several regions as regards cell counts and related DSP toxicity levels. Thus, long periods (3-7 months) of high to very high toxicity are observed yearly on the

following sectors: bay of Douarnenez (station 039) and Languedoc-Roussillon coastline (stations 081 and 088), although cell counts are not excessively high. Conversely, the bay of Vilaine (station 057) shows that the usually higher concentrations do not necessarily imply high toxicity levels. In other sectors, such as the Calvados coast (station 013), Lorient Harbor (station 045), the Penerf river (station 056), average concentrations (usually <1000 cells L^{-1}) lead to variable toxicity levels from one year to the next. Finally, in certain sectors such as the bay of Aiguillon (station 066), both cell counts and toxicity levels remain relatively low.

Alexandrium minutum

Since its first occurrence dating back to 1988 in the Abers (rias of north-western Brittany), where the maximum PSP toxicity was measured at 401 µg STX 100 g^{-1} of mussel flesh and 282 µg STX 100 g^{-1} of oyster flesh, this species has become endemic throughout this area of Brittany (Fig. 4). Thus, in 1989 and 1990 *Alexandrium minutum* was observed in the bay of Morlaix, close to the previous sector, with maximum toxicity levels of 143 (1989) and 151 µg STX 100 g^{-1} (1990) of mussel flesh, while periods of toxicity remained generally short. The overall PSP toxicity recorded is due primarily to gonyautoxins GTX2 and GTX3 [10]. Figure 5 presents the evolution of cell counts and related PSP toxicity levels in the two concerned areas. *Alexandrium minutum*, or a closely-related species, is currently observed along a large portion of the Brittany coastline (see Fig. 4), however PSP toxicity has not been recorded. In 1990, an extensive *A. cf. minutum* bloom (180 x 10^6 cells L^{-1}) occurred in the Toulon Harbor (Mediterranean); toxicity measured 20 days after the bloom reached 80 µg STX 100 g^{-1} of mussel flesh.

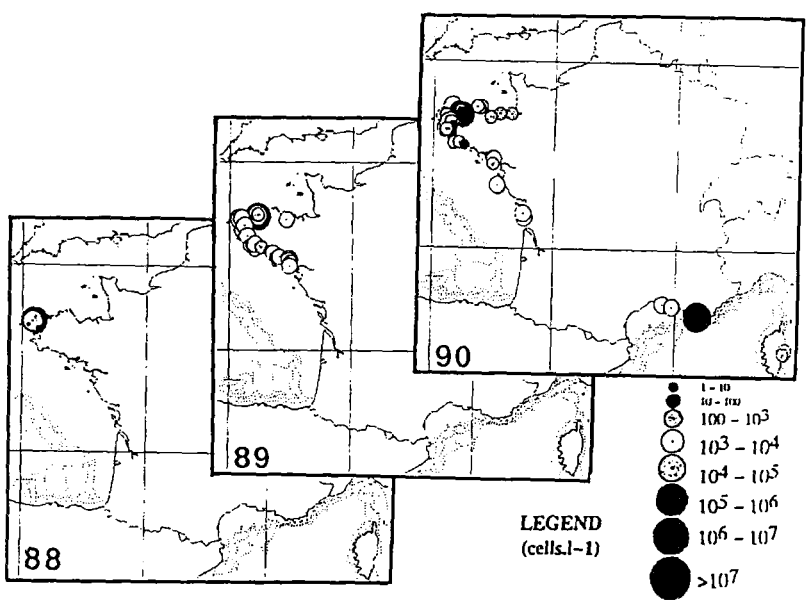
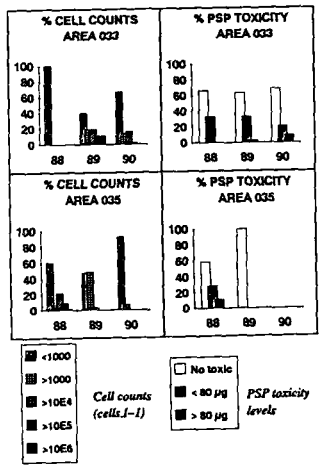


FIG. 4: Maximum cell counts of *Alexandrium* cf. *minutum* along French coasts from 1988-1990 [14].



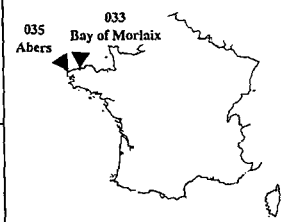


FIG. 5: *Alexandrium* cf. *minutum* cell counts and PSP toxicity levels in two areas of Brittany from 1988-1990: (i) no toxicity, (ii) toxicity, but below the French health threshold-80 µg STX.100 g-1 of flesh-, (iii) toxicity above this threshold.

DISCUSSION - CONCLUSION

These results originate from a geographically dense monitoring network, covering the entire French coastline, but cannot be considered for extreme accuracy since samples are collected only at the surface or subsurface. Nevertheless, they show evidence of a geographical spread of *Dinophysis* spp. along the Atlantic coasts (see Fig. 1): in 1984, the coastline of Normandy and Brittany only were affected, while the area stretching from the Loire estuary to the Gironde estuary has been regularly affected since 1987. Conversely, it should be noted that the species is systematically absent from the coasts of northern France and northern Brittany. The Mediterranean first suffered from a *Dinophysis* spp. invasion in 1987, and since then a major portion of its coastline, except in coastal lakes, has been regularly affected. Additional data regarding the ecology of *Dinophysis* spp. are required to explain its distribution along the coastline. However, studies conducted in several regions have revealed an absence of any direct relationship between nutrient inputs and abundance of *Dinophysis* cells [11]. A number of conditions necessary to *Dinophysis* growth, such as stratification of water masses, were nevertheless identified. Mathematical modelling tests have shown that no significant growth could occur in the absence of any vertical migration [12]. The hypothesis that *Dinophysis* develops offshore before drifting towards the coast, as suggested by its nearly simultaneous occurrence each year at springtime along most of the southern Brittany coastline, was confirmed by several surveys conducted in the Pertuis Charentais region (between the Loire and Gironde estuaries) from 1989 to 1991 [13]. With regard to the geographical variations in the relationship between cell concentration and DSP toxicity level, they could result from differences in the toxins produced by the various dominant *Dinophysis* species, although evidence of such differences remains to be demonstrated. In addition, the excessively long periods (up to eight weeks in 1989) required for decontamination of mussels on offshore ropes submerged off the Languedoc-Roussillon coast (Mediterranean), following disappearance of *Dinophysis* cells, could be explained by the stress suffered by the shellfish in a low nutrient environment [8].

As for *Alexandrium minutum*, a number of resting cysts were found in the sediments surrounding the area where this species proliferates (Abers and Bay of Morlaix) [11]. It is, therefore, likely that this species is now established in that area.

ACKNOWLEDGMENTS

These results were obtained from sample collections and analyses conducted collectively by my colleagues from the coastal laboratories of the Direction de l'Environnement et de l'Aménagement Littoral at IFREMER.

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Toxic Phytoplankton Blooms in the Sea
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DSP CASES ALONG THE COAST OF EMILIA-ROMAGNA (NORTHWESTERN ADRIATIC SEA)

LAURITA BONI[1], ANNA MILANDRI[2], ROBERTO POLETTI[2] and MARINELLA POMPEI[2]
[1]Department of Biology, University of Bologna, Via Irnerio, 42 - 40126 Bologna, Italy; [2]Consorzio di Studi, Ricerche ed Interventi sulle Risorse Marine di Cesenatico, Via Vespucci, 2 - 47042 Cesenatico (FO), Italy

ABSTRACT

Phytoplankton blooms and red tides are common phenomena in the Northwestern Adriatic Sea, their annual occurrences being quite regular since 1975. Potentially toxic species such as *Dinophysis* spp. and *Alexandrium* spp. have been found; the former for many years, the latter only since 1982. However, toxic problems did not appear until summer 1989. The occurrence of DSP in mussels due to *Dinophysis* spp. during 1990 is described.

INTRODUCTION

Since the last century, many phytoplankton-related phenomena (i.e. blooms and mucilage) are known from the Adriatic Sea, but particularly since 1975 red tides caused by dinoflagellates became more and more frequent causing fish kills, bad smell, and strange colors in the sea. Consequently, the tourist and fishery industries of the region suffered heavy financial losses. Phytoplankton have been monitored since 1976, the presence of toxic and potentially toxic species was particularly evaluated. Notwithstanding the presence of *Dinophysis* spp., *Prorocentrum* spp. and, since 1982, *Alexandrium tamarense* no episodes of shellfish poisoning have been observed [1, 2, 3].

In summer 1989, many people suffered from abdominal pains, vomiting and diarrhea after eating mussels (*Mytilus galloprovincialis*) collected from the Emilia-Romagna coast. In both sea water and the digestive tract of the mussels, many *Dinophysis* spp. cells were found. Accordingly, the Regional Government was induced to intensify phytoplankton monitoring and toxicological tests. Biotoxicological tests (for PSP, NSP and DSP [4]) were performed on *Mytilus galloprovincialis* and on some other edible bivalve shellfish, such as *Tapes semidecussatus*, *Venus gallina* and *Ostrea edulis*. DSP only was found in the mussels and their sale forbidden. In 1990, the same tests continued and DSP was detected in mussels and *Dinophysis* spp. Fishing and selling of mussels were prohibited, and only a few cases of DSP occurred.

MATERIALS AND METHODS

Shellfish and water monitoring was carried out on natural banks off-shore of Ravenna and in the breeding areas of Goro, Bellaria and Rimini. Water and mussel samples were collected at three different levels: surface, middle and bottom (Fig. 1). In the present work we present the results from the natural banks Amelia, Agostino B and Antares (artificial islands for gas extraction) and P.C.W.B. (well for gas extraction). Phytoplankton was observed by inverted microscope and counted by Utermöhl's method. Toxicological analyses were carried out using Yasumoto's method [5]. Three acetonic phases from 20g of shellfish hepatopancreas were

evaporated and the residue resuspended in 4 ml of 1% Tween 60. 1 ml aliquots of the solution were injected i.p. into 18-20g Swiss albino mice. Toxicity is expressed as mice death time, human risk level 5 h, following Italian law. The results obtained are shown in Figures 2-5.

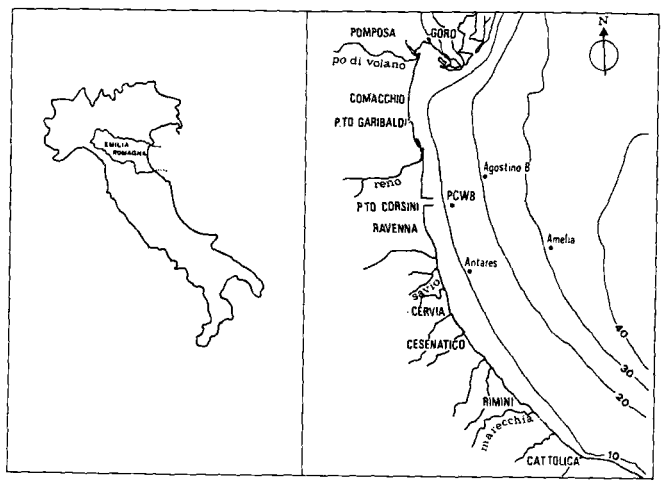

FIG. 1: Sampling area.

RESULTS AND DISCUSSION

In January and February, some mussel samples were affected by DSP before *Dinophysis* appeared. The toxicity was clearly due to the cells present in 1989 [4]: liposoluble toxins are slowly eliminated by mussels. Some cells of *Dinophysis* spp., especially *D. fortii*, were also found during this period in Agostino B, surface and bottom samples, Amelia surface, Antares bottom and P.C.W.B. surface, middle and bottom samples. In March and April, small amounts of *D. fortii* and *D. tripos* were present in the study area (not in P.C.W.B.), but since toxicity disappeared, it was possible to revoke the prohibition of fishing and selling of mussels in force since June 1989. In May, cell numbers of *Dinophysis* spp. increased and DSP in mussels reached dangerous values for consumers; hence, fishing and selling of mussels were again prohibited. DSP was absent or not at dangerous levels sometimes during summer, but it was impossible to revoke any prohibition relative to mussels because a few days later DSP reappeared. We often noted a two-week interval between increase in cell numbers and DSP, in which a decrease in cell numbers was followed by a low level of DSP, but a few days later an increase in cell numbers caused a new, high level of DSP. *Dinophysis* spp. and DSP were present until November.

In all samples, *Dinophysis* spp. were present in low abundance, ranging from 40 to 3400 cells/l, but their abundance varied seasonally and with depth. Maximal abundance occurred from May to July (Figs. 2-5). During summer, cell numbers increased in bottom waters. The most common species were *D. tripos*, *D. fortii*, *D. sacculus*, *D. rotundata* and *D. caudata*. Sometimes, *D. acuta*, *D. diegensis* and *D. hastata* were found, as was a *Dinophysis* sp. which seemed similar both to *D. sacculus* and *D. acuminata*. Due to the impossibility of growing *Dinophysis* in the laboratory, we cannot know if this is a real species or a geographic or seasonal form. It is also very difficult to determine if one or another species is more toxic than the others; rarely is just one species present and toxicity depends also on which species were ingested during the previous days. DSP levels are often very similar in the presence of small or larger amounts of *Dinophysis* cells; ambient conditions probably influence the production of toxins by the algal cells [6]. We think also that mussel toxicity depends on the presence of other palatable phytoplankton. In fact, we note a reduced level of DSP after the presence of large amounts of *Dinophysis* (1300 cells/l of *D. fortii*) in the same period as a *Gonyaulax polyedra* red tide (Agostino B bottom, end of August). The lack of phytoplankton or the presence of cells not palatable because of spines or size force the mussels to filter large volumes of water to obtain a sufficient quantity of phytoplankton; so, even if *Dinophysis* cells are few, mussels may still accumulate many cells.

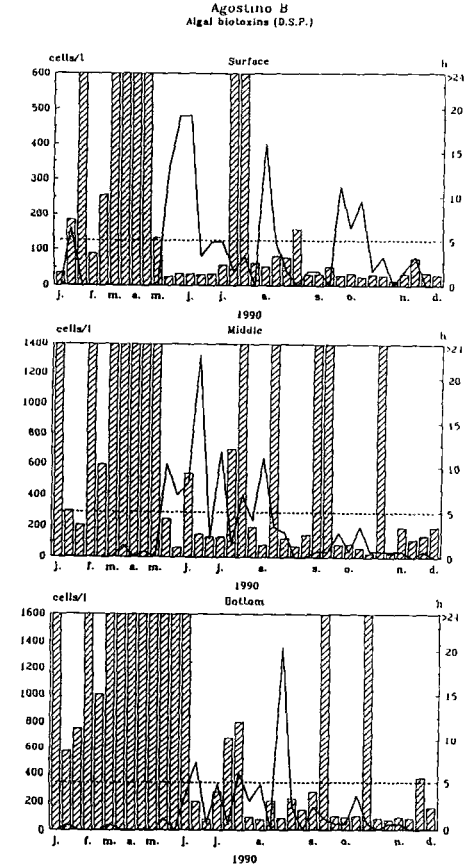

FIG. 2: *Dinophysis* concentrations (- - - -), toxicity levels (▨) as mouse death time and risk level (—): at Agostino B site. A - surface, B - middle, C - bottom.

CONCLUSIONS

The analyses demonstrated that the presence of DSP in mussels was strictly related to the presence of *Dinophysis* spp. Shellfish detoxification required a long period after the disappearance of toxic cells. In order to protect public health, continuous biotoxicological tests are necessary besides phytoplankton monitoring.

The *Dinophysis* species found in 1989 and 1990, when DSP cases occurred, were the same species occurring during years without DSP. They are very common in the Adriatic Sea, but never in high cell numbers. It is really difficult to answer the question: Is there an increase in toxicity in the Adriatic Sea, or were there other causes for gastrointestinal disorders in the past years? We are sure that past intoxication phenomena as heavy as that found in 1989 were not described. In 1991 (un-published data), DSP levels were low and mussels were consumed without problems. Possibly, ambient conditions are important in determining toxin level. We are continuing our research in the same zones and are also evaluating ambient conditions.

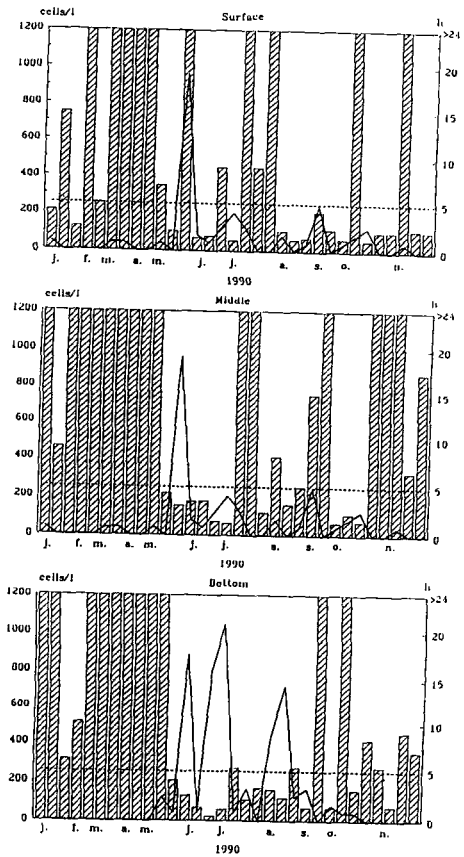

FIG. 3: As in FIG. 2, except at Amerlia site.

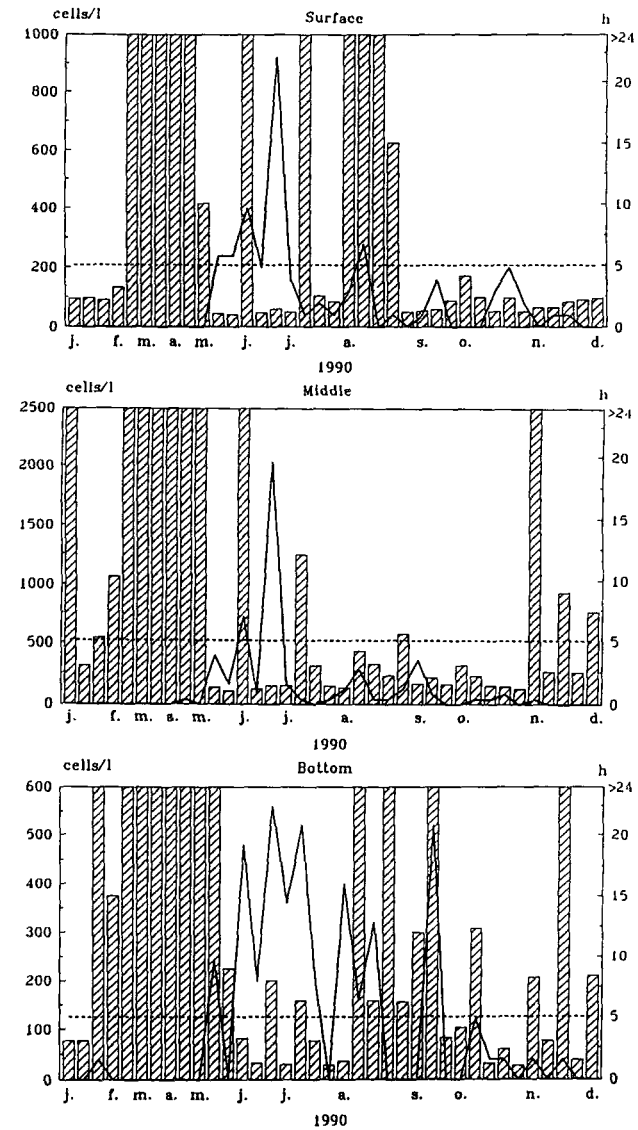

FIG. 4 As in FIG. 2, except at Antares site.

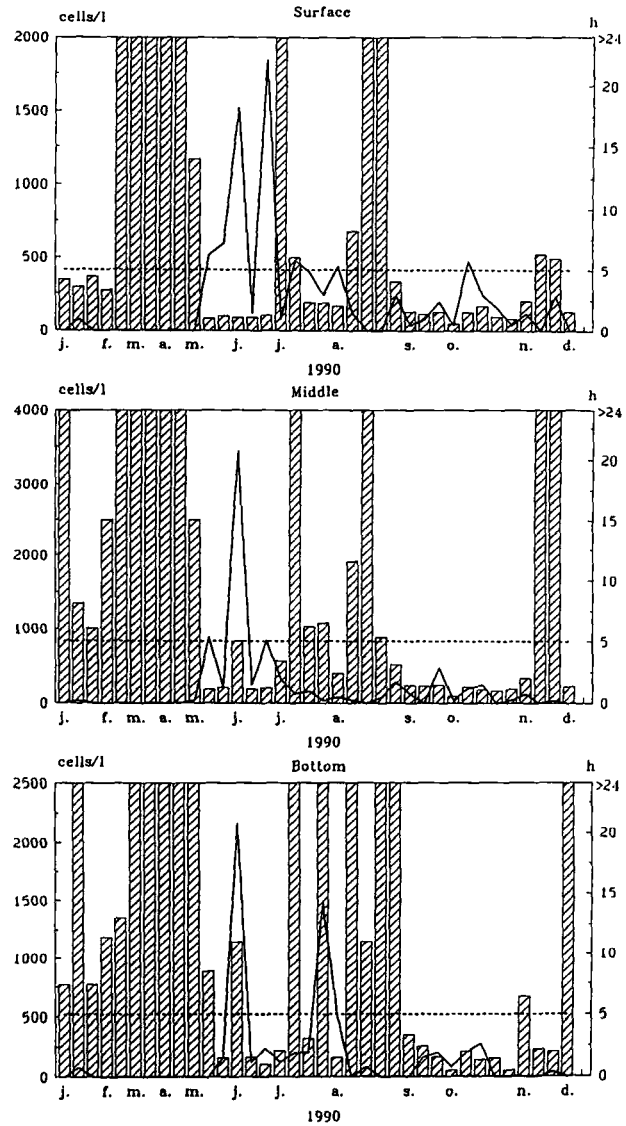

FIG. 5: As in FIG. 2, except at P.C.W.B. site.

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RELATIONSHIP BETWEEN *Dinophysis* spp. IN SEAWATER AND DSP TOXINS IN MUSSELS IN THE NORTHERN ADRIATIC SEA

R. DELLA LOGGIA[1], M. CABRINI[2], P. DEL NEGRO[2], G. HONSELL[3] and A. TUBARO[1]
[1]Institute of Pharmacology and Pharmacognosy, University of Trieste, 34127 Trieste, Italy; [2]Marine Biology Laboratory, 34010 Trieste, Italy; [3]Department of Biology, University of Trieste, 34127, Trieste, Italy

ABSTRACT

From September 1990 to September 1991, mussels and seawater from a mussel farm in the Gulf of Trieste (Northern Adriatic Sea) were checked monthly for DSP toxins and *Dinophysis* cells. The concentration of OA and DTX-1 in mussels was determined by immunoassay using monoclonal antibodies (ELISA). A toxin content higher than 1 µg/g of hepatopancreas was found in September and October 1990 which then decreased to about 0.1 µg/g from January to September 1991. *Dinophysis* concentrations were significant only in September and October 1990 and in May 1991. *D. fortii* was most abundant in autumn and *D.* cf. *acuminata* in spring. Mussel contamination was observed after *D. fortii* exposure, but not *D.* cf. *acuminata* exposure. A relationship between the toxin concentration and the presence of *D. fortii* during the previous month was observed.

INTRODUCTION

Diarrhetic Shellfish Poisoning (DSP) is a gastroenteritis caused by ingestion of shellfish contaminated by toxin-producing dinoflagellates. Three different groups of toxins have been isolated from various Japanese shellfish involved in DSP phenomena [1]. One group consists of okadaic acid (OA) and its derivatives, dinophysistoxin-1 (DTX-1) and dinophysistoxin-3 (DTX-3) [2]. The other two groups consist of macrolides of the pectenotoxin family [2, 3] and the recently isolated toxins named yessotoxins [1, 4].

In 1989, some cases of DSP were observed in Italy. Since the official data of the National Health Service did not indicate a clear correlation between the presence of potentially toxic algae and the mussel toxicity determined by the "mouse bioassay" of Yasumoto, a monitoring program on phytoplankton and on toxin content in *Mytilus galloprovincialis* was started in September 1990 in a mussel growing farm of the Gulf of Trieste (Northern Adriatic Sea). Among the DSP toxins, only the OA group appears to possess diarrheogenic properties [5, 6] and, therefore, may be considered the most relevant for toxicological monitoring [7]. Since DTX-3 is typical of Japanese scallops and only exceptionally is present in European mussels [1], a competitive enzyme immunoassay (ELISA) that determines cumulatively OA and DTX-1 was used. Simultaneously, *Dinophysis* cells present in the mussel farm seawater were identified and counted and temperature and salinity measured.

MATERIAL AND METHODS

From September 1990 until September 1991, *Mytilus galloprovincialis* and seawater samples were collected monthly in a mussel farm located 200 m offshore in the Gulf of Trieste. Temperature and salinity were recorded with a 401 Ocean Seven (Idronaut) at each meter from the surface to 12 m (bottom).

Water samples (500 ml) were collected with a Niskin bottle from the surface at 2, 5 and 10 m and at the bottom and immediately preserved with 4% formaldehyde neutralized with hexamethylentetramine. *Dinophysis* species were identified and counted with a Zeiss IM 35 inverted microscope at 400x magnification, according to the Utermöhl method [8] after sedimentation of 100 ml.

Immunoenzymatic Detection of Okadaic Acid and Dinophysistoxin-1

Monoclonal antibodies against OA and related reagents were purchased from UBE Industries (Tokyo, Japan). The assay was carried out as described by Uda *et al.* [9]. Absorbance was read by a photometer (Ube Handy Reader - Tokyo, Japan). One gram of frozen digestive glands of *Mytilus galloprovincialis* was homogenized by Ultra-Turrax with 5 ml of 90% aqueous methanol for 3 mins at room temperature. The homogenate was centrifuged for 5 mins at 16,000 x g. The supernatant was diluted to double the volume with distilled water and centrifuged again for 5 mins at 16,000 x g. This latter supernatant was used for the assay step of ELISA. The sample extracts were always tested in triplicate.

Using the standard solutions of the ELISA kit, five concentrations of OA (100, 55, 32.5, 18 and 10 ng/ml) were prepared and measured by ELISA. To evaluate the reproducibility of the data, the extraction and measurement procedures were repeated at least four times for each of three different samples. In recovery tests, a known amount of OA was added to one gram of digestive glands of commercial mussels, theoretically free from OA, and to one with a low toxin content. The toxins were then extracted as described previously.

RESULTS

The sensitivity of the kit, declared by the producer, ranged from 0.01 to 0.10 µg/ml of okadaic acid. In this range, the calibration curve for OA is linear (Fig. 1; $r = 0.995$). To determine the reproducibility of the method, a minimum of four separate determinations of the two different contaminated samples (samples 1 and 2) and of sample NO, theoretically free of toxins, were carried out (Table 1). Sample NO shows an OA + DTX-1 concentration of 0.10 ± 0.02 µg/g, just at the lower limit of sensitivity of the method. For the two contaminated samples, OA + DTX-1 concentrations of 0.37 ± 0.05 and 0.69 ± 0.05 µg/g were measured. The coefficient of variation (CV = SD/mean) ranged from 20 to 7%, according to the toxin concentration. The results of the recovery test are reported in Table 2: the recovery was almost complete, ranging from 98.6 to 108.7%.

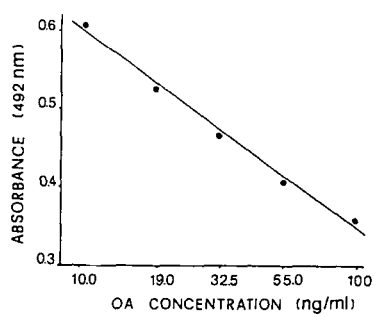

FIG. 1: Calibration curve for okadaic acid (OA). Each point represents the mean value of duplicate assays of three experiments; the deviation from the mean value for each point was within ± 5%.

Table 1. Reproducibility of the Elisa method.

Sample	OA conc.* µg/g	OA conc. mean ± S.D.	CV
N°0	0.08		
	0.11		
	0.09	0.10 ± 0.02	20%
	0.12		
N°1	0.31		
	0.31		
	0.39	0.37 ± 0.05	14%
	0.43		
	0.39		
N°2	0.65		
	0.70		
	0.64	0.69 ± 0.05	7%
	0.76		

* Each value derives from a different extraction and represents the mean value of duplicate assays.

Table 2. Recovery test of okadaic acid.

Concentration of OA (µg/g)				
In Origin	Added	Theoretical	Measured	Recovery %
0.10	0.60	0.70	0.69	98.6
0.39	0.30	0.69	0.75	108.7

Using the ELISA assay method, the concentrations of OA + DTX-1 in mussels were evaluated monthly (Fig. 2). The highest DSP toxin content was observed in September and in October 1990 (about 1.3 µg/g of hepatopancreas), then decreased to around the lower limit of sensitivity of the method starting in January 1991.

The phytoplankton analysis revealed a significant presence of *Dinophysis* spp. in September and October 1990; subsequently, sporadic occurrences were detected until May 1991, when *Dinophysis* spp. were again present and remained until September 1991 (Fig. 2). Six species of *Dinophysis* were identified: *D.* cf. *acuminata, D. caudata, D. fortii, D. rotundata, D. sacculus* and *D. tripos. D. fortii* and *D.* cf. *acuminata* were the most abundant species, with a different seasonal distribution. *D. fortii* dominated (about 80% of the total *Dinophysis* number) during the autumn at each depth, reached their maximum value at 5 m (210 cells/l) in September 1990, then decreased below 100 cells/l in October. During the other months, it was not observed until the end of May 1991 (Figs. 2, 3a). *D.* cf. *acuminata* was not detected from September to April at any depth, but became the most

represented species (60%) during spring and early summer. *D.* cf. *acuminata* was observed mainly at the surface: the maximum value was in May, with 180 cells/l (Figs. 2, 3b). Significant values of the other species were not found at any depth; their presence was sporadic.

Temperature and salinity during this study ranged from 4.9-25.4°C and 23.2-38‰, respectively.

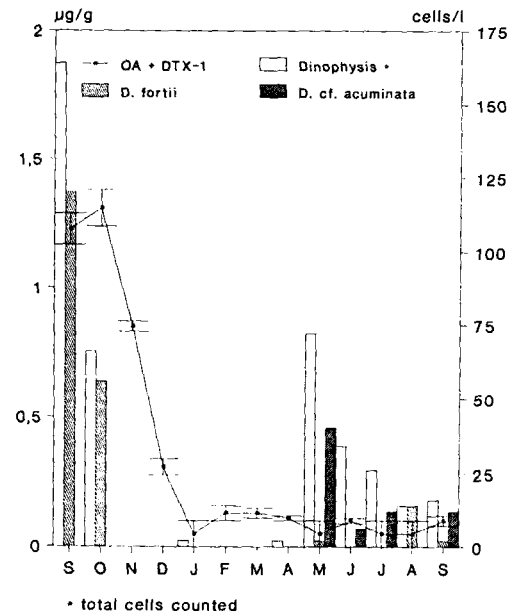

FIG. 2: Seasonal variation of the OA + DTX-1 mussel content (µg/g of hepatopancreas) and the abundance of total *Dinophysis* cells, *D. fortii* and *D.* cf. *acuminata* (cells/l: mean value for the water column).

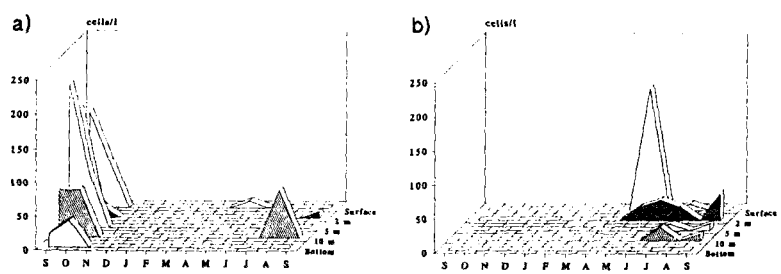

FIG. 3: Vertical distribution of *D.fortii* (a) and *D.* cf. *acuminata* (b) from September 1990 to September 1991.

DISCUSSION

Dinophysis is a common component of the Northern Adriatic Sea phytoplankton [10-16]. No cases of *Dinophysis* - discolored water have been reported. *Dinophysis*

was never observed as the dominant dinoflagellate. Its cell numbers are generally low: the magnitude most frequently observed in the Adriatic Sea ranges from 10 to 10^3 cells/l [16, 17]. The temporal distributional pattern of *Dinophysis* from September 1990 to September 1991 reveals two different seasonal peaks dependent mainly on two species: *D. fortii* is present in autumn and *D.* cf. *acuminata* present mainly in late spring and early summer (Figs.2, 3b). *D. fortii* was distributed throughout the water column; *D.* cf. *acuminata* occurred mainly at the surface (Fig. 3), and with a seasonal distribution pattern similar to that observed along the French and North American Atlantic coasts [18-20].

In autumn 1990, water column temperatures were homogeneous from surface to bottom (about 22-23°C). From May to August 1991, the water mass was thermally stratified (20-24.8°C at surface, 14.8-19°C at bottom). In our data the occurrence of *D.* cf. *acuminata* in spring seems to be related to thermal stratification. A relationship between salinity and *Dinophysis* occurrence was not observed.

The toxin levels in mussels were low (Fig. 2): none of the samples showed a concentration of toxins sufficient to cause gastro-intestinal effects in humans. All values were lower than the official tolerance limits established by the Health Authorities of various countries. The contamination of mussels by OA + DTX-1 is related to the presence of *D.* fortii, but not to *D.* cf. *acuminata* (Fig. 2). A direct relationship between toxin concentration and the presence of *D. fortii* the month before is evident (Fig. 4). Based on the data, we hypothesize that in the Northern Adriatic Sea *D. fortii* is the probable source of diarrhetic shellfish toxins. It is the causative organism of DSP in Japan [21], where it was found to contain DTX-1 and pectenotoxin-2 (PTX-2), but not OA [22, 23]. Assuming that *D. fortii* in the Adriatic Sea contains the same toxins as detected in Japan, we hypothesize that DTX-1 is the diarrhetic shellfish toxin most present in Northern Adriatic Sea mussels. PTX-2, the other toxin produced by *D. fortii*, belongs to the PTX family that does not seem to possess diarrheic properties [7]. To our knowledge, no DSP outbreak in Europe was associated with *D. fortii* before the Adriatic cases in 1989. Until now, the main toxin involved in European DSP phenomena is considered to be OA, related to the presence of *D.* cf. *acuminata* and *D. acuta* [22]. In our case, the lack of mussel contamination observed after exposure to *D.* cf. *acuminata* (Fig.2) can be due to the low concentration of this alga. It is also known that the content of OA *in D.* cf. *acuminata* is very low [23] and, therefore, large numbers of these cells are necessary to induce significant contamination of mussels. In fact, many outbreaks of DSP poisoning in Atlantic and Northern Sea coasts of Europe occurred when densities of *D.* cf. *acuminata* exceeded 20,000 cells/l [24].

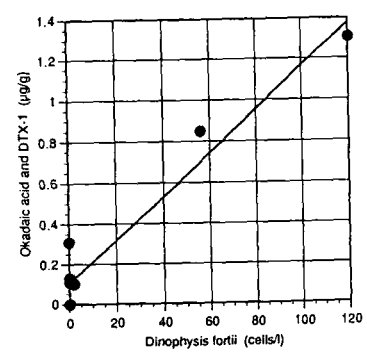

FIG. 4: Relation between the concentration of OA + DTX-1 (µg/g of hepatopancreas) and the population density of *Dinophysis fortii* (cells/l: mean value for the water column). The counts of *D. fortii* were carried out on samples collected one month before the mussels.

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DO *Dinophysis* spp. COME FROM THE "OPEN SEA" ALONG THE FRENCH ATLANTIC COAST?

DANIEL DELMAS, ALAIN HERBLAND and SERGE Y. MAESTRINI
Centre de Recherche en Ecologie Marine et Aquaculture de L' Houmeau (CNRS-IFREMER) B.P. 5, 17136 L'Houmeau, France

ABSTRACT

A study was conducted with the aim of showing which environmental conditions promote an increase in *Dinophysis* cells in a land-locked embayment with shellfish installations. From May 1989 to September 1991, vertical profiles were taken of temperature, salinity, nutrients and chlorophyll-a, and counts of phytoplankton species composition were made during late spring and summer. In the strait separating this area from the open sea, residual tidal currents are from offshore to nearshore. Significant thermocline (Δ t ≈ 5°C) and stable stratification of the water column were required for *Dinophysis* to exceed 1000 cells L^{-1}. *Dinophysis* spp. were more abundant in the "thermocline layer", scarce below this layer and absent near the bottom. These stratified conditions occurred first in the open sea. Denser *Dinophysis* populations were then carried through the strait from offshore to nearshore waters by tidal currents. Growth of *Dinophysis* occurred in nutrient-impoverished waters after the spring diatom bloom, and was not correlated with the measured nutrient concentrations (nutrient inputs from agricultural and domestic origins appeared not to promote growth directly). The maximum apparent *in situ* growth rate was low, 0.25 division day^{-1}, a rate similar to that of other dinoflagellates. In late spring and early summer, dinoflagellate importance relative to diatoms increased in offshore surface waters, as a result of both cell division and diatom sinking. The importance of the nano- and the pico-size classes also increased relative to the micro-size class. Six *Dinophysis* species have been recorded; *D. sacculus, D. acuminata* and *D. rotundata* were the most abundant.

INTRODUCTION

There is provocative evidence of recent, significant increases in algal biomass and production, as well as changes in community structure and species distribution, in some inshore waters worldwide [1, 17]. In French coastal waters, three dinoflagellates now represent a serious potential nuisance or health hazard [2]. Among them, several species of the genus *Dinophysis* seem to have recently changed from low-density occurrence [5] to cell densities high enough to warrant monitoring in programs related to public health [3]. Moreover, occurrences of *Dinophysis* at noxious levels have spread from Brittany, where they were first recorded in 1983, both northward and southward, as well as into the Mediterranean Sea [4, 12]. *Dinophysis* spp. always occur at low densities (not more than a few thousand cells L^{-1}) even in summer [11]. On the other hand, considerable increases in cell density have been recorded in both offshore and coastal Atlantic waters [13]. In contrast, *D. fortii* populations in Japanese waters first develop offshore and then are introduced by currents into nearshore areas where it renders farmed shellfish toxic [7].

An investigation was thus started in May 1989 on the French Atlantic coast, in the vicinity of La Rochelle, with the aims: (i) to point out what environmental conditions lead to increases in *Dinophysis;* (ii) to investigate whether nutrient input of

agricultural origin favours growth; and (iii) to determine where increased cell densities first appear.

STUDY AREA AND METHODS

The area investigated (Fig. 1) is divided into three parts: (i) two land-locked subareas (pertuis Breton; bassin Marennes-Oléron) with important shellfish industries; (ii) an intermediate strait (pertuis d'Antioche); and (iii) the open ocean up to the 100 m isobath. Coastal waters are enriched by agricultural runoff contributed by two rivers. In the strait, tidal currents transfer water from the open sea to the coast, while the displaced water exits through another strait (pertuis de Maumusson) after bathing shellfish installations. The sampling strategy was modified yearly in order to favour spatial distribution and the temporal variations. In May 1989, 24 stations, from the coast to the open sea (-40 m), were visited four times. During May-June 1990, only 9 of the 24 stations were visited eight times, and one station in the strait was monitored continuously for 48 hours. In 1991, from late April to late September, 11 vertical profiles were taken at six stations located from the coast to the open sea (-100 m). In addition, a drogue was deployed just outside the strait and followed for three days, and "METEOSAT" satellite images analyzed to check whether "true" open-sea circulation patterns characterized the most offshore stations. In the continuous vertical profile, temperature, salinity (CTD probe) and irradiance (Licor quanta meter) were recorded before discrete sampling (Niskin bottle) for nutrients, chlorophyll-a and counts of phytoplankton abundance using Utermöhl's method.

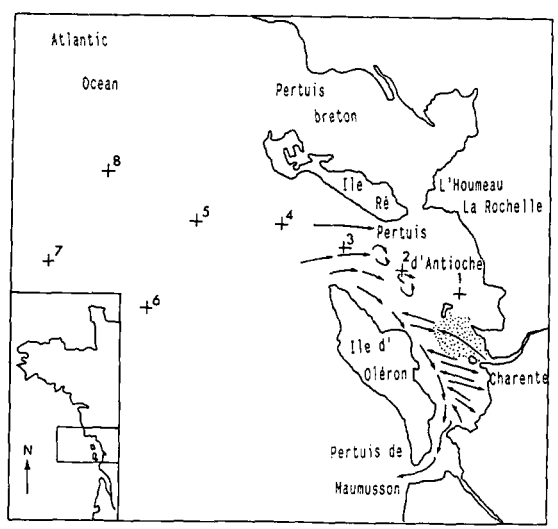

FIG. 1: Study area and sampling stations (+); arrows indicate tidal and residual currents; dotted area, nutrient-rich plume of River Charente.

RESULTS AND DISCUSSION

Highest *Dinophysis* densities were recorded in 1990. Furthermore, over the three-year period the rate of cell increase was always positively related to stable stratification of the water column (Fig. 2). Like many phytoflagellates which accumulate at the thermocline [10, 15, 16], *Dinophysis* spp. were generally more abundant in the water layer limited by the upper and lower values of the thermocline (Fig. 3). They were scarce below this layer and absent near the bottom, as in the case of *D. fortii* observed in Japan [8]. Since the thermal stratification became established first in offshore waters, growth occurred in the open sea before it did in the landlocked areas: again with the same sequence as for *D. fortii* off Japan [7, 9]. Flood-tide currents carried *Dinophysis* cells from offshore to inshore waters, whereas ebb-tide currents advected some cells back out (Figs. 1, 4).

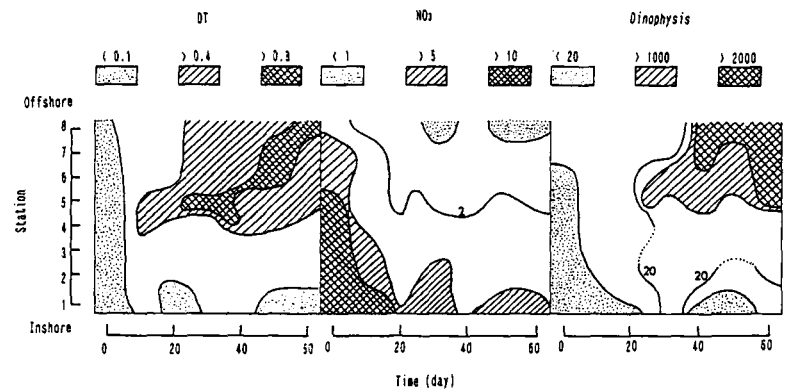

Fig. 2: Temperature gradient (°C m^{-1}) within "thermocline layer", NO_3 concentration (µM) in upper 15 m and *Dinophysis* numbers (cells L^{-1}) from 22 April to 19 June 1990 along nearshore (station 1) to offshore (station 7) gradients.

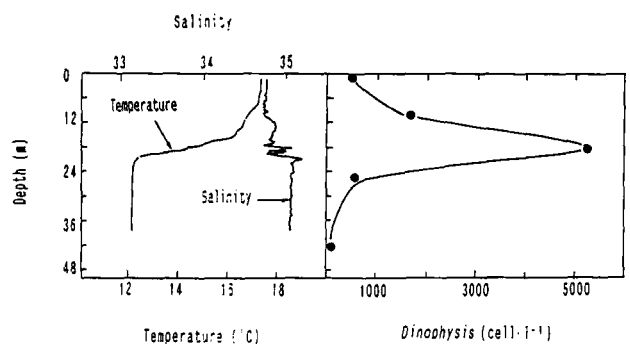

FIG. 3: Variation with depth in temperature, salinity and *Dinophysis* spp. density off the strait of Antioche on 19 June 1990.

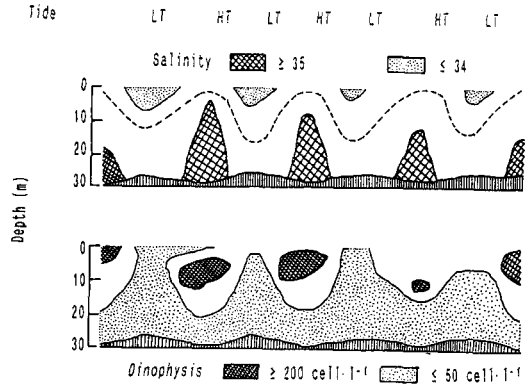

FIG. 4: Evolution, according to tidal salinity cycle, of *Dinophysis* spp. cell density in the "strait of Antioche" on 22-24 May 1990 (48-h continuously monitored station). Flood-tide currents bring high-salinity, offshore water towards the coast; ebb-tide currents return less saline nearshore waters offshore.

Dinophysis spp. growth, which occurred in water nutrient impoverished by previous phytoplankton growth (e.g. NO_3, Fig. 2), was not correlated with any change in nutrient concentrations. Moreover, since no experimental evidence is yet available worldwide, except for *D. rotundata* reported to be phagotrophic [6], it remains unknown whether growth of *Dinophysis* spp. is related to uptake of organic or inorganic nutrients. It is clear from our data (Fig. 2) that nutrient input from agricultural and domestic origins does not directly favor inshore *Dinophysis* growth. Since increased cell densities of *Dinophysis* cf. *acuminata* in the Seine plume, however, parallel increased nitrate concentrations [14], an indirect stimulation accompanying inorganic nutrient enrichment is a possibility.

Dinophysis spp. growth was slow, 0.25 net division day^{-1} during its fastest growth period (Fig. 5), similar to that of other dinoflagellates. During late spring and summer, the importance of dinoflagellates relative to diatoms increased, finally representing 80% of the microphytoplankton in the upper 15 m in offshore waters. This increased relative importance resulted both from their growth and the sedimentation of diatoms; the maximum chlorophyll-a content was recorded deeper as the season progressed. Both the nano- (<20 µm) and picophytoplankton (<3 µm) size classes relative to the micro-size class also increased in the upper layer, finally representing 80% and 40% of total chlorophyll-a content, respectively (Fig. 5). Six *Dinophysis* species were recorded, none of which was present in all samples (detection limit: 7 cells L^{-1}). *D. sacculus* and *D. acuminata* were the most abundant and frequent species, *D. rotundata* occurred similarly, but at markedly lower densities, and *D. acuta*, *D. tripos* and *D. caudata* were scarce and episodic.

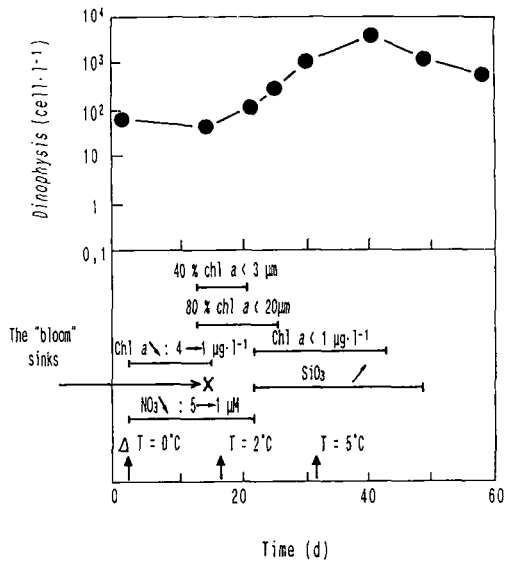

FIG. 5: Summary of changes in main environmental conditions related to *Dinophysis* spp. growth in coastal waters off La Rochelle in 1990. Ordinate value: T_0 = 20 April; T_{60} = 20 June. *Dinophysis* growth started when thermal stratification had established, Spring-diatom bloom had ended, the cells were sinking (chlorophyll-*a* maximum deep), the nutrient reservoir was greatly impoverished, and relative importance of <3 µm and <20 µm size fractions was increasing. Apparent *in situ* growth rate was 0.25 division d^{-1}.

CONCLUSION

Within the "Bay of Biscay" system, the area investigated is but a small component. To the extent that satellite images and discrete measurements (temperature, chlorophyll-a) confirm that farthest offshore stations were truly located in offshore waters and representative of them, it is clear that *Dinophysis* comes from the open sea in this part of the French Atlantic coast. It is also clear that nutrients from land origin do not promote *Dinophysis* growth.

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IMPROVED ANALYTICAL METHODOLOGY FOR THE DERIVATIZATION AND HPLC-FLUOROMETRIC DETERMINATION OF OKADAIC ACID IN PHYTOPLANKTON AND SHELLFISH

ROBERT W. DICKEY, H. RAY GRANADE and F. ALADAR BENCSATH
Division of Seafood Research, U.S. Food and Drug Administration, P.O. Box 158, Dauphin Island, AL 36528

ABSTRACT

A method for the derivatization and liquid chromatographic determination of the marine toxin okadaic acid (OA) was developed. Okadaic acid was extracted from cultured dinoflagellates and from homogenized shellfish with aqueous 80% methanol and partitioned into chloroform from water. OA in the extract was derivatized in acetonitrile with 1-bromoacetylpyrene. The product, pyrenacyl okadaate, was then separated from excess reagent and reaction by-products by solid phase extraction following the method of Lee [1]. Analyses were carried out in acetonitrile solution on a C_{18} reversed-phase column using aqueous 75% acetonitrile as mobile phase with fluorometric detection at 365 nm excitation and 418 nm emission. Deoxycholic acid (DOCA) derivatized and extracted by the same procedure was employed as internal standard. Peak height coefficients of variation for 12 randomized injections of three OA standard concentrations (5, 10 and 50 ng) with fixed DOCA concentrations (40 ng) ranged from 1.72% to 8.65% for OA and 1.82% to 9.23% for DOCA. The relative retention time OA/DOCA was 0.598 $\pm$ 0.001 (n = 13). Standard OA recoveries from shellfish at spike levels of 1.0 μg/g or larger were greater than 95%. The method was linear between 1.0 and 80 ng of OA injected and the lower limit of detection was 0.1 ng.

INTRODUCTION

Okadaic acid (OA) is the parent molecular structure for the polyether toxins responsible for diarrhetic shellfish poisoning (DSP). The assemblage of toxins is comprised of OA, 35-methyl-OA, and several 7-acylesters of OA [2, 3]. The wide distribution of OA and its analogues in the world oceans is highly significant in general because of the numerous opportunities for entry into the marine food web leading to humans. It has been postulated, though not yet substantiated, that OA and related compounds may also be involved in ciguatera fish poisoning, another serious problem in many regions of the world. OA and analogues are produced by dinoflagellates of the genera Dinophysis and Prorocentrum [4, 5, 6, 7, 8]. The distinctive structures of these toxins appear to impart unique physiological activities beyond the gastrointestinal affects by which diarrhetic shellfish poisoning derives its name. The tumor-promotion and protein phosphatase inhibition exhibited by these compounds have led to their use as molecular probes in the study of tumorgenesis and basic cellular metabolism [9]. For the same reason, this group of toxins is viewed as potentially significant human health hazards. The isolation of DSP-toxins for use as molecular probes and as analytical standards in the development of methods for their determination in seafood is presently the focus of much attention world-wide.