Vol. 100: 185–195, 1993 | MARINE ECOLOGY PROGRESS SERIES
Mar. Ecol. Prog. Ser. | Published October 5

Discrimination between domoic-acid-producing and nontoxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence

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ABSTRACT: Separate polyclonal antibodies were developed against cell surface antigens of the 2 forms of the pennate diatom *Pseudonitzschia* (previously known as *Nitzschia*) *pungens*, i.e. forma *multiseries* (the domoic-acid-producing form) and forma *pungens* (the nontoxic form). Positive antigenic reactions were visualized with epifluorescence microscopy, using a fluorescein isothiocyanate (FITC) indirect immunofluorescence assay. The assay successfully distinguished 31 clones of f. *multiseries* from the 17 clones of f. *pungens* tested, with no cross reactions of the antisera between the 2 forms. The antisera were active against *P. pungens* cells isolated from Prince Edward Island and Nova Scotia, Canada, and Massachusetts, Rhode Island, Texas and Washington, USA. Of the 27 other clones tested from the genera *Pseudonitzschia* and *Nitzschia*, 2 other domoic-acid-producing species (*P. australis* and *P. pseudodelicatissima*) and 2 nontoxic species (*P. subcurvata* and *P. fraudulenta*) showed a slight positive response to the antisera, as did the domoic-acid-producing pennate diatom *Amphora coffaeiformis*. These reactions are not great enough to cause concern about misidentification, but pose questions about phylogenic relationships. Other representatives from the class Bacillariophyceae and from 8 other major classes of phytoplankton did not cross react with the antisera. Excellent labelling was obtained with live cells and those frozen at –60°C, or preserved in 2% glutaraldehyde-paraformaldehyde, 2% borate-buffered formalin or 2% paraformaldehyde. Immunofluorescence shows great promise as a technique to distinguish between the 2 forms of *P. pungens* for research and monitoring purposes.

INTRODUCTION

In December 1987, an outbreak of food poisoning due to the consumption of mussels *Mytilus edulis* from Prince Edward Island (PEI), Canada, resulted in 3 deaths and 107 cases of illness (Todd 1993). The syndrome is called Amnesic Shellfish Poisoning (ASP) due to one of the characteristic symptoms, impairment of short-term memory. The neurotoxin in the mussels was identified as domoic acid (Wright et al. 1989), and its source was traced to the pennate diatom *Nitzschia pungens* f. *multiseries* (Bates et al. 1989). Hasle (1993) has recently transferred many of the *Nitzschia* species, including *N. pungens*, that were previously in the section Pseudonitzschia to the genus *Pseudonitzschia*; we have adopted this new nomenclature. The domoic-acid-producing diatom has also been isolated from coastal waters of Texas (Fryxell et al. 1990, Dickey et al. 1992), Massachusetts and Rhode Island, USA. Domoic acid has also appeared on the west coast of the United States: the implicated organism in California is the pennate diatom *P. australis* (Buck et al. 1992, Fritz et al. 1992, Garrison et al. 1992), but the causative organism in Oregon and Washington remains uncertain (Wood & Shapiro 1993). These events have considerably broadened worldwide concern about *Pseudonitzschia* spp. and domoic acid intoxication.

A complicating factor for programs that monitor for toxic phytoplankton has been the occurrence of *Pseudonitzschia pungens* blooms without the accompanying domoic acid (Smith et al. 1990). This is a consequence of the fact that *P. pungens* occurs in 2 forms, one that produces domoic acid and is therefore toxic

(f. *multiseries*) and the other that is nontoxic (f. *pungens*). The 2 forms, moreover, are indistinguishable by optical microscope observations and can only be distinguished after the acid-cleaned silica frustules are examined with an electron microscope; f. *pungens* has 1 to 2 rows of large intercostal poroids rather than the 3 or more rows of smaller poroids found in f. *multiseries* (Hasle 1965). The existence of the 2 forms can pose problems because of the possibility of false positive indications of a toxic event if the nontoxic f. *pungens* is dominant (Smith et al. 1990). It is evident that an alternative method for discriminating between the toxic and nontoxic forms of *P. pungens* would be an advantage over the time-consuming and inconvenient method of scanning electron microscopy. Development of an immunofluorescence assay may prove useful to distinguish between the 2 forms.

Immunochemical techniques are increasingly being used to detect and identify marine phytoplankton. In one approach, the indirect immunofluorescence assay, cells are first incubated with a primary antiserum, and then with a fluorescently tagged secondary antiserum directed against the primary antibody, thus rendering the target cells visible with a fluorescence microscope (Campbell et al. 1988). The primary antibodies are obtained by immunizing rabbits with whole phytoplankton cells, and are thus directed against cell surface antigens. This approach was used to identify ultraplanktonic marine coccoid cyanobacteria (Campbell et al. 1983, Campbell & Carpenter 1987, Campbell 1988), as well as other phytoplankton which are difficult to identify because of their small size and/or lack of characteristic morphological features (Fliermans & Schmidt 1977, Anderson et al. 1988, Shapiro et al. 1989a, b). Taxonomic relationships among marine phytoplankton have been studied by Campbell & Carpenter (1987), Hiroishi et al. (1988), Shapiro et al. (1989a) and Uchida et al. (1989), using immunochemical approaches. Polyclonal (Anderson et al. 1988, Vrieling et al. 1993a, b) and monoclonal (Hiroishi et al. 1988, Uchida et al. 1989, Nagasaki et al. 1991, Sako et al. 1992) antibodies have recently been used to detect nuisance and toxic marine phytoplankton cells. Here we report the results of a fluorescein isothiocyanate (FITC) indirect immunofluorescence assay using polyclonal antibodies able to discriminate between the toxic and nontoxic forms of *Pseudonitzschia pungens*.

MATERIALS AND METHODS

Immunization cultures. Nonaxenic *Pseudonitzschia pungens* f. *multiseries* (clone POM; Seguel 1991) and *P. pungens* f. *pungens* (clone BRUD B; isolated from the Brudenell River, PEI, August 1989) used for immunization were grown in f/2 medium (Guillard & Ryther 1962) at 18°C and at a photon flux density of about 100 $\mu E\ m^{-2}\ s^{-1}$ provided by Cool-White fluorescent bulbs (12:12 h light:dark cycle). Cells were harvested by centrifugation at the end of the exponential growth phase (Day 7), and were thus not yet toxic (Bates et al. 1991) as verified by the fluorenylmethoxycarbonyl (FMOC) fluorescence derivatization technique (Pocklington et al. 1990). The pelleted cells were preserved in 0.6% (v/v, final concentration) paraformaldehyde prepared in seawater, divided into 5 duplicate 1.0 ml aliquots in microcentrifuge tubes, each containing 5.7×10^6 cells of f. *multiseries* or 7.5×10^6 cells of f. *pungens*, and stored at 4°C until used for immunization. The identity of the cells was confirmed by scanning electron microscopy (Bates et al. 1989).

Test cultures. The phytoplankton used for the immunological tests, and their origins, are given in Tables 1 to 4. Test cultures were generally harvested during the late-exponential to early-stationary phase, and preserved in 2 or 5% (v/v, final concentration) paraformaldehyde-glutaraldehyde. This was a 1:1 solution of 1% paraformaldehyde (10 g solid paraformaldehyde per litre distilled water) in 25% (full-strength stock solution) glutaraldehyde. In addition, *Pseudonitzschia pungens* cells were tested either fresh, frozen or preserved, as indicated, to determine the best fixation procedure to use.

Immunization of rabbits. Rabbits were immunized to produce separate polyclonal antibodies against cell surface antigens of the 2 forms of *Pseudonitzschia pungens* at East Acres Biologicals (Southbridge, MA). Cells were washed with phosphate-buffered saline (PBS) to remove the preservative, mixed with Freund's complete adjuvant, and a 0.5 ml mixture was then subcutaneously injected into New Zealand White rabbits (initially, 2 for f. *multiseries* and 2 for f. *pungens*). Subcutaneous primary immunization was chosen in preference to intravenous injections to increase the residence time of the cells for antibody production, and because of the concern that whole *P. pungens* cells (about 70 µm long × 5 µm wide) could become clogged in the rabbit's circulatory system. No attempt was made to break apart the chains, which were less than 4 cells long. A schedule of 5 boosts, with 0.5 ml of cells in Freund's incomplete adjuvant, was carried out at 3 wk intervals. When an acceptable titre was achieved, production bleeds were obtained weekly via the marginal ear vein. The blood was allowed to clot for 1 h in a water bath, and then was centrifuged twice to remove red blood cells, yielding 20 to 25 ml of serum per bleed. Each delivery of serum was tested, divided into 1.0 ml aliquots and stored at -20°C. The rabbits were then boosted monthly with the same cell concentration as was used for the immunization. Different rabbits were

Table 1. Clones of *Pseudonitzschia pungens* f. *multiseries*. Summary of results obtained by indirect immunofluorescence assay using antisera raised against *P. pungens* f. *multiseries* and *P. pungens* f. *pungens*. Immunological cross reactivity is scaled from very strong (+++) to none (–). Clone prefixes are TV: collection of T. A. Villareal; F: collection of G. A. Fryxell; CCMP: Guillard-Provasoli Culture Collection of Marine Phytoplankton

Clone	Date isolated	Location isolated	Isolated by	Antiserum f. *multiseries*	Antiserum f. *pungens*
NPARL	14 Mar 1988	Cardigan Bay, PEI, Canada	S. Bates	++	–
BRUD A	10 Aug 1989	Brudenell River, PEI, Canada	C. Léger	+++	–
TKA-2	25 May 1989	Galveston, TX, USA	T. Ashworth	+	–
POM	Nov 1989	Pomquet Harbour, NS, Canada	M. Seguel	+++	–
KP-59	18 Oct 1991	New London Bay, PEI, Canada	K. Pauley	+++	–
TV-2	2 Mar 1992	Massachusetts Bay, MA, USA	T Villareal	+	–
TV-7	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	+++	–
TV-12	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	+++	–
TV-13	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	++	–
TV-14	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	+++	–
TV-17	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	+++	–
TV-18	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	++	–
B991K	Sep 1991	Narragansett Bay, RI, USA	P. Hargraves	+++	–
892G	Aug 1992	Narragansett Bay, RI, USA	P. Hargraves	+++	–
F279m[a]	13 Aug 1989	Galveston, TX, USA	G. Fryxell	+++	–
F288m[b]	14 Aug 1989	Galveston, TX, USA	G. Fryxell	++	–
F296	10 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
F302	11 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
F307	8 Jun 1990	Galveston, TX, USA	G. Fryxell	+++	–
F340	29 Mar 1991	Galveston, TX, USA	G. Fryxell	++	–
F433	27 May 1992	Galveston, TX, USA	G. Fryxell	+++	–
F445	27 May 1992	Galveston, TX, USA	G. Fryxell	+++	–
F415d	17 Jun 1992	Galveston, TX, USA	G. Fryxell	++	–
F435	27 May 1992	Galveston, TX, USA	G. Fryxell	+++	–
CCMP562	10 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
CCMP565	25 May 1989	Galveston, TX, USA	T. Ashworth	++	–
CCMP566	18 Jul 1989	Galveston, TX, USA	M. Davis	++	–
CCMP567	11 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
CCMP569	11 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
CCMP570	11 Mar 1990	Galveston, TX, USA	G. Fryxell	+++	–
CCMP571	18 Jul 1989	Galveston, TX, USA	M. Davis	+++	–

[a]CCMP573 (originally identified as *P. pungens* f. *pungens*). [b]CCMP572 (originally identified as *P. pungens* f. *pungens*)

immunized with f. *multiseries* on 2 occasions (July 1991 and February 1992).

Immunofluorescence protocol. The indirect immunofluorescence protocol used (Fig. 1) was modified from that of Anderson et al. (1988). All solutions were filter sterilized (0.22 µm Millipore filter). Two incubation approaches were evaluated using *Pseudonitzschia pungens* as the test organism: cells were incubated with the test serum on either 1.0 µm Nuclepore polycarbonate membrane filters (Anderson et al. 1988), or in 1.5 ml microcentrifuge tubes. Both approaches were successful, but the latter was used for the majority of the tests presented here. For the centrifugation approach, 1.5 to 3.0 ml of culture was first rinsed 3 times [Eppendorf Model 5415 microcentrifuge; 2 min at 8000 rpm (5220 × *g*)] with PBS (0.02 M phosphate; 0.15 M NaCl; pH 7.45) to remove the seawater or preservative. Cells were then incubated for 40 min at room temperature with 1.0 ml of a blocking agent, 3% normal goat serum (G-9023, Sigma) in PBS. Cells were centrifuged 3 times in 1.0 ml of PBS as a rinse. This was followed by a 40 min incubation in the primary test antiserum, diluted to 1:10 with PBS; higher dilutions were used in other tests. Parallel samples were incubated for 40 min with normal rabbit serum (from the preimmunization rabbit or R-9133 from Sigma) in place of the antiserum, as a control. After rinsing 3 times with 1.0 ml of PBS, the cells were incubated for 20 min with 1.0 ml of FITC-conjugated goat anti-rabbit antiserum (F-0382, Sigma), diluted to 1:800 with PBS, as the secondary antibody. We later showed that the above incubation times could be shortened to 15 min with the blocking agent and primary antiserum and 10 min with the secondary antiserum, but times should be tested for each new batch of serum. After a final triplicate rinse and resuspension in 0.5 ml of PBS, a drop of sample was placed on a microscope slide and examined at 250× or 400× with a Leitz Orthoplan fluorescence

Table 2. Clones of *Pseudonitzschia pungens* f. *pungens*. Summary of results obtained by indirect immunofluorescence assay using antisera raised against *P. pungens* f. *multiseries* and *P. pungens* f. *pungens*. Immunological cross reactivity is scaled from very strong (+++) to none (–)

Clone	Date isolated	Location isolated	Isolated by	Antiserum f. *multiseries*	Antiserum f. *pungens*
BRUD B	10 Aug 1989	Brudenell River, PEI, Canada	C. Léger	–	+++
BRUD C	24 Aug 1989	Brudenell River, PEI, Canada	C. Léger	–	+++
CARA	17 Nov 1989	Cardigan Bay, PEI, Canada	C. Léger	–	+++
NP90-07	31 Jul 1990	Cardigan Bay, PEI, Canada	C. Léger	–	++
NP90-08	31 Jul 1990	Cardigan Bay, PEI, Canada	C. Léger	–	++
KP-60	18 Oct 1991	New London Bay, PEI, Canada	K. Pauley	–	++
TV-16	2 Mar 1992	Massachusetts Bay, MA, USA	T. Villareal	–	++
B991I	Sep 1991	Narragansett Bay, RI, USA	P. Hargraves	–	–
F378	21 Sep 1991	Galveston, TX, USA	G. Fryxell	–	+
F384	1 Nov 1991	Newport, RI, USA	G. Fryxell	–	++
F386	1 Nov 1991	Newport, RI, USA	G. Fryxell	–	++
F390	1 Nov 1991	Newport, RI, USA	G. Fryxell	–	++
F391	1 Nov 1991	Newport, RI, USA	G. Fryxell	–	+++
F394	1 Nov 1991	Newport, RI, USA	G. Fryxell	–	++
F400	1 Nov 1991	Narragansett, RI, USA	G. Fryxell	–	++
F402	1 Nov 1991	Narragansett, RI, USA	G. Fryxell	–	+++
F403	1 Nov 1991	Narragansett, RI, USA	G. Fryxell	–	+++
ORI-8	14 Dec 1991	Ilwaco, WA, USA	M. Villac	–	++
CCMP572[a]	14 Aug 1989	Galveston, TX, USA	G. Fryxell	++	–
CCMP573[a]	13 Aug 1989	Galveston, TX, USA	G. Fryxell	++	–

[a]Now f. *multiseries* (G. A. Fryxell pers. comm.); see Table 1, Clones F279m and F288m

Table 3. Phytoplankton species of the genera *Pseudonitzschia* (other than *P. pungens*) and *Nitzschia*. Summary of results obtained by indirect immunofluorescence assay using antisera raised against *P. pungens* f. *multiseries* and *P. pungens* f. *pungens*. Immunological cross reactivity is scaled from very strong (+++) to none (–)

Clone	Species	Location isolated	Isolated by	Antiserum f. *multiseries*	Antiserum f. *pungens*
NP90-05	*Nitzschia* sp.	Cardigan Bay, PEI, Canada	C. Léger	–	–
CCMP551	*Nitzschia brevirostris*	Oyster Pond, MA, USA	R. Guillard	–	–
CCMP554	*N. curta*	57° 46' S, 31° 52' W	G. Fryxell	–	–
CCMP555	*N. curvilineata*	New Haven, CT, USA	J. Lewin	–	–
CCMP557	*N. cylindrus*	Antarctica	G. Fryxell	–	–
CCMP558	*N. frustulum*	Prospect, NS, Canada	J. Lewin	–	–
CCMP561	*N. punctata*	San Diego, CA, USA	J. Lewin	–	–
CCMP576	*N.* cf. *constricta*	Coral Sea, Australia	S. Jeffrey	–	–
CCMP1118	*N.* cf. *ovalis*	Magdalena Bay, Mexico	A. Dodson	–	–
CCMP1309	*Pseudonitzschia seriata*	Resolute Bay, NWT, Canada	R. Smith	–	–
CCMP1437	*P. subcurvata*	McMurdo Sound, Antarctica	G. Fryxell	+[a]	+[a]
CCMP1438	*P. subcurvata*	McMurdo Sound, Antarctica	G. Fryxell	–	–
CCMP1443	*P. subcurvata*	McMurdo Sound, Antarctica	G. Fryxell	–	–
CCMP1444	*P. subcurvata*	McMurdo Sound, Antarctica	G. Fryxell	+	+
B991H	*P. fraudulenta*	Narragansett Bay, RI, USA	P. Hargraves	+[a]	–
N1	*P. pseudodelicatissima*	Massachusetts Bay, MA, USA	T Villareal	–	–
F379	*P. pseudodelicatissima*	Galveston, TX, USA	G. Fryxell	+[a]	–
F380	*P. pseudodelicatissima*	Galveston, TX, USA	G. Fryxell	+[a]	–
F397	*P. pseudodelicatissima*	Newport, RI, USA	G. Fryxell	+[a]	–
P:SID	*P. australis*	Monterey Bay, CA, USA	D. Douglas	–	–
MB27	*P. australis*	Monterey Bay, CA, USA	M. Villac	+[a]	+[a]
MB1	*P. australis*	Monterey Bay, CA, USA	M. Villac	+[a]	+[a]
MB7d	*P. australis*	Monterey Bay, CA, USA	M. Villac	+[a]	+[a]
MB8d	*P. australis*	Monterey Bay, CA, USA	M. Villac	+[a]	+[a]
MB33a	*P. australis*	Monterey Bay, CA, USA	M. Villac	+[a]	+[a]
ORI-2	*P. australis*	Ilwaco, WA, USA	M. Villac	+[a]	+[a]
ORI-4	*P. australis*	Ilwaco, WA, USA	M. Villac	+[a]	+[a]

[a]Slight labelling

Table 4. Phytoplankton species representing major classes. Summary of results obtained by indirect immunofluorescence assay using antisera raised against *Pseudonitzschia pungens* f. *multiseries* and *P. pungens* f. *pungens*. Immunological cross reactivity is scaled from very strong (+++) to none (–). Isolation locations are given in the CCMP Catalogue of Strains, except for *Bacillaria paxillifer*, which was isolated in 1990 from Galveston, Texas, USA, and *Amphora coffaeiformis*, which was isolated in 1987 by L. Maranda from Cardigan Bay, PEI, Canada

Clone	Species	Class	Antiserum f. *multiseries*	Antiserum f. *pungens*
CCMP169	*Chaetoceros concavicornis*	Bacillariophyceae	–	–
CCMP398	*Fragilaria pinnata*	Bacillariophyceae	–	–
CCMP543	*Navicula pelliculosa*	Bacillariophyceae	–	–
CCMP630	*Phaeodactylum tricornutum*	Bacillariophyceae	–	–
CCMP974	*Thalassionema nitzschoides*	Bacillariophyceae	–	–
CCMP1049	*Thalassiosira weissflogii*	Bacillariophyceae	–	–
CCMP1442	*Thalassiosira gravida*	Bacillariophyceae	–	–
CCMP1439	*Fragilariopsis ritscheri*	Bacillariophyceae	–	–
CCMP1332	*Skeletonema costatum*	Bacillariophyceae	–	–
JW-1	*Bacillaria paxillifer*	Bacillariophyceae	–	–
BPT-11	*Amphora coffaeiformis*	Bacillariophyceae	+[a]	+[a]
CCMP364	*Dunaliella tertiolecta*	Chlorophyceae	–	–
CCMP452	*Heterosigma akashiwo*	Raphidophyceae	–	–
CCMP462	*Isochrysis galbana*	Prymnophyceae	–	–
CCMP674	*Porphyridium aeruginium*	Rhodophyceae	–	–
CCMP739	*Rhodomonas lens*	Cryptophyceae	–	–
CCMP833	*Synechoccus* sp.	Cyanophyceae	–	–
CCMP878	*Tetraselmis apiculata*	Prasinophyceae	–	–

[a]Slight labelling

microscope (Leitz I2 filter block for excitation light; Block I for transmitted light) or a Zeiss IM35 inverted epifluorescence microscope (Anderson et al. 1988). All tests were read 'blind', i.e. the person reading and scoring the tests did not know the identity of the test material. Each sample was rated for the visual intensity of fluorescence from excellent (+++) to weak (+), with a (–) indicating no antigenic reaction relative to the control incubated with preimmunization rabbit serum.

RESULTS

Titre tests

Usable antisera were obtained 116 d after the primary injection for the first set of rabbits immunized in July 1991 with *Pseudonitzschia pungens* f. *multiseries* or *P. pungens* f. *pungens*, and by Day 52 for the second set immunized in February 1992 with *P. pungens* f. *multiseries*. For f. *multiseries*, an antiserum dilution of 1:1000 was the lowest concentration that produced visible labelling around the perimeter of the cell. The f. *pungens* antiserum had a slightly lower titre, with labelling at a dilution of 1:500. A working dilution of 1:10 to 1:20 was generally used for the tests, as no cross reactivity was observed between the 2 forms, even at these high antibody concentrations. Successful labelling was characterized by a bright green fluorescent band around the outer edge of the cell (Fig. 2), indicating that surface antigens were indeed labelled. We observed no background fluorescence in the cells, other than the occasional yellow-red-orange autofluorescence due to chlorophyll *a*.

Preservation techniques

Several different preservation techniques were evaluated to determine which gave acceptable immunofluorescent results. Cells preserved with solutions of 2% (v/v, final concentration) glutaraldehyde-paraformaldehyde, paraformaldehyde, or borate-buffered formalin gave the strongest fluorescence labelling (Table 5). *Pseudonitzschia pungens* cells frozen at –60 °C with no preservative also gave a bright fluorescence and maintained their shape even after thawing. The labelling intensity of the preserved or frozen cells was about equal to that of 'live' cells. Cells retained their FITC fluorescence for at least 6 mo after being labelled, if kept refrigerated at 4 °C.

Effects of culture age

The time since inoculation into batch culture appeared to have a small, but noticeable, influence on

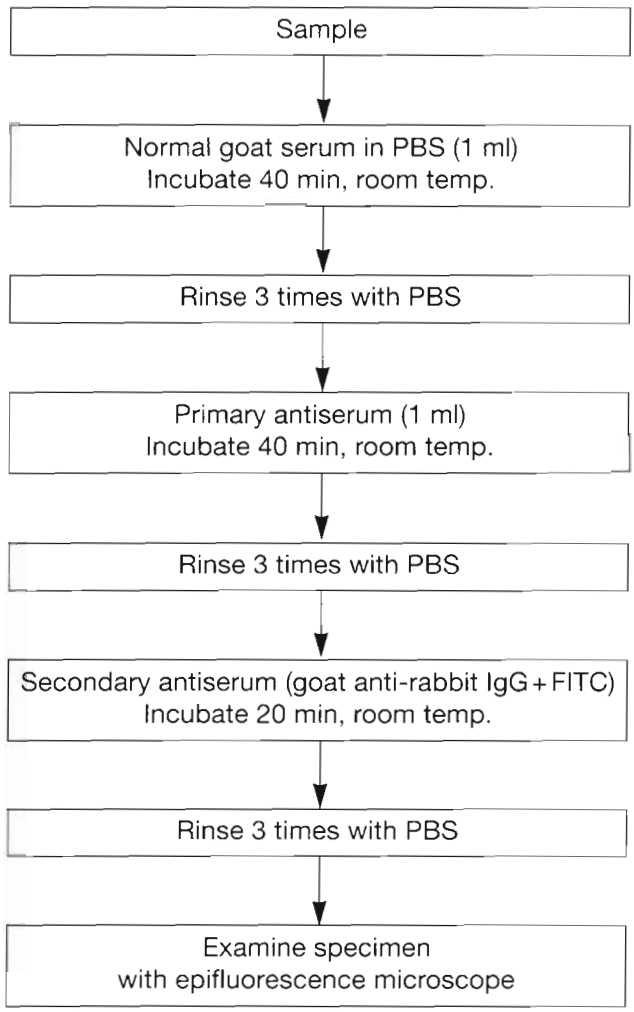

Fig. 1. Immunofluorescence protocol (Anderson et al. 1988), applicable to either the filtration or the centrifugation approach. Incubation times can be shortened (see text)

the strength of the immunological reaction for both f. *multiseries* and f. *pungens* (Table 6). Cultures in the mid- to late-stationary phase appeared to have a greater affinity for the antisera than exponential to early-stationary phase cultures.

Effects of incubation time

Decreasing the incubation time with the sera by 75% (i.e. 10 min each for the blocking agent and primary antiserum, and 5 min for the secondary antiserum) still resulted in good labelling ('++') of the cells. However, a visibly stronger labelling ('+++') was observed with the longer incubation times normally used (i.e. 40 min each for the blocking agent and primary antiserum, and 20 min with the secondary antiserum).

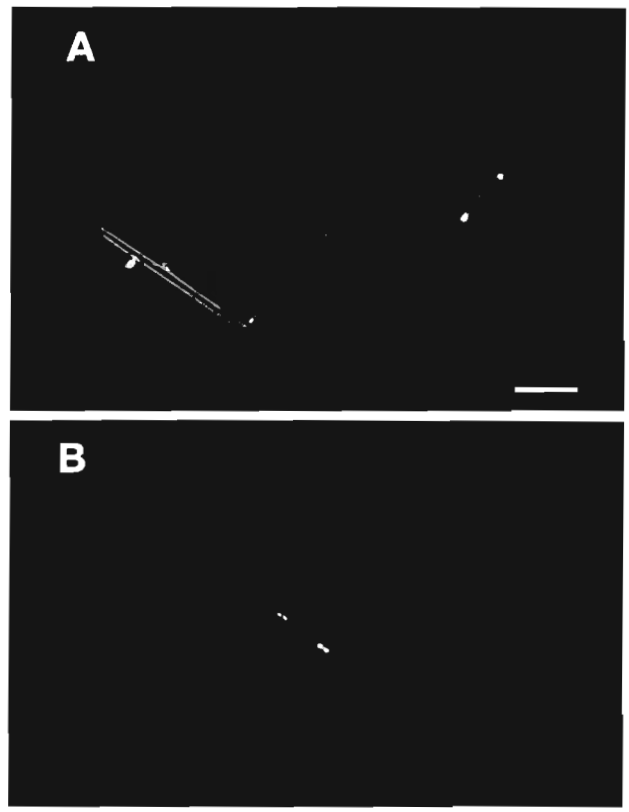

Fig. 2. *Pseudonitzschia pungens*. Epifluorescence photomicrographs of *P. pungens* f. *pungens* (Clone F391). (A) Cells incubated with the f. *pungens* antibody and the FITC-conjugated goat anti-rabbit antibody. The label is confined to the outer cell surface as shown by the bright band around the parameter of the cells; labelling for *P. pungens* f. *multiseries* incubated with the f. *multiseries* antiserum is identical in appearance. Most chains break up into single cells during the centrifugation and incubation steps. (B) Control cells incubated with normal rabbit serum in place of the f. *pungens* antiserum, indicating the absence of labelling. The paired spots are autofluorescence of chlorophyll *a* within the chloroplasts, normally seen as yellow-red-orange rather than as the green fluorescence of FITC. Scale bar = 20 µm

Cross reactivity tests

Over 150 phytoplankton cultures were used to test for specificity and cross reactivity, i.e. reactions of the antiserum against antigen(s) not present in the immunization preparation (Campbell et al. 1988). Separate antibodies were produced against the cell surface of the 2 forms of *Pseudonitzschia pungens*, and each of the resulting antisera were specific to f. *multiseries* (Table 1) or to f. *pungens* (Table 2), with no cross reactivity with the other form (Tables 1 & 2). There was no evident difference in labelling between axenic and nonaxenic test cultures. Positive antibody labelling was found not only with clones of *P. pungens* isolated from eastern PEI, the origin of the cells used

Table 5. Comparison of various preservation techniques, including freezing, on the immunofluorescent reaction between f. *pungens* antiserum (diluted to 1:20) and *Pseudonitzschia pungens* f. *pungens* (Clone BRUD B); samples remained in the fixative or were frozen for 11 d prior to testing. Immunological reactivity is scaled from very strong (+++) to good (++)

Preservation technique	Strength (%)	Immunofluorescent reaction
Glutaraldehyde-paraformaldehyde	2.0	+++
Paraformaldehyde	2.0	+++
Borate-buffered formaldehyde	2.0	+++
Lugol's iodine	4.0	++
Glutaraldehyde	0.5	++
Formalin-acetic acid	2.0	++
Frozen at −60 °C		+++
Frozen at −20 °C		++

Table 6. *Pseudonitzschia pungens*. Effect of culture age on the immunological response of antisera raised against *P. pungens* f. *multiseries* and *P. pungens* f. *pungens*. Samples had been kept frozen at −20 °C for 6 mo prior to the assay. Immunological reactivity is scaled from very strong (+++) to weak but observable (+)

Culture age (d)	f. *multiseries* clone (BRUD A)	f. *pungens* clone (CARA)
4	+	++
9	+	++
16	++	+++
24	++	+++
32	++	+++

for the immunizations, but also with clones from northern PEI, Nova Scotia, Massachusetts, Rhode Island, Texas, and Washington (Tables 1 & 2). There was a slight difference in labelling intensity with clones originating from these different North American locations and at different times of the year from these locations. However, since there were also small differences in fluorescence intensity even between clones isolated from the same location, this is not considered to be a problem. The positive reactions were unquestionable.

In 2 cases (Clones CCMP572 and CCMP573; Table 2), the antibody assays indicated that the diatom was *Pseudonitzschia pungens* f. *multiseries*, but the expected identification given was f. *pungens*. When the same clones (designated as F279m and F288m in Table 1) were received from Dr G. A. Fryxell's collection (Texas A&M University, College Station, TX), a note indicated that they were identified as f. *pungens* when originally isolated, but that recent observation by scanning electron microscopy had, in fact, identified them as f. *multiseries*. Our immunofluorescence results had indeed also identified these clones as f. *multiseries*, not f. *pungens*.

Twenty-seven other pennate diatom clones from the *Pseudonitzschia* and *Nitzschia* genera were examined for cross reactivity (Table 3). No cross reactions were found, with the exception of 4 species. *P.* (= *N.*) *pseudodelicatissima* (Clones F379 and F380) from Galveston, Texas, and *P. pseudodelicatissima* (Clone F397) from Newport, Rhode Island, showed a slight positive response with the f. *multiseries* antiserum. However, *P. pseudodelicatissima* (Clone N1) from Massachusetts Bay showed a negative response with antisera from both f. *multiseries* and f. *pungens*. Other samples of *P. pseudodelicatissima* must be tested to resolve this discrepancy. *P. australis*, believed to be responsible for the domoic acid incident in Monterey Bay, California (Fritz et al. 1992), also gave mixed results; either a negative reaction (for Clone P:SID) with both f. *multiseries* and f. *pungens* antisera or a slight positive reaction with both antisera (clones from Monterey Bay and Ilwaco). The slight positive reaction was not great enough to cause concern about misidentification, but it does pose some interesting questions about the evolutionary relationship between the domoic acid producers *P. pungens* f. *multiseries* and *P. australis*. *P. fraudulenta* (Clone B991H) also gave a slight positive reaction with the f. *multiseries* antiserum. Finally, *P. subcurvata* (Clones CCMP1437 and CCMP1444) gave a slight positive response for both antisera, following triplicate tests. Clones CCMP1438 and CCMP1443 of this same species also gave a slight labelling, but it appeared to be confined to the interior of the cell rather than to the cell surface, and was therefore scored as a negative.

Ten other representatives of the class Bacillariophyceae, including *Fragilariopsis ritscheri* and *Bacillaria paxillifer*, did not cross react with the antiserum from either f. *multiseries* or f. *pungens* (Table 4). However, the domoic-acid-producing *Amphora coffaeiformis* (Clone BPT-11, Maranda et al. 1990) labelled slightly with both antisera. Nevertheless, representative species from 7 other major classes of phytoplankton did not cross react (Table 4), nor did numerous other species, including the dinoflagellates *Dinophysis* spp. and *Prorocentrum* spp. and the diatoms *Skeletonema costatum*, *Chaetoceros* spp., and *Rhizosolenia* spp., in natural samples collected in Cardigan Bay and New London Bay, PEI, in October and November 1992 (S. C. S. Smith unpubl.). These same samples, however, did contain *Pseudonitzschia* sp. cells that reacted strongly with the f. *multiseries* and the f. *pungens* antisera when tested separately, clearly indicating the presence of both forms in a field situation.

DISCUSSION

The indirect immunofluorescence assay, using polyclonal antisera, successfully distinguished the domoic-acid-producing *Pseudonitzschia pungens* f. *multiseries* from the nontoxic *P. pungens* f. *pungens*, with no cross reactions between the 2 forms. Such fine-scale immunospecificity has not been demonstrated previously for marine phytoplankton, which have generally shown genus-specific or species-specific reactions (Anderson et al. 1988, Campbell et al. 1988, Shapiro et al. 1989a, b, Vrieling et al. 1993b). Our results indicate that unique and as yet unidentified epitopes exist on the cell surface of each form, raising some interesting phylogenetic questions. This provides additional evidence, beyond the differences in morphology and the capability to produce domoic acid, that there is genetic separation between these 2 forms (G. A. Fryxell pers. comm.).

Antibody reactions were found not only with clones of *Pseudonitzschia pungens* isolated from eastern PEI (the origin of the cells used for the immunizations), but also with *P. pungens* from northern PEI, Nova Scotia, Massachusetts, Rhode Island, Texas, and Washington. These results indicate that there is genetic continuity of cell surface antigens on *P. pungens* clones isolated from diverse geographical areas. Similar immunological results were described by Shapiro et al. (1989b) for other cosmopolitan species. On the other hand, slight clonal variations in labelling intensity may be expected (Wood & Leatham 1992). The only exception to the labelling pattern was Clone B991I, identified as *P. pungens* f. *pungens*, but which did not react with either of the antisera (Table 2). This clone will be examined in more detail.

The potential utility of the assay was demonstrated when repeated tests indicated Clones CCMP572 and CCMP573 to be f. *multiseries* (Table 2), although they were originally identified as f. *pungens* in the CCMP Catalogue of Strains. Updated information received from Dr G. A. Fryxell showed that these clones (also designated as F279m and F288m, respectively, in Table 1) were initially identified as f. *pungens*, but were in fact now f. *multiseries* (probably due to contamination). In another instance, our immunofluorescence assay correctly showed that the identifications of 2 *P. pungens* clones from New London Bay, PEI, had inadvertently been reversed. Such assays may therefore also serve as a check on the taxonomic identifications of phytoplankton, analogously to the way in which RNA or DNA sequences can be used to separate *Alexandrium* species (Scholin & Anderson 1993). Immunofluorescence assays may be especially useful for diatoms, whose taxonomy is based primarily on frustule morphology. Frustule morphology, however, can change with environmental (Schmid 1979, Mizuno 1987) and culture conditions. For example, cells in some of our *P. pungens* cultures have become so deformed (cf. Takano & Kikuchi 1985, Subba Rao & Wohlgeschaffen 1990) and short (because sexual reproduction has not taken place) that they no longer fit the species definition for *P. pungens* (Hasle 1965). Nevertheless, all of our f. *multiseries* and f. *pungens* clones reacted with the appropriate antiserum, regardless of cell size or the presence of deformities.

As promising as this immunoassay is for distinguishing the 2 forms of *Pseudonitzschia pungens*, no assay is completely infallible. Slight labelling was found with 3 other species of domoic-acid-producing pennate diatoms: *P. pseudodelicatissima* (Martin et al. 1990), *P. australis* (Buck et al. 1992, Fritz et al. 1992, Garrison et al. 1992), and *Amphora coffaeiformis* (Maranda et al. 1990). Again, it would be interesting to study the phylogeny of these diatoms, for example with RNA probes (cf. Scholin & Anderson 1993). *P. subcurvata* (Clones CCMP1437 and CCMP1444) also labelled slightly, but at least 1 of these clones (CCMP1437 = MC-2) did not produce domoic acid at a detection limit of 3.28 fg cell^{-1} (Fryxell et al. 1991). It is unlikely that *P. subcurvata* could be confused with *P. pungens* in North American waters because of its mainly Antarctic distribution (Fryxell et al. 1991).

The working titre found in this study (10 to 200) was lower than that reported for other phytoplankton: 3200 for a chrysophyte; 12800 for a prymnesiophyte (Campbell et al. 1988); and 12800 for the 'brown tide' chrysophyte *Aureococcus anophagefferens* (Anderson et al. 1988). Nevertheless, the antibodies were relatively easily, rapidly and consistently produced by at least 4 rabbits used thus far. It is interesting to note that there were no cross reactions between the 2 forms, even at the high antibody concentration of 1:10 used for the majority of the tests reported here. A working dilution of 1:50 is recommended for field and laboratory tests, as this concentration adequately labels the cells and conserves antiserum. It is also likely that the slight cross reactions seen with *Pseudonitzschia pseudodelicatissima*, *P. fraudulenta*, and *P. australis* would not be visible at the 1:50 dilution.

Labelling was good to excellent with the 6 preservatives tested (Table 5), and each preservative also maintained structural integrity of the cells. We did not observe the nonspecific staining seen by Campbell et al. (1988) and Shapiro et al. (1989b) for glutaraldehyde-preserved cells. Cells frozen at −20 and −60°C also gave good to excellent labelling, even after 6 mo (Table 6). The fluorescence yield did not appreciably increase by adding glycerol to the microscope slide (cf. Anderson et al. 1988), nor by raising the pH of the mounting medium (cf. Goldman 1968, Ward 1982,

Campbell et al. 1983). The capability of the immunofluorescence assay described here to discriminate between the toxic and nontoxic forms of *Pseudonitzschia pungens* in glutaraldehyde-paraformaldehyde-preserved field samples was also demonstrated.

The slightly better labelling of *Pseudonitzschia pungens* cells during the stationary phase (Table 6) suggests that the quantity and/or quality of epitopes on the cell surface changes over time. Although this may be expected, it is difficult to explain the apparently stronger reactions with stationary-phase than with exponential-phase cells, because the cells used for immunization were in fact harvested during the late-exponential phase (Day 7). It is interesting to note the relationship between the strength of the labelling and the production of domoic acid, both of which are most evident during the stationary phase (Bates et al. 1991).

Incubation times were taken from Anderson et al. (1988) and thus were not necessarily optimized. They could be considerably reduced from the 40 min used, as evidenced by good labelling even after a 10 min incubation period with the primary antiserum and a 5 min period with the secondary antiserum. A 15 min period for the blocking agent and primary antiserum is probably sufficient, but these times should be optimized for each new bleed and rabbit. The assay has also not yet been optimized to increase the number of laboratory or field samples processed at one time. Up to 18 incubations could easily be carried out simultaneously with the centrifugation approach, which was convenient for laboratory cultures requiring only 1.5 to 3.0 ml of sample because of their high biomass. However, if larger volume, low biomass 'whole water' field samples are to be processed, the cells must first be concentrated. This is most conveniently done by filtration (Anderson et al. 1988), as was also demonstrated in our study, provided that the filtration apparatus has a sufficient capacity for filters. Sample throughput could be increased if cells could be made to stick onto histological microscope slides (e.g. Ward & Perry 1980), which would then be incubated and rinsed in batches.

The number of samples processed could be doubled by simultaneously using 2 secondary antibodies conjugated to fluorochromes that emit at different wavelengths. For example, the currently used green-fluorescing fluorescein isothiocyanate (FITC) could be used to visualize f. *pungens*, and the red-fluorescing tetramethylrhodamine isothiocyanate (TRITC) could be used for f. *multiseries*. In this way, only 1 sample need be incubated to distinguish the 2 forms of *Pseudonitzschia pungens*. However, for the 2 secondary antibodies to recognize the primary antibodies, the latter must be developed in 2 different animal species (e.g. f. *pungens* in rabbits and f. *multiseries* in goats), and the animal species in which 1 of the secondary antibodies originates must also be different. Alternatively, a direct immunofluorescence assay could be used in which the 2 fluorochromes are conjugated directly to the respective primary antibodies produced in the same animal species.

It was important for the development of this assay to be able to visualize which cells were labelled, using a fluorescence microscope. Aside from being able to look for cross reactions, this approach also has the advantage that cell counts of each form of *Pseudonitzschia pungens* can be made. In future developments of the technique, the fluorescence intensity could be quantified by conventional fluorometers or by flow cytometry (Cucci & Robins 1988, Shapiro et al. 1989b, Vrieling et al. 1993a). The secondary antibody could be conjugated to a molecule other than a fluorochrome, e.g. an enzyme or a radioisotope. This would enable the assay to be run as an immunodot blot, enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA), increasing the throughput of samples via automation. Finally, the success shown by these polyclonal antibodies suggests that it may be worthwhile to develop monoclonal antibodies against *P. pungens*, although this approach may also have some disadvantages, e.g. a loss of sensitivity (Campbell et al. 1988).

There are numerous benefits of an immunochemical assay capable of detecting and distinguishing toxic and nontoxic forms of *Pseudonitzschia pungens*. Such a technique will: (1) provide a research tool to carry out laboratory experiments, e.g. on the competitive growth of the 2 forms of *P. pungens* growing in the same culture flask; (2) enable toxic phytoplankton monitoring programs (including those currently run by the Department of Fisheries and Oceans, Canada) to more rapidly and accurately determine the presence of toxic and nontoxic forms of *P. pungens*; (3) allow a comparison of *P. pungens* cells in natural samples collected from different oceans of the world to evaluate the potential for those waters to have an outbreak of domoic acid poisoning, given a sufficient increase in *P. pungens* cell concentration; and (4) eventually lead to the development of easy-to-use 'test kits' that could be used by aquaculturalists, regulatory agencies, and others concerned with determining the presence of the toxic form of *P. pungens*.

It is clear that the international scientific community and government regulatory agencies are becoming increasingly aware that the problem of harmful algal blooms, i.e. both the number of phytoplankton species implicated and the number of reported events, are spreading globally (Anderson 1989, Smayda 1990). The use of immunology as a tool for studying the presence, identity and distribution of toxic algae will likely increase as concern about their harmful impact on human health and the economy increases.

Acknowledgements. We thank M. Laflamme for excellent technical assistance and timely advice. G. A. Fryxell, D. M. Jacobson, T. A. Villareal, P. E. Hargraves, K. E. Pauley, D. J. Douglas and L. Maranda kindly provided preserved samples of phytoplankton for testing from their culture collections. K. E. Pauley and J. C. Smith carried out scanning electron microscopy on several of the *Pseudonitzschia pungens* clones, using facilities at the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia. This work was supported in part by grant OCE89-11226 (to D.M.A.) from the U.S. National Science Foundation. Contribution no. 8235 from the Woods Hole Oceanographic Institution.

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This article was presented by D. A. Caron, Woods Hole, Massachusetts, USA

Manuscript first received: December 11, 1992
Revised version accepted: June 22, 1993