

## *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health

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### ABSTRACT

Over the last decade, our understanding of the environmental controls on *Pseudo-nitzschia* blooms and domoic acid (DA) production has matured. *Pseudo-nitzschia* have been found along most of the world's coastlines, while the impacts of its toxin, DA, are most persistent and detrimental in upwelling systems. However, *Pseudo-nitzschia* and DA have recently been detected in the open ocean's high-nitrate, low-chlorophyll regions, in addition to fjords, gulfs and bays, showing their presence in diverse environments. The toxin has been measured in zooplankton, shellfish, crustaceans, echinoderms, worms, marine mammals and birds, as well as in sediments, demonstrating its stable transfer through the marine food web and abiotically to the benthos. The linkage of DA production to nitrogenous nutrient physiology, trace metal acquisition, and even salinity, suggests that the control of toxin production is complex and likely influenced by a suite of environmental factors that may be unique to a particular region. Advances in our knowledge of *Pseudo-nitzschia* sexual reproduction, also in field populations, illustrate its importance in bloom dynamics and toxicity. The combination of careful taxonomy and powerful new molecular methods now allow for the complete characterization of *Pseudo-nitzschia* populations and how they respond to environmental changes. Here we summarize research that represents our increased knowledge over the last decade of *Pseudo-nitzschia* and its production of DA, including changes in worldwide range, phylogeny, physiology, ecology, monitoring and public health impacts.

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### 1. Introduction

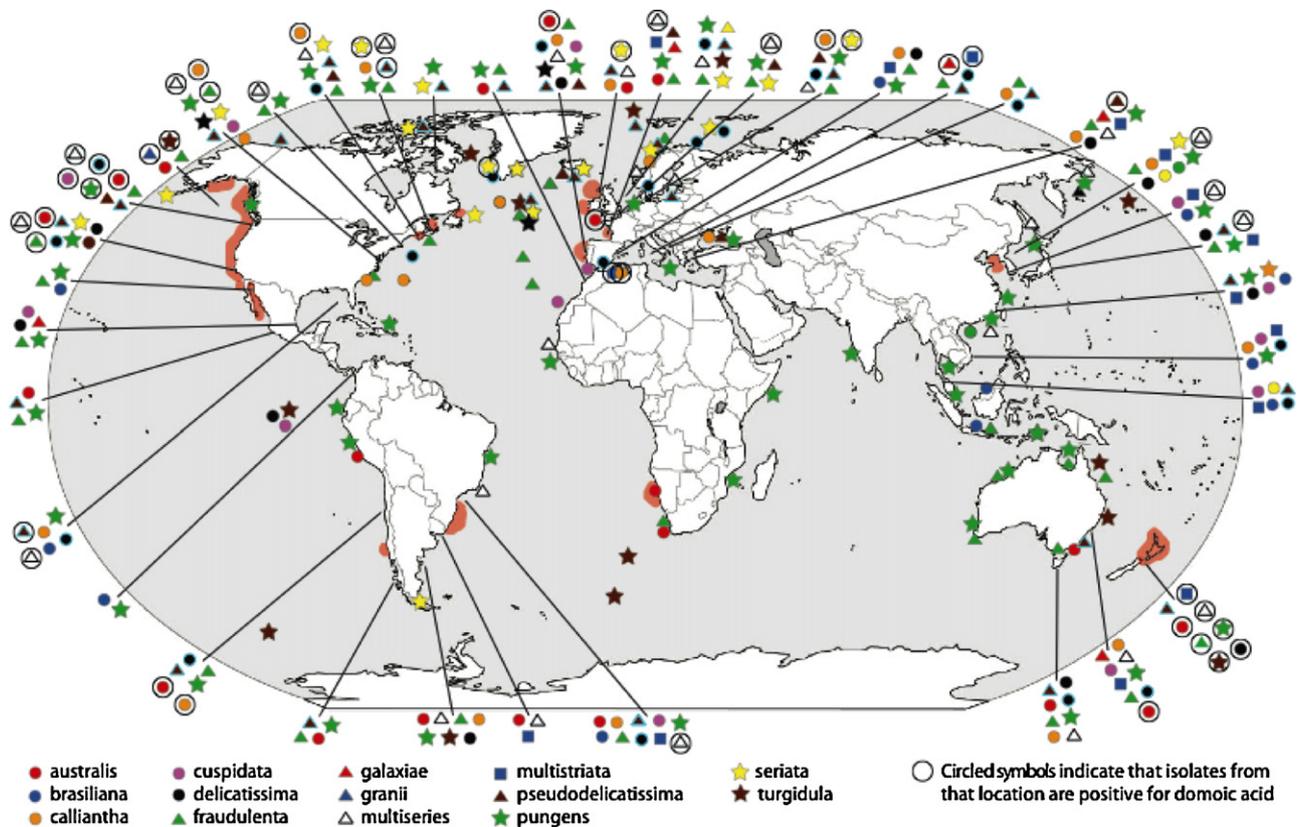
Since the discovery of domoic acid (DA) as a human health problem, causing amnesic shellfish poisoning (ASP), off Prince Edward Island, Canada, in 1987 (Bates et al., 1989) and as a potent killer of wildlife in Monterey Bay, USA, in 1991 (Work et al., 1993a,b) and thereafter (Scholin et al., 2000; reviewed by Bejarano et al., 2008; Bargu et al., in press), the monitoring of *Pseudo-nitzschia* and its toxin, DA, has expanded to more coastlines of the world. Thus, the number of described *Pseudo-nitzschia* species has climbed from 15 in the 1980s to ~37 in the 2010s, and the number of species confirmed to produce DA has risen to 12 (Trainer et al., 2010; Lundholm, 2011; listed in Fig. 1). The presence of

*Pseudo-nitzschia* in high numbers during open ocean iron-fertilization experiments (Trick et al., 2010) and the quantification of DA in both preserved and live samples from open ocean regions (Silver et al., 2010) have enhanced our understanding of the importance and wide distribution of *Pseudo-nitzschia*. It is found at a small scale, e.g. in thin layers (Rines et al., 2002), or at a large scale, covering wide expanses of our coastlines (e.g. Trainer et al., 2009a,b). *Pseudo-nitzschia* can form intense, high-density, visible blooms in bays, such as Monterey Bay, whereas offshore it more often forms less dense blooms that are not visible to the naked eye. Environmental cues causing toxic blooms of *Pseudo-nitzschia* are complex, and can be unique to the bays, coastal, or open ocean regions where they occur.

The cosmopolitan nature of most toxigenic *Pseudo-nitzschia* species (Hasle, 2002) argues for widespread human health effects of this toxin. However, since the deaths of humans as a result of the 1987 ASP event, no human mortalities have been confirmed,

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**Fig. 1.** Toxicogenic species of *Pseudo-nitzschia*. Symbols of those species that have been demonstrated to produce domoic acid in culture are circled, and are shown at the locations from which they were isolated. Areas along coastlines marked with red are locations where closures of shellfish harvesting due to elevated levels of domoic acid ( $>20 \mu\text{g DA g}^{-1}$  wet weight of shellfish tissue), or animal mortalities, have occurred. Modified and updated from Thessen (2007) and Trainer et al. (2008). Symbols outlined in blue indicate a species description that was made before major taxonomic revisions were implemented for *P. delicatissima* and *P. pseudodelicatissima*.

thanks to effective implementation of monitoring programs. The presence of DA can nevertheless have negative economic consequences for harvesters of wild and aquacultured molluscan shellfish. Impacts of DA on marine wildlife at various levels within the food web have also been observed, and this toxin is known to contribute to the deaths of seabirds, sea otters, sea lions and whales. The adsorption of DA, especially to sediments (Burns and Ferry, 2007), facilitates its potentially long-lasting impacts, particularly to the benthic food chain. Here we summarize those impacts of DA on marine wildlife as well as our increased knowledge about *Pseudo-nitzschia* and DA over the last decade, including changes in worldwide range and impacts, phylogeny, physiology, ecology, monitoring and public health impacts.

## 2. Worldwide range and impacts

It is remarkable that, almost without exception, the impacts of DA have been described primarily in eastern boundary upwelling systems (Fig. 1; Trainer et al., 2008, 2010). Although *Pseudo-nitzschia* and low levels of DA are found in other regions, e.g. the Mediterranean, Russia, Japan and Vietnam, damaging blooms have generally not been noted. Therefore, this section will focus primarily on those systems in which DA has caused negative effects on human health or marine wildlife.

### 2.1. United States

#### 2.1.1. Fisheries and organisms affected

Over the last decade, several cases of DA poisoning of finfish, marine mammals and birds have been documented on the U.S. West Coast, while human deaths have been prevented through

routine monitoring of shellfish. In U.S. waters, a number of commonly harvested shellfish and finfish have been reported as vectors of the toxin, including blue mussels, clams (razor, littleneck, geoduck and manila), Pacific oysters, crabs (Dungeness, rock and pelagic red), spiny lobster viscera, anchovies, and sardines (Lewitus et al., in press; Table 1), each with differing rates of toxin retention and release. Marine animals, e.g. sea lions, sea otters and marine birds, have also been found to accumulate DA, leading to widespread transfer through marine food webs (e.g. Scholin et al., 2000; Lefebvre et al., 2002; Bargu and Silver, 2003; Bargu et al., 2008, 2010; Kvittek et al., 2008). Since the first recorded DA-related mortality of sea lions and seabirds in 1991 (Work et al., 1993a,b), death or illnesses have been reported in California sea lions, northern fur seals, harbour porpoises, common dolphins, sea otters, gray whales, brown pelicans, Brandt's cormorants, black-crowned night herons, Pacific loons, red-throated loons, common loons, rhinoceros auklets, Cassin's auklets, ruddy ducks, surf scoters, short-tailed shearwaters, western grebes and Clark's grebes (F.M.D. Gulland, unpubl. data for marine mammals; D.A. Caron, unpubl. data for birds; from Lewitus et al., in press; Bejarano et al., 2008; Bargu et al., in press).

DA has been detected in seafood species in Washington State and California almost every year (Trainer and Suddleson, 2005; Langlois, 2007). In 2007, the highest DA concentration ( $610 \mu\text{g DA g}^{-1}$ ) on the U.S. west coast was detected in mussels in California (Langlois, 2007). An expansion of *Pseudo-nitzschia*-related shellfish closures into Puget Sound was reported in 2003 (Bill et al., 2006) and 2005 (Trainer et al., 2007), when blooms of *P. pseudodelicatissima* and *P. australis* caused significant commercial, recreational and tribal shellfish harvest losses. These closures led to concerns that DA events in northern Puget Sound might expand

**Table 1**  
Evidence for the impact of *Pseudo-nitzschia* and domoic acid in the food web.

Organism		Location	Evidence	Reference
Common name	Scientific name			
<b>Protists</b>				
Dinoflagellate	<i>Gyrodinium</i> sp.	Monterey Bay, CA	Frustules in fecal pellets	Buck et al. (2005)
Dinoflagellate	<i>Noctiluca scintillans</i>	NW Spain	Cells in food vacuoles	Escalera et al. (2007)
Dinoflagellate	<i>Protoperdinium pellucidum</i>	Pacific NW coast, WA	Grazed on DA in laboratory	Olson and Lessard (2010)
Dinoflagellate	<i>Protoperdinium</i> spp.	Oslofjord, Norway	Abundances of <i>Protoperdinium</i> spp. tracked abundances of <i>Pseudo-nitzschia</i>	Kjæret et al. (2000)
<b>Echinoderms</b>				
Sand dollar	<i>Dendraster excentricus</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003) and Kvitek et al. (2008)
<b>Mollusks</b>				
Furrow shell	<i>Scrobicularia plana</i>	Portugal	DA in tissue	Vale et al. (1998)
Olive snail	<i>Olivella biplicata</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003) and Kvitek et al. (2008)
Chilean blue mussel	<i>Mytilus chilensis</i>	Chile	DA in tissue	López-Rivera et al. (2009)
Channeled basket whelk	<i>Nassarius fossatus</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003) and Kvitek et al. (2008)
California mussel	<i>Mytilus californianus</i>	San Diego, CA	DA in tissue	Busse et al. (2006)
Mediterranean mussel	<i>Mytilus galloprovincialis</i>	Riá de Muros, Spain; Croatia; Portugal	DA in tissue	Vale and Sampayo (2001), Blanco et al. (2002), and Ujević et al. (2010)
Blue mussel	<i>Mytilus edulis</i>	New Brunswick, Canada; Nantucket Shoals; Georges Bank; Washington coast; Argentina; Japan; Ireland; Aviero Lagoon, Portugal	DA in tissue, fed on <i>Pseudo-nitzschia</i> in the lab and accumulated DA; also in field samples	Haya et al. (1991), Wohlgeschaffen et al. (1992), Douglas et al. (1997), Horner and Postel (1993), Kawatsu and Hamano (2000), Vale and Sampayo (2001), Negri et al. (2004), and James et al. (2005)
Ribbed mussel	<i>Aulacomya ater</i>	Chile	DA in tissue	López-Rivera et al. (2009)
Greenshell™ mussel	<i>Perna canaliculus</i>	New Zealand	fed on <i>Pseudo-nitzschia</i> in lab and accumulated DA	Mackenzie et al. (1993)
Horse mussel	<i>Modiolus modiolus</i>	Atlantic Canada	DA in tissue	Gilgan et al. (1990)
Mediterranean cockle	<i>Acanthocardia tuberculatum</i>	Algarve, Portugal	DA in tissue	Vale and Sampayo (2001)
Common cockle	<i>Cerastoderma edule</i>	Mondego estuary and Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo (2001)
King scallop	<i>Pecten maximus</i>	Scotland, Ireland; Morocco	DA in tissue	Tahri-Joutei et al. (2003), Smith et al. (2006), Campbell et al. (2003), and James et al. (2005)
Mediterranean scallop	<i>Pecten jacobaeus</i>	Croatia	DA in tissue	Ujević et al. (2010)
Sea scallop	<i>Placopecten magellanicus</i>	New Brunswick, Canada; Eastport, Maine	DA in digestive gland	Shumway (1989), Haya et al. (1991), Wohlgeschaffen et al. (1992), and Douglas et al. (1997)
New Zealand scallop	<i>Pecten novaezealandiae</i>	New Zealand	DA in digestive gland	Rhodes et al. (1996), and Hay et al. (2000)
Soft-shell clam	<i>Mya arenaria</i>	New Brunswick, Canada; Atlantic Canada	DA in tissue	Haya et al. (1991), and Gilgan et al. (1990)
Spondylus clam	<i>Spondylus versicolor</i>	Vietnam, Philippines	DA in tissue	Dao et al. (2009)
Razor clams	<i>Siliqua patula</i>	California/Washington/Oregon coasts	DA in tissue	Horner et al. (1993), Horner and Postel (1993), and Wekell et al. (1994)
Razor clam	<i>Ensis siliqua</i>	Ireland	DA in tissue	James et al. (2005)
Razor clam	<i>Ensis</i> spp.	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo (2002)
Clam	<i>Ruditapes decussata</i>	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo (2002)
Carpet shell	<i>Venerupis pullastra</i>	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo (2002)
Calico clam	<i>Tapes decussatus</i>	Portugal	DA in tissue	Vale et al. (2008)
Clam	<i>Protothaca thaca</i>	Chile	DA in tissue	López-Rivera et al. (2009)
Proteus scallop	<i>Flexopecten proteus</i>	Croatia	DA in tissue	Ujević et al. (2010)
European flat oyster	<i>Ostrea edulis</i>	Aveiro Lagoon, Portugal; Croatia	DA in tissue	Vale and Sampayo (2002), and Ujević et al. (2010)
Pacific oyster	<i>Crassostrea gigas</i>	Puget Sound, WA	DA in tissue	Horner and Postel (1993)
Common octopus	<i>Octopus vulgaris</i>	Portuguese coast	DA in gut, digestive gland, brachial heart	Costa et al. (2004)
Squid	<i>Loligo opalescens</i>	Monterey Bay, CA	DA in tissues, frustules in stomach	Bargu et al. (2008)
Common cuttlefish	<i>Sepia officinalis</i>	Portuguese coast	DA in digestive gland	Costa et al. (2005)
<b>Annelids</b>				
Polychaete worm	<i>Poebius meseres</i>	Monterey Bay, CA	frustules found in gut	Uttal and Buck (1996), and Buck et al. (1992)
<b>Echiurans</b>				
Fat innkeeper worm	<i>Urechis caupo</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003), and Kvitek et al. (2008)
<b>Crustaceans</b>				
Sand crab	<i>Emerita analoga</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003), and Kvitek et al. (2008)

Table 1 (Continued)

Organism		Location	Evidence	Reference
Common name	Scientific name			
Swimming crab	<i>Polybius henslowii</i>	Portuguese coast	DA in viscera, tissue	Costa et al. (2003)
Blue band hermit crab	<i>Pagurus samuelis</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003), and Kvitek et al. (2008)
Blue crab	<i>Callinectes sapidus</i>	US Pacific Coast	DA in tissue	Altwein et al. (1995)
Rock crab	<i>Cancer pagurus</i>	US Pacific Coast	DA in tissue	Altwein et al. (1995)
Dungeness crab	<i>Cancer magister</i>	California/Oregon/Washington coasts	DA in tissue	Horner and Postel (1993), and Wekell et al. (1994)
Stone crab	<i>Menippe adina</i>	US Pacific Coast	DA in tissue	Altwein et al. (1995)
Ghost shrimp	<i>Neotrypaea californiensis</i>	Monterey Bay, CA	DA in tissue	Goldberg (2003), and Kvitek et al. (2008)
Copepod	<i>Acartia tonsa</i>		Grazed on <i>Pseudo-nitzschia</i> in the lab, DA in tissue	Lincoln et al. (2001)
Copepod	<i>Acartia clausi</i>		Grazed on <i>Pseudo-nitzschia</i> in the lab, DA in tissue	Maneiro et al. (2005)
Copepod	<i>Calanus finmarchicus</i>		Grazed on <i>Pseudo-nitzschia</i> in the lab, DA in tissue	Leandro et al. (2010a)
Copepod	<i>Temora longicornis</i>		Grazed on <i>Pseudo-nitzschia</i> in the lab, DA in tissue	Lincoln et al. (2001), and Windust (1992)
Copepod	<i>Calanus glacialis</i>		Grazed on <i>Pseudo-nitzschia</i> in the lab	Windust (1992)
Krill	<i>Euphausia pacifica</i>	Monterey Bay, CA	Frustules in gut and DA in tissue	Bargu et al. (2002), and Bargu and Silver (2003)
Spiny lobster	<i>Palinurus elephas</i>	US Pacific Coast	DA in tissue	Altwein et al. (1995)
Gooseneck barnacle	<i>Pollicipes pollicipes</i>	Portugal	DA in tissue	Vale and Sampayo (2002)
<b>Eurochordates</b>				
Tunicate	<i>Pyura chilensis</i>	Chile	DA in tissue, feces and gut; frustules in gut	López-Rivera et al. (2009)
<b>Fishes</b>				
Northern anchovy	<i>Engraulis mordax</i>	Monterey Bay, CA	Frustules and DA in gut, DA in tissues	Fritz et al. (1992), Work et al. (1993a), Lefebvre et al. (1999, 2002), Scholin et al. (2000), and Wekell et al. (1994)
European anchovy	<i>Engraulis encrasicolus</i>	Portugal	DA in tissue	Vale and Sampayo (2002)
Argentine anchovy	<i>Engraulis anchoita</i>	Argentina	DA in gastrointestinal	Negri et al. (2004)
Petrale sole	<i>Eopsetta jordani</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Sand sole	<i>Psettichthys melanostictus</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Slender sole	<i>Eopsetta exilis</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Rex sole	<i>Errex zachirus</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Dover sole	<i>Microstomas pacificus</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver, 2007
English sole	<i>Pleuronectes vetulus</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Curlfin turbot	<i>Pleuronectes decurrens</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Sand sole	<i>Psettichthys melanostictus</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
Pacific halibut	<i>Hippoglossus stenolepis</i>	Monterey Bay, CA	DA in viscera	Vigilant and Silver (2007)
White croaker	<i>Genyonemus lineatus</i>	Santa Cruz, CA	DA in viscera	Fire and Silver (2005)
Staghorn sculpin	<i>Leptocottus armatus</i>	Santa Cruz, CA	DA in viscera	Fire and Silver (2005)
Jack mackerel	<i>Trachurus symmetricus</i>	Monterey Bay, CA	DA in viscera	Busse et al. (2006)
Albacore	<i>Thunnus alalunga</i>	Monterey Bay, CA	DA in viscera	Lefebvre et al. (2002)
Mackerel	<i>Scomber japonicus</i>	Cabo San Lucas, Mexico; Monterey Bay, CA, San Diego, CA	frustules and DA in gut	Sierra-Beltrán et al. (1997), Lefebvre et al. (2002), and Busse et al. (2006)
Jack mackerel	<i>Trachurus symmetricus</i>	San Diego, CA	DA in viscera	Busse et al. (2006)
Herring		Monterey Bay, CA	frustules and DA in gut	Naar et al. (2002)
Pilchard	<i>Sardinops sagax</i>	Baja California peninsula	frustules and DA in gut	Sierra-Beltrán et al. (1998), and Lefebvre et al. (2002)
Pilchard	<i>Sardina pilchardus</i>	Portuguese coast; Namibian coast	frustules and DA in gut, DA in tissues	Costa and Garrido (2004), Vale and Sampayo (2002), D. Louw, B. Currie, G. Doucette (pers. comm.)
Pacific sanddab	<i>Citharichthys sordidus</i>	Monterey Bay, CA, San Diego, CA	DA in viscera and tissue	Lefebvre et al. (2002), Busse et al. (2006), Goldberg (2003), Kvitek et al. (2008), and Vigilant and Silver (2007)
Albacore	<i>Thunnus alalunga</i>	Monterey Bay, CA	DA in viscera	Lefebvre et al. (2002)
Jack smelt	<i>Atherinopsis californiensis</i>	Monterey Bay, CA	DA in viscera	Lefebvre et al. (2002)
Gulf Menhaden	<i>Brevoortia patronus</i>	Gulf of Mexico	frustules and DA in gut	Del Rio et al. (2010)
Longspine combfish	<i>Zaniolepis latipinnus</i>	San Diego, CA	DA in viscera	Busse et al. (2006)
<b>Birds</b>				
Brown pelican	<i>Pelecanus occidentalis</i>	Monterey Bay, CA; Cabo San Lucas, Mexico	Frustules and DA in gut, death, neurological disorder	Fritz et al. (1992), Work et al. (1993a,b), and Sierra-Beltrán et al. (1997)
Brandt's cormorant	<i>Phalacrocorax penicillatus</i>	Monterey Bay, CA	Frustules and DA in gut, death, neurological disorder	Fritz et al. (1992), and Work et al. (1993a,b)
Double-crested cormorant	<i>Phalacrocorax auritus</i>	Monterey Bay, CA	Frustules and DA in gut, death, neurological disorder	Fritz et al. (1992), and Work et al. (1993a,b)
Pelagic cormorant	<i>Phalacrocorax pelagicus</i>	Monterey Bay, CA	Frustules and DA in gut, death, neurological disorder	Fritz et al. (1992), and Work et al. (1993a,b)

Western gull	<i>Larus occidentalis</i>	Monterey Bay, CA	Frustrules and DA in gut, death, neurological disorder	Fritz et al. (1992), and Work et al. (1993a,b)
King penguin	<i>Aptenodytes forsteri</i>	Kentucky Zoo	Death, frustrules and DA in their food	Naar et al. (2002)
Common loon	<i>Gavia immer</i>	Baja California peninsula	Death	Sierra-Beltrán et al. (1998)
<b>Mammals</b>				
Common dolphin	<i>Delphinus capensis</i>	Baja California peninsula	Frustrules in gut, DA in tissues, brain lesions, death	Sierra-Beltrán et al. (1998)
Humpback whale	<i>Megaptera novaeangliae</i>	Monterey Bay, CA	Frustrules and DA in feces	Lefebvre et al. (2002)
Blue whale	<i>Balaenoptera musculus</i>	Monterey Bay, CA	Frustrules and DA in feces	Lefebvre et al. (2002)
Right Whale	<i>Eubalaena glacialis</i>	North Atlantic	DA present in body and feces, frustrules in feces	Bates and Trainer (2006), and Leandro et al. (2010b)
California sea lion	<i>Zalophus californianus</i>	Monterey Bay, CA	Death, neurological disorder, frustrules and DA in feces, DA in body fluids	Scholfin et al. (2000), Gulland et al. (2002), and Bargu et al. (2010)
Pygmy sperm whale	<i>Kogia breviceps</i>	U.S. southeast coast	DA in urine and feces	Fire et al. (2009)
Bottlenose dolphin	<i>Tursiops truncatus</i>	Gulf of Mexico	DA in urine, feces and blood	Schwacke et al. (2010), Fire et al. (2011), and Twiner et al. (2011)
Harbour seal	<i>Phoca vitulina</i>	Scotland	DA in urine and feces	Hall and Frame (2010)
Minke whale	<i>Balaenoptera acutorostrata</i>	Southern California, USA	frustrules in gut and feces, DA in feces	Fire et al. (2010)
Dwarf sperm whale	<i>Kogia sima</i>	U.S. southeast coast	DA in urine and feces	Fire et al. (2009)
Southern sea otter	<i>Enhydra lutris nereis</i>	California coast	DA in urine	Kreuder et al. (2003)
Human	<i>Homo sapiens</i>	Eastern and central Canada	Death, neurological disorder, DA in food, brain lesions	Perl et al. (1990), Teitelbaum et al. (1990), and Cendes et al. (1995)

into areas further south, paralleling the expansion of paralytic shellfish toxicity into south Puget Sound in the 1990s (Trainer et al., 2003).

Further to the north, in the State of Alaska, shellfish are not routinely tested for DA; however, an intensive study in the 1990s found measurable levels of this toxin. In 1997, 11.5 µg DA g<sup>-1</sup> was measured in razor clams from Katchemak Bay and low levels were detected in Pacific anchovies from southeast Alaska (Alaska Dept. of Environmental Conservation database).

### 2.1.2. *Pseudo-nitzschia* species

*Pseudo-nitzschia* species are most often a minor component of the phytoplankton assemblage, contributing <17% of the total carbon biomass, even in the most intense bloom observed off the Washington State coast (Trainer et al., 2009a). *Pseudo-nitzschia* spp. are common members of the coastal phytoplankton community and have been present in the California Current System at least since the 1930s (Gran and Thompson, 1930). The species associated with DA production are frequently present, albeit in low numbers, in most water samples (e.g. Walz et al., 1994). Of the 12 species of *Pseudo-nitzschia* known to produce DA around the world (Lundholm, 2011), 10 have been reported from the U.S. West Coast waters (Horner et al., 1997; Anderson et al., 2008). *Pseudo-nitzschia* spp. occur along the entire Alaskan coast (Bursa, 1963; Horner, pers. obs. as *Nitzschia seriata*), throughout the Bering Sea (Schandelmeier and Alexander, 1981 as *Nitzschia* spp., section *Pseudo-nitzschia*), and into the Gulf of Alaska (Cupp, 1943) and south-central Alaska (Horner et al., 1973; summarized in Lewitus et al., in press). *Pseudo-nitzschia australis* and *P. multiseriata* are most commonly associated with toxic events throughout this region, with *P. pseudodelicatissima* and *P. cuspidata* also implicated in toxic events in Washington State waters (Adams et al., 2000; Trainer et al., 2009a). The most problematic species in California appear to be *P. australis* and *P. multiseriata* (Trainer et al., 2000).

### 2.1.3. Known “hot spots”

Areas impacted by *Pseudo-nitzschia* span coastal Washington State, with a demonstrated “hot spot” in the Juan de Fuca eddy area, where *Pseudo-nitzschia* blooms occur relatively frequently, associated with high DA concentrations (Trainer et al., 2002). For DA to reach coastal beaches from the eddy region, toxic cells must first become concentrated enough to form a bloom, then must escape the eddy and travel southward toward the razor clamming beaches, move onto coastal beaches, and remain for a long enough period in the surf zone for a significant amount of the toxic cells to be ingested by the clams. The highest toxic cell concentrations, as well as onshore transport, occur during periods of downwelling winds (storms), whereas escape from the eddy and travel southward occur during periods of upwelling winds (MacFadyen and Hickey, 2010). Another initiation site, or “hot spot”, for toxigenic *Pseudo-nitzschia* blooms is the Heceta Bank, off the Oregon coast (Trainer et al., 2000). The impact of DA toxicity on razor clam, mussel and Dungeness crab industries appears to be increasing in recent years, with exceptionally extensive and prolonged closures occurring from 2003 to 2005 (Tweddle et al., 2010). Additional hot spot areas in California include Monterey Bay, San Luis Obispo and Point Conception (Trainer et al., 2000).

An exceptional closure of DA-associated razor clam harvesting in Washington occurred in 2002–2003, for ~1.5 y, resulting in an estimated \$10.4 million loss in revenue (HAB Report, 2009). A record high concentration of DA in razor clams (295 µg DA g<sup>-1</sup>) was measured in 1998 (Adams et al., 2000). The total estimated impact of a hypothetical coast-wide seasonal closure of the recreational razor clam fishery for 2008 was estimated to be \$21.9 million and the income impact of the recreational razor clam fishery in Washington State for 2008 was estimated at \$13.5

million (Dyson and Huppert, 2010). Therefore, the economic impact of these toxic episodes on the U.S. west coast appears to have become more severe over the last decade.

While the impact of DA and occurrence of toxigenic *Pseudo-nitzschia* are greatest on the U.S. west coast where shellfish closures or animal mortalities occur frequently, other regions of the U.S. are also vulnerable to the same consequences. In the Gulf of Mexico, abundances of *Pseudo-nitzschia* have been increasing since the 1950s (Parsons et al., 2002) and time series of *Pseudo-nitzschia* spp. from sediment cores document increasing cell abundance in association with increased nutrient input from Mississippi River runoff (Parsons et al., 2002). Low levels of DA have been detected in menhaden (Table 1) and seawater samples off the Louisiana coast (Del Rio et al., 2010), where *P. pseudodelicatissima*, *P. pungens*, *P. americana* and *P. calliantha* are the dominant *Pseudo-nitzschia* species. High *Pseudo-nitzschia* abundance ( $>10^6$  cells  $l^{-1}$ ) has been associated with submarine ground discharge of nutrients off the coast of nearby Alabama (Liefer et al., 2009; MacIntyre et al., 2011). Toxic *Pseudo-nitzschia* spp. are present in the Chesapeake Bay, although their abundance and toxin production are highly variable. Six species of *Pseudo-nitzschia* have been identified in this area: *P. pungens*, *P. calliantha*, *P. subpacific*, *P. cuspidata*, *P. fraudulenta* and *P. multiseri*. Compared to other diatom species, *Pseudo-nitzschia* abundances were low, rarely exceeding 1000 cells  $ml^{-1}$ , and they did not occur as monospecific blooms. DA concentrations were generally low, ranging from 4 to 1037  $\mu g$  DA  $ml^{-1}$ . Low *Pseudo-nitzschia* abundances and low, irregular DA concentrations may partially explain the lack of documented toxic events in the Chesapeake Bay (Thessen and Stoecker, 2008).

At least 9 *Pseudo-nitzschia* species are found in the Gulf of Maine, including *P. delicatissima*, *P. fraudulenta*, *P. pungens*, *P. cf. subpacific* and *P. delicatissima/pseudodelicatissima* (Fernandes et al., 2009). In July, 2003,  $>20$  whales, mostly humpbacks, were found dead near Georges Bank; DA was detected in most whales sampled, with several containing high concentrations (D.M. Anderson, pers. comm.). Later, DA was detected in fecal samples of the highly endangered North Atlantic right whale (*Eubalaena glacialis*) in the Gulf of Maine and Georges Bank, and in its food source (krill and copepods), from April to September of 2005 and 2006 (Leandro et al., 2010b).

## 2.2. Canada

### 2.2.1. East coast

DA, produced by *P. multiseri*, was identified as the source of the toxicity from Cardigan Bay (Prince Edward Island, eastern Canada), that resulted in the first ASP incident in the world, in 1987 (review by Bates et al., 1998). It is also the first time that a diatom produced a phycotoxin. The toxin reached among the highest levels recorded (790  $\mu g$  DA  $g^{-1}$ ), in blue mussels (*Mytilus edulis*), and at lower levels (38  $\mu g$  DA  $g^{-1}$ ), in soft-shell clams (*Mya arenaria*) (Bates et al., 1989). An expanded shellfish monitoring program, implementing regulatory closures of harvesting sites when DA levels reach the regulatory limit (20  $\mu g$  DA  $g^{-1}$ ; now adopted internationally), has subsequently prevented any further cases of ASP in Canadian consumers. However, there have been subsequent harvesting closures along the eastern Canadian coastline (Table 2). The original 1987 event was followed by closures in Cardigan Bay in 1987, 1988 and 1999, but with decreasing concentrations of *P. multiseri* and of DA in mussels (summarized in Bates et al., 1998). Northern Prince Edward Island experienced short closures of harvesting in 1990, 1991, 1992, 1993 and 1999 (Bates, 2004). The presence of *P. multiseri* diminished after 1999, being replaced by nontoxic *P. pungens* and *P. calliantha*. The last harvesting closure was in 2002, covering most of the

southern Gulf of St. Lawrence, but this time it was caused by a massive bloom of toxic *P. seriata* (Table 2) (Bates et al., 2002). Since then, there have been no shellfish harvesting closures due to toxic *Pseudo-nitzschia* blooms in the Gulf of St. Lawrence.

On the southwest shore of Nova Scotia, DA was found in June 1995 at levels near or exceeding the action level in some samples of soft-shell (*M. arenaria*), propeller (*Cyrtodaria siliqua*) and surf (*Spisula solidissima*) clams, blue mussels (*M. edulis*), quahaugs (*Mercenaria mercenaria*), Stimpson's surf clam (*Mactromeris polymya*), Jonah crabs (*Cancer borealis*), American lobster (*Homarus americanus*) hepatopancreas and sea scallop (*Placopecten magellanicus*) digestive glands collected along the southwest coast of Nova Scotia (unpublished data). The toxin source is unknown.

Just prior to the above incidence, high levels of DA were found in sea scallops (*P. magellanicus*) from Georges, German, and Browns Banks. The sample with the highest toxin concentration analyzed showed the following tissue distribution: digestive gland (3400  $\mu g$  DA  $g^{-1}$ ), roe (55  $\mu g$  DA  $g^{-1}$ ), gills plus mantle (19  $\mu g$  DA  $g^{-1}$ ) and adductor muscle (1  $\mu g$  DA  $g^{-1}$ ) (unpublished data). Again, the DA source is not known, but it is possible that a bloom of toxic *Pseudo-nitzschia* growing in the upper, sunlit layers of the ocean settled to the benthos, where it was fed upon by the scallops.

The second location in the world where shellfish harvesting was closed due to DA was in the Bay of Fundy, eastern Canada, during September 1988 to December 1988 (Gilgan et al., 1990; Haya et al., 1991). Soft-shell clams (*M. arenaria*) were contaminated by DA from blooms of *P. pseudodelicatissima*-complex species; it may have been *P. calliantha* (Lundholm et al., 2003) but its identity is still in dispute (Lundholm et al., in press). Also in the Bay of Fundy, elevated concentrations of DA ( $\sim 100$   $\mu g$  DA  $g^{-1}$ ) were found in the digestive gland of cultured sea scallops (*P. magellanicus*) from Digby, Nova Scotia, in July 1996 (unpublished data). DA concentrations and toxic *Pseudo-nitzschia* species have not been detected at any significant levels since then.

### 2.2.2. West coast

In British Columbia, the first recorded incidence of DA was in 1992 (Forbes and Chiang, 1994). DA has been found in razor clams (*Siliqua patula*), native littleneck clams (*Protothaca staminea*), manila clams (*Tapes philippinarum*), horse clams (*Tresus capax* and/or *T. nuttalli*), Pacific oysters (*Crassostrea gigas*), California mussels (*Mytilus californianus*), blue mussels (*M. edulis*), geoducks (*Panope abrupta*), Dungeness crabs (*Cancer magister*) and red rock crabs (*Cancer productus*). Between August 1992 and May 1993, the main areas affected were along the west coast of Vancouver Island, primarily in Quatsino, Clayoquot and Barkley Sounds, with additional isolated locations on Graham Island in the Queen Charlotte Islands, the Central Coast, and the Desolation Sound area of the inner South Coast. After September 1993, DA became even more widely distributed, occurring at additional locations, e.g. the Queen Charlotte Islands, on the North Coast and in inner South Coast waters. Recorded peak concentrations of DA are 277  $\mu g$  DA  $g^{-1}$  (in crab viscera; September 1992, Quatsino Sound) and 112  $\mu g$  DA  $g^{-1}$  (in razor clams; September 1992, Cox Bay, near Tofino) (Department of Fisheries and Oceans, 1992). Since then, the highest DA level was 106  $\mu g$  DA  $g^{-1}$ , on the north central west coast of Vancouver Island during March 2002 (Lewitus et al., in press). Quatsino Sound was closed to harvesting of crabs in August 1992, and again October 1993, due to excessive levels of DA. As well, portions of Barkley Sound were closed to harvesting of crabs and all molluscan shellfish in November 1993. Low levels of DA from the second event persisted in razor clams from the Queen Charlotte Islands and in molluscan shellfish from Barkley Sound

**Table 2**

Closures of molluscan shellfish harvesting, caused by domoic acid. nr: not reported.

Location	Dates	Organisms affected	Dominant species of <i>Pseudo-nitzschia</i>	Reference
Eastern Prince Edward Island, Canada	December 1987–January 1988	Blue mussel ( <i>Mytilus edulis</i> )	<i>P. multiseriis</i>	Bates et al. (1989)
Northern Prince Edward Island, Canada	Autumn of 1991, 1992, 1994, 2000, 2001	Blue mussel ( <i>Mytilus edulis</i> )	<i>P. multiseriis</i>	Bates (2004)
Bay of Fundy, Canada	September 1988–December 1988	Soft-shell clam ( <i>Mya arenaria</i> )	<i>P. pseudodelicatissima</i> <sup>a</sup>	Gilgan et al. (1990), and Haya et al. (1991)
Coastal Washington, USA	1991, 1993, 1995, 1998–1999, 2002, 2003, 2004, 2005, 2006	Razor clam ( <i>Siliqua patula</i> )	<i>P. cf. pseudodelicatissima</i> , <i>P. australis</i>	Adams et al. (2000), Wekell et al. (2002), Trainer and Suddleson (2005), and Washington State Department of Health database.
Quatsino Sound, British Columbia, Canada	August 1992, October 1993	Dungeness crab ( <i>Cancer magister</i> )	nr	Department of Fisheries and Oceans (1992)
Barkley Sound, British Columbia, Canada	November 1993	Razor clam ( <i>Siliqua patula</i> ); Dungeness crab ( <i>Cancer magister</i> )	nr	Department of Fisheries and Oceans (1992)
New Zealand	Various dates between 1994 and 1999; review pending for 2000–2010	New Zealand scallop ( <i>Pecten novaezealandia</i> ); Greenshell <sup>TM</sup> mussel ( <i>Perna canaliculus</i> )	<i>P. australis</i>	Hay et al. (2000), L. Rhodes (pers. comm.)
Galicia, Spain	1995, 1996	Scallop ( <i>P. maximus</i> , <i>P. jacobus</i> )	<i>P. australis</i>	Arévalo et al. (1998), and Fraga et al. (1998)
West coast of Scotland	June 1999–April 2000	King scallop ( <i>Pecten maximus</i> )	<i>P. australis</i>	Gallacher et al. (2001)
Southeast coast of Ireland	December 1999–May 2000	King scallop ( <i>Pecten maximus</i> )	<i>P. australis</i>	James et al. (2005), and Bogan et al. (2007a,b)
Coastal Portugal	Periodic closures lasting 1 week; mostly in May of various years	Common cockle ( <i>Cerastoderma edule</i> ); Peppery furrow shell ( <i>Scrobicularia plana</i> ); blue mussel ( <i>Mytilus edulis</i> )	<i>P. australis</i>	Vale and Sampayo (2001, 2002)
California, USA	2000–2009	Blue mussel ( <i>Mytilus edulis</i> ), lobster viscera	<i>P. australis</i> , <i>P. multiseriis</i>	Trainer et al. (2000), and Langlois (2001–2009)
Southern Gulf of St. Lawrence, Canada	April 2002–June 2002	Blue mussel ( <i>Mytilus edulis</i> )	<i>P. seriata</i>	Bates et al. (2002)
Baie de Seine, France	November 2004–“several months”	King scallop ( <i>Pecten maximus</i> )	<i>P. australis</i> or <i>P. multiseriis</i>	Nézan et al. (2006)
West coast of Ireland	April 2005	Blue mussel ( <i>Mytilus edulis</i> )	nr	Bogan et al. (2006)
Puget Sound, Washington, USA	September 2003; October 2005–November 2005	Blue mussel ( <i>Mytilus edulis</i> )	<i>P. australis</i> , <i>P. pseudodelicatissima</i>	Bill et al. (2006), and Trainer et al. (2007)
Oregon, USA	1991, 1998, 2003, 2004, 2005	Razor clam ( <i>Siliqua patula</i> ), blue mussel ( <i>Mytilus edulis</i> )	<i>P. australis</i>	Tweddle et al. (2010)
Denmark, various locations	March–April 2005	Blue mussel ( <i>Mytilus edulis</i> )	<i>P. seriata</i>	Lundholm et al. (2005a)
Santa Catarina State, Brazil	January 2009–February 2010	Brown mussel ( <i>Perna perna</i> )	nr	Reported in Fernandes and Brandini (2010)

<sup>a</sup> The identity of this species is still in dispute (Lundholm et al., in press).

until the autumn of 1994. In the fall of 1995, razor clam harvesting was again suspended in the northern Queen Charlotte Islands, due to high levels of DA. Incidences of DA contamination have since decreased.

The responsible organism(s) in coastal British Columbia waters has not yet been determined. However, the known DA producers *P. multiseriata*, *P. australis*, *P. pseudodelicatissima* and *P. seriata* are present (Forbes and Denman, 1991; Taylor et al., 1994; Taylor and Haigh, 1996). *Pseudo-nitzschia* spp. are usually a minor component of the phytoplankton population off the southwest coast of Vancouver Island in late summer, but there is substantial variability and species may occur throughout the year. Species are also found in the Strait of Georgia and north of Vancouver Island. *Pseudo-nitzschia* spp. are present and often abundant in all coastal waters throughout the summer and fall, e.g. Sechelt Inlet (Taylor et al., 1994), but the most intense blooms have occurred over the outer continental shelf (Forbes and Denman, 1991). Taylor and Haigh (1996) observed that blooms enter Barkley Sound from the open coast and then appear to sink.

### 2.3. Mexico

Several countries in Latin America are surrounded by two oceans, so it is not surprising that they are vulnerable to HABs, including those caused by toxigenic species of *Pseudo-nitzschia*. Considering the wide-spread presence of these diatoms, there have been few negative impacts that can be linked specifically to DA.

Of the ~21 described *Pseudo-nitzschia* species in Mexican waters, at least 10 are toxic or potentially toxic. On the Pacific coast: *P. australis*, *P. brasiliensis*, *P. fraudulentula* and *P. pungens*. On the Gulf of Mexico coast: *P. australis*, *P. calliantha*, *P. cuspidata*, *P. galaxiae*, *P. australis*, *P. pseudodelicatissima* (which may be any from the *P. pseudodelicatissima* complex) and *P. pungens*. Nontoxic species include: *P. americana*, *P. inflatula*, *P. lineola*, *P. roundii* (although not yet tested), *P. subfraudulenta*, *P. arenysensis*, *P. cacciantha*, *P. decipiens*, *P. subfraudulenta* and *P. subpacifica* (Hernández-Becerril, 1998; Hasle, 2002; Hernández-Becerril and Díaz-Almeyda, 2006; Hernández-Becerril et al., 2007; Quijano-Scheggia et al., 2011).

The first indication that DA was a problem was in 1996, when 150 brown pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*) from the southern Baja California peninsula showed abnormal behaviors or died (Sierra-Beltrán et al., 1997). This bird mortality event was similar to the one described by Work et al. (1993a,b) in Monterey Bay, USA. Unidentified *Pseudo-nitzschia* spp. and DA were detected in the birds' stomachs; stomach extracts caused the characteristic abnormal behavior and death in mice injected intraperitoneally. DA was also found in the birds' food, mackerel (*Scomber japonicus*). Several other mortality events have since occurred, although it was not possible to make a definitive link to DA. For example, another massive mortality of sea birds and marine mammals occurred in the winter of 1997, on the east coast of the Baja California peninsula (Ernesto García-Mendoza, pers. comm.). In 2002, *Pseudo-nitzschia* spp. were associated with the sickness or death of 87 sea lions (*Zalophus californianus*) on the beaches in northern Baja California (Hernández-Becerril et al., 2007). Additional events consistent with DA toxicity have been identified. In 2002, 87 sea lions were found stranded on beaches from the border of the USA to Ensenada, on the Baja peninsula, and it was assumed that DA toxicity was the cause of this event (Hernández-Becerril et al., 2007). As well, there were mass mortalities of sea mammals and birds in 1995 and 2004, in the Gulf of California. However, there was no hard evidence to pinpoint DA as the cause of the mortalities (Ochoa et al., 2002; Sierra-Beltrán et al., 2005).

At times *Pseudo-nitzschia* spp. have bloomed, but without significant accumulation of DA or death of animals. DA was detected in net phytoplankton samples dominated by *P. fraudulentula* during upwelling in June–July 2006, at La Paz, southern Gulf of California (Gárate-Lizárraga et al., 2007). Strains of this species have been shown to produce low levels of DA only in New Zealand (Rhodes et al., 1998), Monterey Bay, California, USA (Wells et al., 2005) and Chesapeake Bay, Maryland, USA (Thessen et al., 2009), but nowhere else. No *P. fraudulentula* isolates from the Gulf of California were available for toxicity testing. Only low levels of DA (0.55 µg DA g<sup>-1</sup>) were detected in chocolate clams (*Megapitaria squalida*). Another toxic bloom occurred in Todos Santos Bay, on the Pacific coast of the Baja California peninsula, during upwelling in April 2007 (García-Mendoza et al., 2009). A maximum DA concentration of 0.86 µg DA l<sup>-1</sup> in the plankton was associated with the presence of *P. australis* that reached a maximum abundance of 3 × 10<sup>5</sup> cells l<sup>-1</sup>, and a maximum calculated specific toxicity of 42 pg DA cell<sup>-1</sup>. No adverse biological effects were detected. This is the southernmost report of the presence of DA in the California Current System. The above events suggest that the incidences and geographic extent of animal mortalities and toxic *Pseudo-nitzschia* blooms may be increasing in Mexican waters.

### 2.4. South America

In South America, *Pseudo-nitzschia australis* was one of the first known *Pseudo-nitzschia* species, recorded in 1939 by Joaquín Frenguelli, from the Golfo de San Matías, Argentina (Sar et al., 1998). Since then, it has been found not just in the southern hemisphere, but in most coastal areas, curiously with the exceptions of the eastern Atlantic and Asia. It is thus considered cosmopolitan (Hasle, 2002) and is the most common toxic *Pseudo-nitzschia* species in South America. What had been called *P. seriata* in Californian waters is actually *P. australis*; the two had been confused up until the 1960s, because they both have wide cells. *P. australis*, along with several others, continues to be problematic in waters of several South American countries. Although *P. australis* has been reported in Peruvian waters (Hasle, 1965), there have thus far been no reports of toxic blooms or DA in the food web.

#### 2.4.1. Chile

DA was first discovered in South America near Puerto Montt, south-central Chile, in March 1997, in clams (*Venus antiqua*; 15.7 µg DA g<sup>-1</sup>), at levels just below the regulatory limit (reported in Ferrario et al., 2002). Later, in 1999–2000, DA was found in scallops (*Argopecten purpuratus*; 62.4 µg DA g<sup>-1</sup>) and clams (*Protothaca taca*; 330 µg DA g<sup>-1</sup>) in central Chile (Suárez-Isla et al., 2002). Each event was associated with blooms of *P. australis*. Strains of this species, as well as of *P. calliantha*, from Northern Chile, were confirmed to produce DA in culture (Álvarez et al., 2009; Table 3). In the Magellan region of southern Chile, *P. australis* and *P. pseudodelicatissima* (which may actually be any of the *P. pseudodelicatissima*-complex species) have been registered during monitoring activities since 1994, along with trace levels of DA found in phytoplankton samples, although not associated with any ASP events (Suárez-Isla et al., 2002). Significant DA concentrations (up to ~70 µg DA g<sup>-1</sup> whole animal; 15.5 µg DA g<sup>-1</sup> in edible tissues) were later found in the tunicate *Pyura chilensis*, in Bahía Inglesa, central Chile, during a bloom of *P. australis* (López-Rivera et al., 2009). This is the first report of DA in a tunicate, which is important because some of its tissues are consumed by humans. Other bivalve molluscs (*Mytilus chilensis*, *Aulacomya ater*, *Protothaca thaca*) at this aquaculture region contained sub-toxic levels of DA (Table 1). The presence of the above toxigenic *Pseudo-nitzschia* species, in addition to the potentially toxigenic

**Table 3**Species of *Pseudo-nitzschia* proven to produce domoic acid (DA) in laboratory culture. nr: not reported.

Species	Max DA concentration (pg DA cell <sup>-1</sup> )	DA quantitation method used	Origin of isolate	Reference
<i>P. australis</i>	37	HPLC-UVD <sup>d</sup>	Monterey Bay, CA, USA	Garrison et al. (1992)
	2.0	HPLC-UVD	Bream Bay, New Zealand	Rhodes et al. (1996)
	nr	HPLC-FMOC <sup>e</sup>	Ría de Vigo, Spain	Fraga et al. (1998)
	nr	ELISA <sup>f</sup>	Australia	Lapworth et al. (2001)
	3.5	HPLC-UVD	Colonsay, Scotland	Campbell et al. (2001)
	27	LC-MS <sup>g</sup>	Waterford Harbour, Ireland	Cusack et al. (2002)
	1.1	LC-MS/MS <sup>h</sup>	Bay of Plenty, New Zealand	Rhodes et al. (2004)
	1.68	HPLC-FMOC	Lynn of Lorne, Scotland	Fehling et al. (2004b)
	3	ELISA	Monterey Bay, CA, USA	Wells et al. (2005)
	0.026	RBA <sup>i</sup>	Offshore Washington State, USA	Baugh et al. (2006)
	1.74	LC-MS/MS	Bahía La Herradura, Chile	Álvarez et al. (2009)
	19.5	cELISA <sup>j</sup>	Point Reyes, California, USA	Bill (2011)
	21.85		Monterey Wharf, CA, USA	Guannel et al. (2011)
3.3	ELISA	Todos Santos Bay, Mexico	Santiago-Morales and García-Mendoza (2011)	
<i>P. brasiliana</i>	0.0095	HPLC-FMOC	Bizerte Lagoon, Tunisia	Sahraoui et al. (2011)
<i>P. calliantha</i>	0.098	HPLC-UVD	Bay of Fundy, Canada	Martin et al. (1990) <sup>a</sup>
	0.221	HPLC-FMOC	Limfjord, Denmark	Lundholm et al. (1997) <sup>a</sup>
<i>P. cuspidata</i>	0.01	LC-MS/MS	Bahía Tongoy, Chile	Álvarez et al. (2009)
	nr	HPLC-FMOC	Bizerte Lagoon, Tunisia	Sahraoui et al. (2009)
	0.0057	cELISA	Chesapeake Bay, Maryland, USA	Thessen et al. (2009)
	0.029	cELISA	Offshore Washington State, USA	Auro (2007)
	0.019	cELISA	Offshore Washington State, USA	Trainer et al. (2009a,b)
<i>P. delicatissima</i>	0.031	cELISA	Offshore Washington State, USA	Lundholm et al. (in press)
	0.005	HPLC-FMOC	Prince Edward Island, Canada	Smith et al. (1991)
<i>P. fraudulenta</i>	0.12	ELISA	Blueskin Bay, New Zealand	Rhodes et al. (1998)
	0.0002	RBA	Offshore Washington State, USA	Baugh et al. (2006) <sup>b</sup>
	0.03	ELISA	Bay of Plenty, New Zealand	Rhodes et al. (1998)
	nr	cELISA	Monterey Bay, California, USA	Wells et al. (2005)
<i>P. galaxiae</i>	0.0008	cELISA	Chesapeake Bay, Maryland, USA	Thessen et al. (2009)
	0.00036	LC-MS	Gulf of Naples	Cerino et al. (2005)
<i>P. granii</i>	0.000004	cELISA	Ocean Station Papa, North Pacific	Trick et al. (2010) <sup>c</sup>
<i>P. multiseriata</i>	21	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (1989)
	9.6	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (1991)
	5.0	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (1993)
	12.5	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (1996)
	18.99	HPLC-UVD, FMOC	Wadden Sea, Holland	Vrieling et al. (1996)
	1.03	HPLC-UVD	Jinhae Bay, South Korea	Lee and Baik (1997)
	67	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (1999)
	46	HPLC-FMOC	Prince Edward Island, Canada	Bates et al. (2001)
	nr	HPLC-UVD	Les Glénan, Brittany, France	Amzil et al. (2001)
	2.4	HPLC-FMOC	Chinhae Bay, South Korea	Cho et al. (2001)
	0.021	HPLC-FMOC	Monterey Bay, California, USA	Maldonado et al. (2002)
	1.15	HPLC-FMOC	Okkiray Bay, Japan	Lundholm et al. (2004)
	0.7	HPLC-FMOC	Bay of Fundy, Canada	Lundholm et al. (2004)
	0.225	ELISA	Offshore Washington State, USA	Wells et al. (2005)
	0.261	RBA	Offshore Washington State, USA	Baugh et al. (2006)
	nr	Mouse bioassay	Paranaguá State, Brazil	Mafra et al. (2006)
	0.006	cELISA	Sequim Bay, WA	Radan (2008)
	140?	LC-MS/MS	Okkiray Bay, Japan	Trimborn et al. (2008)
	20.8	HPLC-FMOC	Peter the Great Bay, Russia	Orlova et al. (2008)
	11.2	RBA	Monterey Bay, California, USA	Doucette et al. (2008)
	0.80	HPLC-UVD	Northland, New Zealand	Rhodes et al. (1998)
	5.0	HPLC-UVD	Thames Estuary, UK	Calu et al. (2009)
	1.807	cELISA	Chesapeake Bay, Maryland, USA	Thessen et al. (2009)
	23.6	HPLC-UVD	Lisbon Bay, Portugal	Amorim et al. (2009)
	3.45	LC-MS/MS	Coastal Brazil	Hagström et al. (2011)
	10	cELISA	Eastern Canada	Sun et al. (2011)
	0.71	cELISA	Eastern Canada	Guannel et al. (2011)
<i>P. multistriata</i>	0.645	LC-MS	Gulf of Naples	Sarno and Dahlmann (2000)
	0.697	LC-MS	Gulf of Naples	Orsini et al. (2002)
	0.28	HPLC-FMOC	Gulf of Naples	Amato et al. (2010)
<i>P. pseudodelicatissima</i>	0.04	LC-MS	New Zealand	L. Rhodes (pers. comm.)
	nr	RBA	Northern Gulf of Mexico	Parsons et al. (1999)
	0.0078	HPLC-UVD	Thermaikos Gulf, Greece	Moschandreu et al. (2010)
<i>P. pungens</i>	0.47	HPLC-UVD	Marlborough Sounds, NZ	Rhodes et al. (1996)
	0.070	HPLC-FMOC-RBA	Offshore Washington State, USA	Trainer et al. (1998)
	0.0018	RBA	Coastal Washington State, USA	Baugh et al. (2006)
<i>P. seriata</i>	0.2	HPLC-UVD	Bay of Crozon, France	Calu et al. (2009)
	33.6	HPLC-FMOC	Nivå Bugt, Denmark	Lundholm et al. (1994)
	0.8	HPLC-FMOC	St. Lawrence Estuary, Canada	Couture et al. (2001)
	7.0	HPLC-FMOC	Gulf of St. Lawrence, Canada	Bates et al. (2002)
	14.7	HPLC-FMOC	Lynn of Lorne, Scotland	Fehling et al. (2004b)
1.93	HPLC-FMOC	Nuuk (Godthåbsfjord), Greenland	Hansen et al. (2011)	

Table 3 (Continued)

Species	Max DA concentration (pg DA cell <sup>-1</sup> )	DA quantitation method used	Origin of isolate	Reference
<i>P. turgidula</i>	0.033 0.0000052	HPLC-UVD cELISA	Tauranga Harbour, New Zealand Ocean Station PAPA (NE Pacific)	Rhodes et al. (1996) Trick et al. (2010)
<i>P. sp. 233</i>	0.09 13.48	cELISA cELISA	Ocean Station PAPA (NE Pacific) Sequim Bay, WA, USA	Bill (2011) Guannel et al. (2011)

<sup>a</sup> Indicated as *P. pseudodelicatissima*; however, Lundholm et al. (in press) indicate that “the identity of the cells causing the 1988 toxic bloom is still not settled and must be reconsidered”.

<sup>b</sup> Belongs to the “*P. delicatissima*” complex.

<sup>c</sup> Shipboard batch culture of natural seawater containing *P. granii* as the only species of *Pseudo-nitzschia*.

<sup>d</sup> HPLC-ultra-violet detection.

<sup>e</sup> HPLC fluorenylmethoxycarbonyl derivative.

<sup>f</sup> Direct enzyme-linked immunosorbent assay.

<sup>g</sup> Liquid chromatograph–mass spectrometry.

<sup>h</sup> Tandem liquid chromatograph–mass spectrometry.

<sup>i</sup> Receptor binding assay.

<sup>j</sup> Competitive enzyme-linked immunosorbent assay.

*P. delicatissima*, *P. fraudulenta* and *P. pungens*, suggests the potential for harvesting closures.

#### 2.4.2. Argentina

DA was recorded for the first time in the Argentine Sea in July 2000, at Mar del Plata. The toxin was detected in plankton, mussels (*M. edulis*; 7.7 µg DA g<sup>-1</sup>) and stomachs of anchovy (*Engraulis anchoita*; 76.6 µg DA g<sup>-1</sup>), during a bloom of *P. australis* (Negri et al., 2004). There were no negative human health consequences during this, or any other subsequent events in Argentina (Ferrario et al., 2002). However, the presence of DA in the food web is cause for concern. As well, several other potentially toxic species are present along the Argentine coast and farther south, in the Drake Passage: *P. multiseriata*, *P. fraudulenta*, *P. pungens* and *P. turgidula* (Negri and Inza, 1998; Ferrario et al., 2004; Almandoz et al., 2007).

The presence of *P. pseudodelicatissima* in Argentinean waters is not yet confirmed as species previously thought to be *P. pseudodelicatissima* are actually *P. calliantha* (Almandoz et al., 2007; Table 4). It should be noted that Almandoz et al. (2007) did not find *P. seriata* during their study, supporting Ferrario et al.

(2002), who considered the records of *P. seriata* in Argentinean waters to be a misidentification, and the identification should actually be *P. australis*. *P. seriata* has only been identified without doubt in cold to temperate waters of the Northern Hemisphere (Hasle, 1964; Hasle and Lundholm, 2005). However, *P. seriata* with only two rows of poroids was observed recently in samples from the Beagle Channel at the southernmost tip of South America (Almandoz et al., 2009; see also Hansen et al., 2011).

#### 2.4.3. Uruguay

The only DA event reported for Uruguay was in mussels from Punta del Este, in December 2001, but toxicity (9.9 µg DA g<sup>-1</sup>) was below the regulatory level; the toxin source was *P. multiseriata* (Medina et al., 2003). The potentially toxic *P. multistriata* is also found in Uruguayan waters (Méndez and Ferrario, 2009).

#### 2.4.4. Brazil

DA was not a human health problem in Brazil until January 2009, when toxin was reported in brown mussels (*Perna perna*; up to 20 µg DA g<sup>-1</sup>) at two mussel farms in Santa Catarina State

Table 4

Amended identifications of *Pseudo-nitzschia* species. n.a.: not available.

Original identification	Reference	Emended identification	Reference
<i>P. pseudodelicatissima</i>	Martin et al. (1990)	<i>P. calliantha</i> , <i>P. pseudodelicatissima</i> or other	Lundholm et al. (2003) Kaczmarek et al. (2005), and Lundholm et al. (in press)
<i>P. pseudodelicatissima</i>	Hallegraef (1994)	<i>P. dolorosa</i>	Jameson and Hallegraef (2010)
<i>P. pseudodelicatissima</i>	Lundholm et al. (1997)	<i>P. calliantha</i>	Lundholm et al. (2003)
<i>P. pseudodelicatissima</i>	Davidovich and Bates (1998)	<i>P. calliantha</i> and <i>P. pseudodelicatissima</i> or <i>P. cuspidata</i>	Lundholm et al. (2003)
<i>P. pseudodelicatissima</i>	Rhodes et al. (1998)	n.a.	
<i>P. pseudodelicatissima</i>	Parsons et al. (1999)	Unknown	Lundholm et al. (2003)
<i>P. pseudodelicatissima</i>	Adams et al. (2000)	<i>P. pseudodelicatissima</i> or <i>P. cuspidata</i>	Bates and Trainer (2006)
<i>P. sp. cf. pseudodelicatissima</i>	Pan et al. (2001)	<i>P. pseudodelicatissima</i> or <i>P. cuspidata</i>	Lundholm et al. (2003)
<i>P. pseudodelicatissima</i> (NWFSC-047)	Stehr et al. (2002)	<i>P. fryxelliana</i>	Lundholm et al. (in press)
<i>P. cf. pseudodelicatissima</i>	Baugh et al. (2006)	n.a.	
<i>P. pseudodelicatissima</i>	Trainer et al. (2007)	<i>P. pseudodelicatissima</i>	Trainer et al. (2007)
<i>P. calliantha</i> 2	Amato et al. (2007)	<i>P. mannii</i>	Amato and Montresor (2008)
<i>P. delicatissima</i> del1	Amato et al. (2007)	<i>P. arenysensis</i>	Quijano-Scheggia et al. (2009b)
<i>P. turgidula</i>	Hallegraef (1994)	<i>P. dolorosa</i>	Lundholm et al. (2006)
<i>P. delicatissima</i>	No known record	<i>P. dolorosa</i> and <i>P. decipiens</i>	Lundholm et al. (2006)
<i>P. pseudodelicatissima</i>	Ferrario et al. (1999)	<i>P. calliantha</i>	Almandoz et al. (2007)
<i>P. calliantha</i> 2	Amato et al. (2007)	<i>P. mannii</i>	Amato and Montresor (2008)
<i>P. delicatissima</i> del1	Amato et al. (2007)	<i>P. arenysensis</i>	Quijano-Scheggia et al. (2009b)
<i>P. pseudodelicatissima</i> ?	Leandro et al. (2010b)	<i>P. hasleana</i>	Lundholm et al. (in press)
<i>P. pseudodelicatissima</i> -complex (Ner-D8)	Orive et al. (2010)	<i>P. hasleana</i>	Lundholm et al. (in press)
<i>P. calliantha</i> (IEO-PS50V)	Penna et al. (2007)	<i>P. hasleana</i>	Lundholm et al. (in press)
<i>P. pseudodelicatissima</i> (CAWB82)	Rhodes et al. (1998)	<i>P. caciaantha</i>	L. Rhodes (pers. comm.)

Modified and updated from Moschandreu et al. (2010).

(Fernandes and Brandini, 2010). This led to closure of the farms for 25 days (Table 2). The responsible organism was not given, but the following potentially toxic species are present in the region: *P. australis*, *P. calliantha*, *P. lineata*, *P. multiseriata*, *P. fraudulenta*, *P. pungens* var. *pungens* and *P. pungens* var. *cingulata* (Odebrecht et al., 2001; Fernandes and Brandini, 2010). Because *P. australis* was reported exclusively in May and July, and was also absent in Santa Catarina State during a later study (Fernandes and Brandini, 2010), it likely was not the source of DA. Rather, it may have been *P. multiseriata*, *P. calliantha* (two morphotypes) or *P. pungens*, which were found in Santa Catarina only, and abundantly from November to February (Fernandes and Brandini, 2010). The potentially toxic *P. brasiliensis* and toxic *P. multistriata* were also found farther north, in the state of Rio de Janeiro (Villac et al., 2004, 2005). The toxigenic *P. seriata* is also described in the state of Paraná, adjacent to the north of Santa Catarina State (Procopiak et al., 2006). However, because of the possible misidentification of this species in South America (see Ferrario et al., 2002 and Almandoz et al., 2007, above), it is possible that this was actually *P. australis*.

Earlier, in adjacent (to the north) Paranaguá State, during August 2002 to October 2003, no DA was detected in harvested mangrove mussels (*Mytella guyanensis*), despite the presence of *P. multiseriata*, *P. calliantha*, and *P. pungens*, sometimes at elevated concentrations (Mafra et al., 2006). Furthermore, one isolate of *P. multiseriata* produced DA in culture (Table 3). It was postulated that the cell toxicity was low, or that the cells accounted for only a small fraction of the mussels' food intake, therefore accounting for the low levels of toxin in the shellfish.

Because DA has already been demonstrated to toxify aquacultured mussels in Brazil, and several toxigenic and potentially toxic *Pseudo-nitzschia* spp. are present, the establishment of a monitoring program in the molluscan aquaculture sectors has been advocated (Mafra et al., 2006). Such a program is currently in effect only in the State of Santa Catarina, the main bivalve producer, where biweekly surveys of several potentially harmful microalgal species, including *Pseudo-nitzschia* spp., and their toxins are carried out in 40 bivalve culture locations (L. Mafra, pers. comm.).

## 2.5. Australia

Although there are toxigenic *Pseudo-nitzschia* species in Australian waters and traces of DA have been detected in molluscan shellfish, there have been no reported incidences of ASP in Australia (Lapworth et al., 2001; Takahashi et al., 2007). Toxic or potentially toxic species include: *P. australis*, *P. calliantha*, *P. cuspidata*, *P. delicatissima* (Lundholm et al., 2003; Jameson and Hallegraef, 2010), *P. galaxiae* (Lundholm and Moestrup, 2002), *P. multiseriata* (Lapworth et al., 2001; Jameson and Hallegraef, 2010), *P. pungens*, *P. fraudulenta* (Takahashi et al., 2007) and *P. multistriata* (Jameson and Hallegraef, 2010). Significant levels of DA (up to 500 ng DA ml<sup>-1</sup>) were confirmed in *P. australis* cultures tested (Table 3), indicating a potential threat in Australian waters (Lapworth et al., 2001), although this has not yet been realized. *P. multiseriata* is present less often, and only at low concentrations, and isolates have not yet been tested for toxicity, but isolates of *P. pungens*, and of the *P. pseudodelicatissima*-complex species, were nontoxic (Hallegraef, 1994; Lapworth et al., 2001). Other nontoxic species include: *P. americana*, *P. dolorosa*, possibly *P. heimii*, *P. lineola*, *P. subfraudulenta* and possibly *P. subpacificus* (Hallegraef, 1994; Lapworth et al., 2001; Jameson and Hallegraef, 2010; G. Hallegraef, pers. comm.).

## 2.6. New Zealand

Diatoms of the genus *Pseudo-nitzschia* have been observed in New Zealand's coastal waters for at least four decades, but no toxic

species or DA contamination in bivalve shellfish were reported until 1993 (Rhodes et al., 1996), when phytoplankton and biotoxin monitoring programs were implemented. The first indication of toxicity was in November, 1993, in Whangaroa Harbour, when DA was found in the digestive gland of the New Zealand scallop (*Pecten novaezealandiae*; 270 µg DA g<sup>-1</sup>) (Rhodes et al., 1996). That same year, toxins causing DSP, PSP and NSP, and their microalgal producers, were detected. There is no evidence that the considerable expansion in Greenshell™ mussel (*Perna canaliculus*) farming in the Marlborough Sounds (the major shellfish growing area) has resulted in any increased incidence of HABs (Rhodes et al., 2001). From then on, DA has been recorded in various shellfish species (Table 1).

The most detailed information on DA in aquacultured bivalves is given in Hay et al. (2000), but only for the years 1994–1999. *Pseudo-nitzschia* species and DA have been detected at all monitoring sites, on both the North and South Islands, although all closures of shellfish harvesting, except one (in the Marlborough Sounds aquaculture area), have occurred on the North Island. The highest level was 600 µg DA g<sup>-1</sup>, in the digestive gland of *Pecten novaezealandiae* from Doubtless Bay, on November 1994. Only a low percentage of the commercially harvested bivalve samples had DA levels above the regulatory limit: 1.64% for scallops and 0.02% for Greenshell™ mussels. Two instances of DA above the limit for mussels were in Marlborough Sounds in December 1994 (187 µg DA g<sup>-1</sup>), and Houhora, in Northland (33 µg DA g<sup>-1</sup>).

In New Zealand, species of *Pseudo-nitzschia* that have been shown to produce DA in culture (Table 3) include: *P. australis*, *P. multiseriata*, *P. multistriata*, *P. pungens*, *P. fraudulenta*, *P. delicatissima*, *P. turgidula* and *P. pseudodelicatissima*; *P. cf. heimii* was present at only low concentrations (Rhodes et al., 1996, 1998, 2000, 2004). It should be noted that clarifications are still needed regarding the status of some species within the *P. pseudodelicatissima* and *P. delicatissima* complexes, and with *P. cf. heimii*; this is being accomplished with molecular data (L. Rhodes, pers. comm.).

## 2.7. Africa

### 2.7.1. South Africa and Namibia

Whereas several *Pseudo-nitzschia* spp. responsible for ASP are known to occur in the Benguela, for example *P. australis* (Marangoni et al., 2001), shellfish toxicity has yet to be recorded in this region (Pitcher and Calder, 2000). However, DA has recently been measured in seawater samples containing *Pseudo-nitzschia* cells, although the toxigenic species were not identified (Fawcett et al., 2007). This provided the first conclusive evidence for the presence of ASP toxins in the Benguela system. During this study, particulate DA concentrations derived from filtered plankton samples ranged from 0.1 to 3 µg DA l<sup>-1</sup> and closely tracked the total cell concentrations of *Pseudo-nitzschia* species. In 2004, DA in phytoplankton (2–200 ng DA l<sup>-1</sup>) and the viscera of planktivorous pilchard (*Sardina pilchardus*; 120 ng DA g<sup>-1</sup>) were found off the Namibian coast (D.C. Louw, B. Currie, G. Doucette, pers. comm.).

### 2.7.2. Tunisia, Algeria and Morocco

In northern Africa, bordering the Mediterranean Sea, several species of *Pseudo-nitzschia* have the risk of becoming problematic, although no instances of DA contamination have yet been reported. Bizerte Lagoon, northern Tunisia, is an important region for molluscan aquaculture that is particularly vulnerable. The bloom dynamics of several *Pseudo-nitzschia* species has been studied, and isolates of *P. calliantha* (Sahraoui et al., 2009) and *P. brasiliensis* (Sahraoui et al., 2011) have been shown to produce DA (Table 3). Additional species reported include: *P. pseudodelicatissima*, *P. multistriata*, *P. delicatissima*-complex species and *P. cf. seriata*.

In Algeria, there have been no reported incidences of DA contamination, although at least two toxigenic species are present: *P. multiseriis* (Hasle, 2002) and *P. calliantha* (Illoul et al., 2008). On Morocco's Mediterranean coast, a bloom of *Pseudo-nitzschia* spp. coincided with the detection of low levels of DA in king scallops (*Pecten maximus*; 0.2 µg DA g<sup>-1</sup>), but not in any other shellfish species (reported in Tahri-Joutei et al., 2003). Subsequent blooms of *Pseudo-nitzschia* spp. (10<sup>4</sup> cells l<sup>-1</sup>) occurred in February 1997, and of *P. cf. delicatissima* (5 × 10<sup>4</sup> cells l<sup>-1</sup>) in April–May 2000 (Tahri-Joutei et al., 2003). On the Atlantic coast, potentially toxic *Pseudo-nitzschia* species include: *P. australis*, *P. fraudulenta* and *P. pseudodelicatissima*-complex species (Ennaffah, 2007).

## 2.8. Asia

*Pseudo-nitzschia* species have been documented in Vietnam, Japan, Korea and Russia, with *P. pungens* dominating in Korea, China and Russia. No human poisonings due to DA in shellfish have been reported in these countries (Skov et al., 2004; NOWPAP, 2005; Huang et al., 2009).

Significant levels of DA (145 µg DA g<sup>-1</sup> whole tissue) have been detected in the spiny oyster (*Spondylus*), collected from the Philippines and Vietnam (Dao et al., 2009). However, the responsible species of *Pseudo-nitzschia* have not yet been identified. The population dynamics of *P. pungens* was studied in Zhelin Bay, China in the early 2000s. In that study, an isolate of *P. cf. pseudodelicatissima* from Lampung Bay, China, showed no production of DA by HPLC–UV (Panggabean, 2007). Likewise, *P. pungens*, *P. cuspidata*, *P. brasiliensis*, *P. multistriata* and *P. multistriata* isolated from the along Chinese coast did not show detectable DA by HPLC–UV (R. Liu, pers. comm.).

In Russia, light and electron microscopical examination of 314 phytoplankton field samples, collected from 1995 to 2006, revealed the presence of eleven *Pseudo-nitzschia* species: *P. americana*, *P. cf. cacciantha*, *P. calliantha*, *P. delicatissima/arenysensis*, *P. fraudulenta*, *P. cf. heimii*, *P. multiseriis*, *P. multistriata*, *P. obtusa*, *P. pungens* and *P. seriata* (Stonik et al., 2011b). Blooms of *P. calliantha*, *P. multiseriis*, *P. multistriata* and *P. pungens* have been observed in summer and autumn in Peter the Great Bay in the northwestern Sea of Japan, with abundances exceeding 10<sup>6</sup> cells l<sup>-1</sup> and constituting 75–98% of the total density of the phytoplankton assemblage. The dominant species in the Sea of Okhotsk were *P. pungens*, *P. calliantha* and *P. americana*, with abundances exceeding 2 × 10<sup>5</sup> cells l<sup>-1</sup> and constituting 30–75% of the total phytoplankton assemblage in summer and autumn (Stonik et al., 2011b).

DA was found for the first time in stationary-phase *P. multiseriis* cells isolated from Peter the Great Bay, at concentrations varying between 180 and 5390 ng DA ml<sup>-1</sup> or 2–21 pg DA cell<sup>-1</sup> (Orlova et al., 2008; Table 3). No DA was detected (<2 ng ml<sup>-1</sup>) in cultures of *P. calliantha* and *P. multistriata* from the same locality. A gamma proteobacterium (tentatively identified as *Alteromonas macleodii*) was isolated from *P. multiseriis* strain PM-02 (Orlova et al., 2008), and was earlier shown to significantly enhance the DA concentration of an axenic culture of *P. multiseriis* (S.S. Bates, I. Kaczmarek, C. Léger, J. Ehrman and D.H. Green, unpubl. data). Concentrations of DA in tissues of the bivalves, *Mytilus trossulus*, *Crenomytilus grayanus* and *Mizuhopecten yessoensis*, from Peter the Great Bay, ranged from 0.01 to 0.5 µg DA g<sup>-1</sup>, well below permissible regulatory limits (Stonik et al., 2011a).

## 2.9. Europe

During the last decade, it was realized that DA can also pose a problem in European shellfish. The toxin has now been detected in shellfish from many European countries, particularly UK, Ireland and France (EFSA, 2009). European strains of several *Pseudo-nitzschia*

species have been found to be toxic: *P. australis*, *P. calliantha*, *P. galaxiae*, *P. multiseriis*, *P. multistriata*, *P. pseudodelicatissima*, *P. pungens* and *P. seriata* (Table 3; Lundholm et al., 1994; Sarno and Dahlmann, 2000; Cusack et al., 2002; Orsini et al., 2002; Fehling et al., 2004a,b; Moschandreu et al., 2010; E. Keady, pers. comm.). DA has especially impacted scallop fisheries in European waters (EFSA, 2009), and accumulation and prolonged retention of DA has resulted in closures of harvesting in e.g. UK, Ireland (Bogan et al., 2007a,b), France (Nézan et al., 2006; Klein et al., 2010), Denmark (Lundholm et al., 2005a), Portugal (Vale and Sampayo, 2001) and Spain (Arévalo et al., 1998; Fraga et al., 1998) (Table 2; James et al., 2005). On average, 59% of scallops tested contained DA above the EU regulatory limit (EFSA, 2009). Particularly in Scotland, accumulation of DA resulted in long-term closures of scallop fisheries from the Scottish west coast (Gallacher et al., 2001; Smith et al., 2006). Since the EU directive for monitoring ASP in scallops changed in 2005, DA has not been a major problem in Scottish waters, as the processors now sell only the adductor muscle of the scallops (which is usually free of DA) and there are thus no closures of scallop harvesting areas (E. Bresnan, pers. comm.). DA in mussels, however, still results in short closures every year in Europe. There have not been any reported human illnesses associated with DA in Europe, but due to lack of a formal reporting system, mild cases could have gone un-noticed (EFSA, 2009).

*Pseudo-nitzschia* has been present in European waters for more than a century (e.g. Hasle et al., 1996; Churro et al., 2009; Lundholm et al., 2010) and is frequently reported as abundant or dominating in coastal areas in certain seasons (Fehling et al., 2004b, 2006; Zingone et al., 2006; Spatharis et al., 2007; Besiktepe et al., 2008; Quijano-Scheggia et al., 2008; Orive et al., 2010). There are few studies on long-term trends of occurrence of *Pseudo-nitzschia*. On the Atlantic coast of Spain, a weakening in the coastal upwelling was observed over the last two decades (Perez et al., 2010). In spite of a simultaneous increase in percentage of dinoflagellates, and a decrease in total diatoms, they found a highly significant increase in numbers of *Pseudo-nitzschia* spp. Sediment core studies are excellent for exploring long-term trends of occurrence and abundance of *Pseudo-nitzschia*. A sediment-core study in Mariager Fjord, Denmark, found no indication of an increase in the total relative abundance of *Pseudo-nitzschia* during the last hundred years (Lundholm et al., 2010). A similar study in the Gulf of Mexico revealed an increasing relative abundance of total *Pseudo-nitzschia* over time (Parsons et al., 2002).

Changes in species composition and distribution of *Pseudo-nitzschia* have been observed in Europe. In the Skagerrak, *P. multiseriis* declined in abundance after the late 1960s and 1970s, when it was the predominant *Pseudo-nitzschia* species (Hasle et al., 1996). Since the early 1990s, it has been recorded less frequently in the Northern European coastal waters (Hasle et al., 1996; Hasle, 2002; Bresnan, 2003; Fehling et al., 2006; Lundholm et al., 2010). At the same time, *P. pungens* has changed from being observed mainly during autumn before 1986, to now occurring all year round (Hasle et al., 1996). In a study of sediment-cores spanning at least a 100 year period, a similar shift in relative species abundance of *Pseudo-nitzschia* was recorded, with *P. multiseriis* being dominant before 1958 (making up >90% of the *Pseudo-nitzschia* community), to *P. pungens*, especially, and recently also *P. americana*, dominating the *Pseudo-nitzschia* community (Lundholm et al., 2010).

*Pseudo-nitzschia australis* seems to have extended its distribution in Europe. Previously, *P. australis* was not recorded from Northern European waters (Hasle et al., 1996). Then, the sudden findings of *P. australis* in European waters in 1994–1995, in Galicia, northwest Spain (Miguez et al., 1996) and in 1999–2000, in Scottish waters (Gallacher et al., 2001), indicate a spreading of *P. australis* northward in Atlantic waters. Long-term studies are

needed to confirm this. The distributional patterns of other *Pseudo-nitzschia* species are still not fully established.

### 3. Molecular studies of *Pseudo-nitzschia*

During the last decade, an increasing number of studies combining morphological and molecular characters for studying taxonomy and evolution of *Pseudo-nitzschia* have been published and species have been newly described or emended (e.g. Lundholm et al., 2003, 2006, in press; Amato et al., 2007; Amato and Montresor, 2008; Churro et al., 2009). This has revealed that cryptic and pseudo-cryptic species are a more commonly encountered phenomenon than previously considered (Orsini et al., 2004; Lundholm et al., 2003, 2006, in press; Amato et al., 2007; Quijano-Scheggia et al., 2009b). Cryptic species are morphologically identical, but genetically different, whereas pseudo-cryptic species, apart from the genetic diversity, show minor morphological differences that are only detected by detailed examination (Mann and Evans, 2007). The description of cryptic and pseudo-cryptic species might appear to make species identification more cumbersome. However, correct species assignment provides a possibility for understanding complex patterns of physiological parameters, e.g. toxin production, biogeographical patterns and species succession in field studies. Species identification will become easier in the future with further developments of PCR tools, e.g. microarray or simpler methods like ARISA (Automated Ribosomal Intergenic Spacer Analysis) analyses (McDonald et al., 2007; Hubbard et al., 2008; Medlin and Kooistra, 2010; reviewed by Kudela et al., 2010). However, development of such molecular methods relies on a thorough understanding of the underlying taxonomy.

#### 3.1. Phylogeny

Different regions of the genome have been targeted to assess the phylogeny of *Pseudo-nitzschia*: LSU, ITS1, 5.8S and ITS2 of the ribosomal DNA and rbcL, the large subunit of RuBisCo (Lundholm et al., 2002a,b, 2006; Orsini et al., 2004; Amato et al., 2007). The nuclear-encoded internal transcribed spacers (ITS), comprising ITS1, 5.8S and ITS2, have been the most widely used, as they give highly supported separation of species, and ITS2 is useful as a barcode sequence (Evans et al., 2007; Moniz and Kaczmarek, 2009). Furthermore, a correlation between mating compatibility and base changes in ITS2 makes the region a useful molecular tool. After sexual reproduction of *Pseudo-nitzschia* was first described (Davidovich and Bates, 1998), it then became possible to include mating compatibility in studies of taxonomic relatedness. Mating of strains has since been included in several phylogenetic studies (Amato et al., 2007; Casteleyn et al., 2008; Quijano-Scheggia et al., 2009b; Lundholm et al., in press). Another way of assessing mating compatibility is to explore the secondary structure of ITS2. The DNA strand of ITS2 folds to form a complex secondary structure with stems (pairings) and loops (no pairings), which play a role in the construction of ribosomes (Tschochner and Hurt, 2003). Base changes in both or one of the bases in the basepairs of the stems, known as CBCs (compensating base changes) and hemi-CBCs, respectively, have been found to correlate with mating compatibility of *Pseudo-nitzschia* strains. This finding supported Coleman's (2000, 2003, 2007, 2009) general observations of ITS2 and mating compatibility and makes ITS2 an excellent tool for studies of species delineation.

A number of species complexes have been identified in *Pseudo-nitzschia*, with the help of molecular tools. What were formerly regarded as *P. pseudodelicatissima* and *P. cuspidata* have now been shown to comprise the morphological, molecular and reproductively well-separated entities: *P. calliantha*, *P. caciaantha*, *P. manni*,

*P. hasleana*, *P. fryxelliana* and *P. cuspidata*/*P. pseudodelicatissima* (Table 4; Lundholm et al., 2003, in press; Amato et al., 2007; Amato and Montresor, 2008). However, there is still a taxonomic problem with *P. cuspidata* and *P. pseudodelicatissima*, as the only morphological difference between the two species is valve shape. Moreover, in all phylogenetic studies, *P. pseudodelicatissima* and *P. cuspidata* strains cluster within one highly supported clade (e.g. Lundholm et al., 2003, in press; Amato and Montresor, 2008; Moschandreou et al., 2010). Studies on the secondary structure of strains from the clade have revealed that CBCs and hemi-CBCs exist among the strains in the group (Amato et al., 2007; Moschandreou et al., 2010), but any subdivision of the clade is premature (Lundholm et al., 2003, in press; Amato and Montresor, 2008; Moschandreou et al., 2010). The type locality of *P. cuspidata* is Gran Canary (most closely represented by the strain "Tenerife8"), whereas the type locality of *P. pseudodelicatissima* is the subarctic Denmark Strait (Hasle, 1965), for which no strains are available. Future studies that include phylogenies on more genes, secondary structure analyses, mating studies comprising several strains, and inclusion of a strain of *P. pseudodelicatissima* from the type locality, are needed to finally settle the taxonomic problem.

*Pseudo-nitzschia delicatissima* has similarly been split into well-separated pseudo-cryptic species: *P. delicatissima*, *P. decipiens* and *P. dolorosa*. However, it was recently recognized that *P. delicatissima*, as previously defined, actually consists of two separate clades, representing two cryptic species: *P. delicatissima* and *P. arenysensis* (Orsini et al., 2004; Lundholm et al., 2006; Amato et al., 2007; Quijano-Scheggia et al., 2009b). The two cryptic species are morphologically indistinct, but comparisons of the secondary structure of ITS2 support that the species are separate entities. Furthermore, the species differ with respect to physiological parameters, e.g. temperature required to sexualize the genotypes and differences in growth rates (Kaczmarek et al., 2008; Quijano-Scheggia et al., 2009b).

*Pseudo-nitzschia americana* and the pseudo-cryptic *P. brasiliiana* and *P. linea* also represent a morphological species complex (Lundholm et al., 2002b). *P. americana* had previously been observed only as epiphytic single cells, in contrast to other *Pseudo-nitzschia* species, but has now also been found forming the chains typical of *Pseudo-nitzschia* (Kaczmarek et al., 2005). Another unusual *Pseudo-nitzschia* species is *P. galaxiae*, which has a completely different stria structure than the other species. Therefore molecular data were important for assessing its phylogenetic relationship (Lundholm and Moestrup, 2002). It has been found with three different morphotypes in the Gulf of Naples, having the same ultrastructure, but differing in size (Cerino et al., 2005). The three morphotypes bloom at different times of the year (Cerino et al., 2005). Parallel analyses on ITS, rbcL and LSU showed a seasonal segregation among these distinct genotypes, which matched the succession of different size classes (McDonald et al., 2007).

The cosmopolitan *P. pungens* has been split into three variants, based on morphological, molecular and reproductive data: var. *pungens*, var. *cingulata* and var. *aveirensis* (Casteleyn et al., 2008; Churro et al., 2009). These represent two reproductively isolated groups. Although two of the variants (*P. pungens* var. *pungens* and *P. pungens* var. *aveirensis*) are well-differentiated, occasional hybrids between the variants have been found in the field (Casteleyn et al., 2008; Holtermann et al., 2010), suggesting that they are not reproductively separated. This is supported by the analyses of the secondary structure of ITS2. Based on similar analyses of ITS2, it is hypothesized that the third variant (*P. pungens* var. *aveirensis*) is reproductively isolated from the other two (Churro et al., 2009). Within the one variant (*P. pungens* var. *pungens*), significant geographical population differentiation has been revealed on a global scale, with very restricted gene flow between the different

geographical populations (Casteleyn et al., 2010). This finding demonstrates that geographic distances play a role in speciation, even in highly dispersal phytoplankton organisms in the ocean. It is thus an excellent example of how detailed knowledge of harmful species like *Pseudo-nitzschia* can be of general importance for our understanding.

The genus *Pseudo-nitzschia* harbors even more diversity than presently described, as morphological variation that does not agree with any present species description continues to be detected (e.g. Lundholm et al., 2003; Kaczmarek et al., 2005; Sahraoui et al., 2009; Lundholm et al., in press). Similarly, the intrageneric and intraspecific diversity has been found to be greater than presently recorded (Stehr et al., 2002; McDonald et al., 2007); sequencing of LSU rDNA of strains from coastal waters of Washington State showed the presence of new ribotypes in the *P. pseudodelicatissima* group (Stehr et al., 2002). Likewise, application of genus-specific primers for LSU rDNA to field samples from the Gulf of Naples revealed three new genotypes not corresponding to any described species (McDonald et al., 2007).

### 3.2. Molecular tools

Within the last decade, there have been advances in the development of molecular tools for identifying *Pseudo-nitzschia* species within mixed assemblages and for determining population structure for species of *Pseudo-nitzschia*. An automated ribosomal intergenic spacer analysis (ARISA) was developed by Hubbard et al. (2008) for the rapid identification of *Pseudo-nitzschia* species in environmental samples. Microsatellite markers have been developed for *P. pungens* (Evans and Hayes, 2004), *P. multiseriata* (Evans et al., 2004), *P. multistriata* (Tesson et al., 2011), and *P. australis* (N.G. Adams, unpubl. data).

#### 3.2.1. ARISA

The ARISA assay allows for rapid identification of *Pseudo-nitzschia* species in environmental samples, using PCR primers predicted to amplify ITS1 fragment lengths for 11 known and one unknown species of *Pseudo-nitzschia* based on sequences in GenBank. Hubbard et al. (2008) used the ARISA to analyze environmental samples from Vancouver Island, Canada and Puget Sound, WA, USA. They detected nine distinct fragment lengths in clone libraries derived from the environmental samples in which eight of the fragment lengths corresponded to nine known or putative *Pseudo-nitzschia* species. In addition to analyzing fragment size, the fragments from the clone libraries can also be sequenced in order to differentiate species or individuals within a species. Hubbard et al. (2008) defined different genotypes of a single species as those that had identical ITS1 fragment lengths but <3% sequence diversity. Additionally, isolates with different ITS1 fragment lengths as well as sequence identity <3% (excluding insertions/deletions) were defined as different species types. For example, while *P. australis* and *P. seriata* shared the same fragment length, the sequences of the ITS1 fragments for these two species are different (7.8%), allowing for differentiation of the two species. The most frequently detected species within the clone library was *P. pungens*, which also had the greatest number of distinct genotypes (12).

Marchetti et al. (2008) used the ARISA to identify *Pseudo-nitzschia* species during an iron enrichment experiment in the northeast subarctic Pacific Ocean, near Ocean Station Papa. They compared the results from the ARISA (performed on bulk seawater samples) to clone libraries developed from the same samples. Their analysis detected seven different ITS1 variants. Two of the fragments detected corresponded to *P. granii* and *P. heimii*, while another fragment corresponded to an unidentified *Pseudo-nitzschia* species that was also present in clone libraries. The remaining four

observed fragments were likely putative, unidentified *Pseudo-nitzschia* ITS1 variants that were not detected in the clone libraries.

The ARISA can be an effective and more rapid method for detecting and identifying *Pseudo-nitzschia* species in environmental samples than traditional microscopic approaches. However, the accuracy of ARISA depends upon a complete understanding of the underlying taxonomy and the acquisition of correct sequence information with confirmatory culture validation. The development of clone libraries is also necessary in order to differentiate between species and to determine genotypes within a species. The assay is limited in that it may identify fragments that correspond to sequences for *Pseudo-nitzschia* species or species types that have not been previously identified. Additionally, the PCR primers may amplify fragments for organisms other than *Pseudo-nitzschia* that have not previously been tested for cross reactivity.

#### 3.2.2. Microsatellite markers

Microsatellite markers can be used to identify distinct populations of *Pseudo-nitzschia* species and to help determine if those populations are made up of cryptic species, different varieties of the same species, or contain hybrid forms of species varieties. Although microsatellite markers are powerful tools for analyzing populations of *Pseudo-nitzschia* species, they rely on isolates and culturing techniques that may introduce some bias, as analysis is performed only on those isolates that survive the culturing process.

Six polymorphic microsatellite markers were characterized by Evans and Hayes (2004) for *P. pungens*. A subsequent study by Evans et al. (2005) in the German North Sea, showed very high levels of genetic and clonal diversity along with weak temporal and spatial genetic structuring among 464 isolates genotyped from two locations ~100 km apart. Evans et al. (2005) suggested that, given the low levels of genetic differentiation and the high level of genetic diversity in their samples, there were no major barriers to gene flow in the German North Sea and that the 464 isolates originated from a single large, well-mixed population.

A study by Casteleyn et al. (2009b) used the microsatellite markers developed by Evans and Hayes (2004) to examine the population structure of 310 *P. pungens* isolates from water bodies with varying environmental conditions in the Southern Bight of the North Sea. The results of Casteleyn et al. (2009b) were similar to those of Evans et al. (2005) in that their data revealed no significant population structuring. When Casteleyn et al. (2009b) expanded their analysis to include isolates from the German, French and Dutch parts of the North Sea, they still did not observe any significant spatial differentiation over an area of ~650 km. However, the analysis included relatively few isolates from the areas outside of the Belgian North Sea.

Adams et al. (2009) used microsatellite markers designed by Evans and Hayes (2004) to analyze the population structure of *P. pungens* from the Pacific Northwest (PNW) coast of the USA and to compare that to the North Sea *P. pungens* using a subset of the isolates from the Evans et al. (2005) and Casteleyn et al. (2009b) studies. Analysis of the PNW samples revealed an admixture of two distinct populations of *P. pungens*. When compared to the North Sea population, the two PNW populations were more different from each other than either PNW population was from the North Sea population. The two PNW populations likely were representative of *P. pungens* clades I (var. *pungens*) and II (var. *cingulata*) that were described by Casteleyn et al. (2008); further analyses on a subset of isolates from these populations supported this hypothesis (Casteleyn et al., 2009a).

Casteleyn et al. (2010) examined *P. pungens* var. *pungens* from various locations worldwide using the microsatellite markers characterized by Evans and Hayes (2004) and found significant geographical genetic structuring. Population structure analysis

revealed six genetic clusters where some isolates showed a more admixed background (northeastern Atlantic) and some isolates clustered mainly according to geographic location (northwestern Atlantic and Pacific). Casteleyn et al. (2010) suggested that gene flow among populations of planktonic diatoms like *P. pungens* var. *pungens* can be restricted by oceanographic features such as fronts and oceanic gyres, as well as continental land masses, resulting in genetic divergence of populations.

Nine polymorphic microsatellite markers were developed by Evans et al. (2005) for *P. multiseriis*. These markers were used to genotype 25 field isolates of *P. multiseriis* as well as six of their descendants that were the products of mating experiments. Twenty-two of the field isolates were from eastern Canada, two from Europe and one from Russia. They found that 23 of the 25 field isolates were distinct and the Russian isolate was the most genetically distinct. Significant genetic differences were observed among isolates from eastern Canada; however, some of those isolates were capable of interbreeding. Evans et al. (2005) suggest that the significant differentiation between the Russian isolate and many of the Canadian isolates indicates that discrete populations of *P. multiseriis* exist; however, more extensive sampling is necessary.

Recently, microsatellite markers have been characterized for *P. multistriata* (Tesson et al., 2011). The authors described PCR primers for seven polymorphic loci and used the primers to amplify the microsatellite loci in 66 isolates of *P. multistriata* collected in the Gulf of Naples, Italy, from three different time periods (22 isolates each) in 2008. Based on their results, all of the loci deviated from Hardy–Weinberg equilibrium and two loci showed a small excess of homozygosity. The authors propose to use these markers in future studies to identify natural populations, infer their dynamics over seasonal blooms, analyze genetic structure over spatial scales and track changes in genetic diversity across sexual reproduction events.

#### 4. Life stages – implications in bloom ecology

*Pseudo-nitzschia*, like many pennate diatoms, can reproduce sexually (Geitler, 1935). Cells in clonal cultures of *Pseudo-nitzschia* gradually decrease in cell size over time and eventually die if they do not undergo sexual reproduction (also called auxosporulation) to regenerate their large cell size. This is due to vegetative cell division and splitting of the silicon frustule between two daughter cells. The halves of the frustule (thecae) fit together like a glass Petri dish, with the hypotheca slightly smaller than the epitheca. The daughter cell that receives the smaller of the two thecae produces a new hypotheca inside the first, larger epitheca. This cell is thus smaller than the initial parent cell. In this way, the average length (but not usually the width) of the cells gradually decreases until they become so small that the culture, and presumably natural populations, can no longer survive. However, if cells undergo sexual reproduction, the large cell size is restored.

A *Pseudo-nitzschia* cell becomes sexualized when cell length decreases below a threshold size, known as the first cardinal point (Geitler, 1935). In *P. multiseriis*, this is ~63% of the length of largest cells (Bates and Davidovich, 2002) and in *P. multistriata* it is 39–71% (D'Alelio et al., 2009). Sexual reproduction must occur before the cells reach a minimum length, which is ~30  $\mu\text{m}$  in *P. multiseriis* (Bates and Davidovich, 2002), 19–80  $\mu\text{m}$  in *P. delicatissima* (Amato et al., 2005) and 39–55  $\mu\text{m}$  in *P. multistriata* (D'Alelio et al., 2009). During this size window, cultures of *Pseudo-nitzschia* can be mixed together to stimulate sexual reproduction. *Pseudo-nitzschia* is generally dioecious (heterothallic), meaning that male and female gametes are produced by separate clones. These “sexes” are referred to as mating types “+” and “–” in *Pseudo-nitzschia* (Chepurnov et al., 2005). However, monoecious (homothallic)

reproduction has been reported in *P. brasiliiana* (Quijano-Scheggia et al., 2009a).

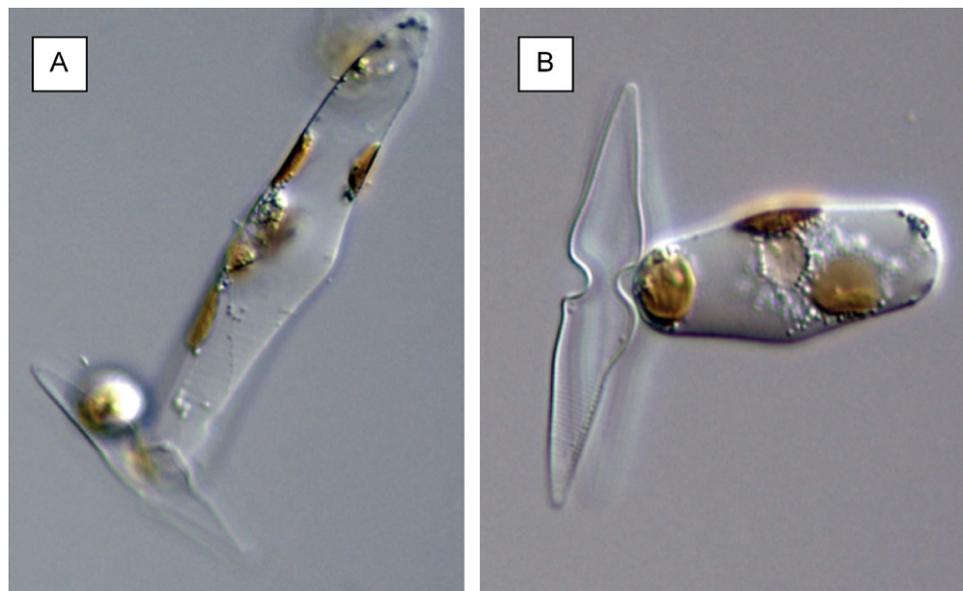
Mating in *Pseudo-nitzschia* can be achieved simply by mixing clones of the same species, but of opposite sex. Clones must be in good physiological condition to undergo sexual reproduction. Thus, clones must be mixed during the exponential growth phase, which can be from 3 to 6 d after inoculation of a batch culture. Temperature is an important factor as different species mate at different temperatures (Kaczmarek et al., 2008; Quijano-Scheggia et al., 2009b). Clones must receive a sufficient amount of light during a 24 h period. A photoperiod length up to 16:8 L:D, the maximum studied, increased gamete and auxospore production (Davidovich and Bates, 1998; Hiltz et al., 2000). These results suggest that parent cells must be healthy and photosynthesizing to produce energy for sexual reproduction.

Sexual reproduction has been described with photographic evidence and/or mating compatibility experiments in 14 species and one variety: *P. subcurvata* (Fryxell et al., 1991), *P. multiseriis* (Davidovich and Bates, 1998; Kaczmarek et al., 2000), *P. pseudodelicatissima* (which may be *P. calliantha*; Lundholm et al., 2003; Davidovich and Bates, 1998), *P. fraudulenta* (Chepurnov et al., 2004), *P. pungens* (Chepurnov et al., 2005; Casteleyn et al., 2008), *P. delicatissima* (Amato et al., 2005, 2007; Kaczmarek et al., 2008; Quijano-Scheggia et al., 2009b), *P. pseudodelicatissima sensu stricto* (Amato et al., 2007), *P. calliantha* (Amato et al., 2007; Lundholm et al., in press), *P. dolorosa* (Amato et al., 2007), *P. mannii* (Amato and Montresor, 2008), *P. brasiliiana* (Quijano-Scheggia et al., 2009a), *P. multistriata* (D'Alelio et al., 2009), *P. arenysensis* (Quijano-Scheggia et al., 2009b), *P. pungens* var. *aveirensis* (Churro et al., 2009), *P. australis* (Holtermann et al., 2010) and *P. cuspidata* (Lundholm et al., in press).

Despite some differences in the time necessary to complete sexual reproduction, the mating process is similar in all *Pseudo-nitzschia* species tested. The first step in sexual reproduction is the pairing between parent cells of opposite sex (gametangiogamy). Two cells generally pair valve to valve, lying parallel with close alignment of the cells. The next stage is gametogenesis. The paired cells divide meiotically and the content of each cell is partitioned along the transapical plane to form spherical gametes, two per cell. These gametes are identical in appearance (isogamous) and non-flagellated, but the behavior of the gametes differs between sexes (physiological anisogamy). One cell produces two active gametes (“+”, “male”) and the other two passive gametes (“–”, “female”). The frustules of both cells open, permitting the active gametes to enter and fuse with the passive gametes (plasmogamy). This is not always successful in both pairs of gametes or in all pairings of parent cells. When it is successful, fusion takes only 1–2 min. After gamete fusion, the resulting zygote expands to form a long auxospore (Fig. 2), inside of which the large initial cell is formed that then divides vegetatively and exits the auxospore. The entire process, from gamete production to formation of initial cells takes 2–4 d.

Preliminary work has shown an interesting relationship between epibiont bacteria and *Pseudo-nitzschia* sexual reproduction. Some axenic clones of *P. multiseriis* would not undergo sexual reproduction until bacteria were reintroduced (Thompson, 2000). Other mixtures of axenic clones did undergo sexual reproduction, but it is possible that these were contaminated by bacteria. Further work is required to determine the role of bacteria in sexual reproduction in this species.

There is also evidence that a type of “pheromone” or other chemical is being produced by sexually active *Pseudo-nitzschia*. Filtrates of sexually reproducing clones induced higher gamete production in other clones, even in the absence of cells of the opposite mating type (Haché, 2000). These results suggest that a chemical signal may stimulate gamete production and perhaps



**Fig. 2.** Auxospores of the pennate diatom *Pseudo-nitzschia australis*, in culture. Each auxospore is attached to an empty parental valve, which has become short and slightly deformed over time in culture. (A) One un-expanded auxospore is also visible above the expanded auxospore. (B) Upon plasmogamy, the primary organic wall is formed around the auxospore. Transverse bands composed of silica are laid down during the auxospore's bipolar expansion. This network of bands, plus the cell wall, is called the perizonium. A large initial cell is formed within the perizonium, restoring the large cell size of the diatom.

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also synchronize gamete production in already sexualized cells. It is not known if such a signal would also assist cells of opposite sex to locate each other.

Sexual reproduction in *Pseudo-nitzschia* may be important for DA production. Clonal cultures of *Pseudo-nitzschia* decrease in size over time (see above), and also lose their ability to produce DA (Bates, 1998). However, when parental cells of *P. multiseries* that have lost their ability to produce DA are mated, their offspring can be toxic, sometimes even more so than their parents were initially (Bates et al., 1999; Lundholm et al., in press). Sibling clones can have significant variability in DA production, which could be accounted for by genetic differences or by the presence of different types and numbers of free-living or epibiont bacteria.

Information on environmental cues that induce sexual reproduction in diatoms *in situ* is almost nonexistent (Chepurnov et al., 2004). However, recent reports have documented the sexual reproduction in natural populations of *P. cf. delicatissima* and *P. cf. calliantha* in the Gulf of Naples, Italy (Sarno et al., 2010), and *P. australis* and *P. pungens* in coastal Washington State, USA (Fig. 1; Holtermann et al., 2010). Sex is likely not a rare event but may be constrained in time and may involve a very limited fraction of the population (Sarno et al., 2010), which may account for the scarcity of observation of sexual stages in nature. In the Gulf of Naples, during a bloom of both *P. cf. delicatissima* ( $9.1 \times 10^6$  cells  $l^{-1}$ ) and *P. cf. calliantha* ( $7.0 \times 10^6$  cells  $l^{-1}$ ) in September 2006, only 9.2% and 14.3% of each species, respectively, were observed to undergo auxosporulation. Observations during the coastal Washington State *Pseudo-nitzschia* bloom (up to  $9.3 \times 10^5$  cells  $l^{-1}$ ) in June 2006, showed that auxosporulation began when the lowest size classes of both *P. pungens* and *P. australis* reached their maximum cell concentrations. In contrast to the bloom in Italy, the number of auxosporulating cells of *P. australis* exceeded the number of vegetative cells at the decline of the bloom. The highest rate of increase in auxosporulation and production of large new cells for both species occurred within a relatively narrow window of time and coincided with the decline in nutrients after the bloom had peaked (Holtermann et al., 2010). The significant relationship between the presence of large new cells resulting from

auxosporulation and DA concentrations in razor clams at the coastal beach calls for further investigation.

## 5. Nutritional physiology

Despite the widespread interest in *Pseudo-nitzschia* and the potential environmental triggers of DA production, much of our understanding of the nutritional physiology of these toxigenic diatoms remains relatively unaltered from the seminal studies conducted by early investigators; their results have strongly influenced the interpretation of many field studies conducted in the past decade. Early studies reported on the relationship between growth rate and DA production, examined primarily with laboratory cultures of *P. multiseries* (reviewed by Bates, 1998; Bates and Trainer, 2006) and often extrapolated to other species. These demonstrated that DA production is usually minimal or non-detectable during exponential growth in batch cultures, and increases during the stationary phase as cell division slows as a result of either Si or P limitation. Similarly, studies have shown a reciprocal relationship between growth rate and DA production in Si- (Bates et al., 1996; Pan et al., 1996b; Kudela et al., 2004a) or P-limited continuous cultures of this species (Pan et al., 1996c; Hagström et al., 2011). An exception to the pattern of minimal or non-detectable DA during fast growth is *P. cf. pseudodelicatissima* (which may be *P. cuspidata*; Lundholm et al., 2003) from the Gulf of Mexico (Pan et al., 2001). Here, DA production and cellular DA concentrations were greatest when the growth rate was high and cell density was low during the early exponential phase, and no net DA production was determined during stationary phase. Other studies also have observed measurable levels of DA prior to the onset of stationary growth, e.g. in *P. australis* (Garrison et al., 1992), *P. multiseries* (Pan et al., 1996a; Radan, 2008; Thessen et al., 2009) and *P. fraudulenta* (Thessen et al., 2009). *P. cuspidata* even exhibited greater cellular DA in the exponential phase relative to stationary phase when N, not Si or P, was the macronutrient inducing the onset of stationary growth (Auro, 2007). These studies suggest that macronutrient stress (or limitation) and the resultant slowing of division rate, rather than cessation of cell growth, is the factor

responsible for increased DA production (see also discussion in Section 6.4).

Field studies have also suggested that macronutrient limitation, most notably Si, as a possible trigger for DA production by *Pseudo-nitzschia* assemblages. During blooms of *P. australis* in coastal waters of the Southern California Bight, significant negative correlations were reported between cell abundance and particulate DA, and the ambient concentrations of silicic acid ( $\text{Si}[\text{OH}]_4$ ), nitrate ( $\text{NO}_3^-$ ) and orthophosphate ( $\text{PO}_4^{3-}$ ) (Anderson et al., 2006; Schnetzer et al., 2007). In Los Angeles coastal waters, these, together with significant correlations between high cellular DA and both low  $\text{Si}[\text{OH}]_4:\text{PO}_4^{3-}$  and  $\text{NO}_3^-:\text{PO}_4^{3-}$  ratios (but not  $\text{NO}_3^-:\text{Si}[\text{OH}]_4$ ), were interpreted as possible enhancement of DA production by P- or Si-limitation (Schnetzer et al., 2007). However, in the Santa Barbara Channel region, no significant relationships were observed for cellular DA and the absolute concentrations or ratios of nutrients. Nevertheless, the significant negative correlations between particulate DA and both the ratio of  $\text{Si}[\text{OH}]_4:\text{PO}_4^{3-}$  and  $\text{Si}[\text{OH}]_4:\text{NO}_3^-$ , together with the observation of the highest cellular DA coincident with the lowest ambient  $\text{Si}[\text{OH}]_4$  concentrations, led the authors to suggest that Si stress plays a role, albeit a complex one, in DA production by natural assemblages (Anderson et al., 2006). Such apparent enhancement of DA production, however, is not uniformly seen for all west coast North American *Pseudo-nitzschia* assemblages. Indeed, macronutrient-replete (including Si) waters of the Pacific Northwest, including the well-known Juan de Fuca Eddy “hot spot”, support healthy-growing *Pseudo-nitzschia* cells producing DA in the absence of measurable Si stress or limitation (e.g. Cochlan et al., 2006a,b; Marchetti et al., 2004; Trainer et al., 2009a).

Nitrogen, unlike Si or P, is required for DA production (cf. review by Bates, 1998), and growth limitation by N is directly unfavorable for DA production in batch cultures of *P. multiseriis* (Bates et al., 1991). However, measurable DA production has been observed under N-limiting conditions in both continuous cultures of *P. australis* and *P. multiseriis* (Kudela et al., 2004a; Radan, 2008; Hagström et al., 2011) and field assemblages of *P. australis* (Kudela et al., 2004b). There is increasing evidence that the N substrate fueling growth may influence both the exponential growth rate and the DA production rate achieved by various species of *Pseudo-nitzschia*, prior to either Si- or P-induction of the stationary phase. Initial studies conducted by Bates et al. (1993) first demonstrated differential growth and toxin response to N substrates, where ammonium ( $\text{NH}_4^+$ ) concentrations of 220 and 440  $\mu\text{mol N}$  promoted greater DA production than the same concentrations of  $\text{NO}_3^-$  during the stationary phase for two strains of *P. multiseriis*, whereas at lower concentrations (110 and 55  $\mu\text{M N}$ ) the cell yield and DA production rates were equivalent for both N substrates. Recent studies have confirmed differential exponential growth rates as a function of N substrate for *P. multiseriis* (Hillebrand and Sommer, 1996), *P. australis* isolates and field assemblages from Monterey Bay, CA (Cochlan et al., 2006a; Howard et al., 2007), and *P. cuspidata* from coastal Washington State (Auro, 2007). These studies also report that toxicity during the exponential phase was inversely related to growth rate; in other words the N substrate supporting the slowest growth (i.e. the least preferred N substrate; Dortch, 1990) will likely produce the most toxic (cellular DA) cells during exponential phase. However this trend is not uniform, as cultures of *P. multiseriis* isolated from coastal Washington State (Sequim Bay) showed no such N preference for growth. Instead, they are consistently more toxic when grown on urea during N-replete batch cultures and also in N-limited continuous cultures where their growth rate does not vary as a function of N source but rather is controlled by the dilution rate of the limiting N source (Radan, 2008).

Light, besides being required for the production of DA (Bates et al., 1991; Whyte et al., 1995), can also alter the N preference for growth, as initially reported for *P. multiseriis* (Hillebrand and Sommer, 1996) and confirmed recently for *P. cuspidata* (Auro, 2007). Auro (2007) demonstrated that, for *P. cuspidata* grown at high light, neither cellular toxicity nor exponential growth rates showed a strong N preference for one N substrate over another, whereas at low light the slower  $\text{NO}_3^-$ -grown cells were significantly more toxic than  $\text{NH}_4^+$ - or urea-grown cells, although urea supported the fastest growth. These more recent studies employed ecologically relevant N concentrations (50–88  $\mu\text{M N}$ ) and have confirmed differential growth as a function of N substrate, with the slower growing cells generally being the most toxic. However, interspecies differences and variability preclude any generalization attributable to the N substrate fueling growth rate or cellular toxicity.

Intrastrain variability has only recently been examined with respect to N use and the resulting toxicity, in studies using two strains of *P. multiseriis*, five of *P. fraudulenta* and two of *P. calliantha*, isolated from the mid-Atlantic coastal region of the U.S. (Thessen et al., 2009). Here, the only observable trends were higher growth rates achieved on ammonium and lower growth rates on urea, for 8 of 9 strains tested. However, toxicity (measured as total DA, particulate DA and dissolved DA), examined during Si-induced stationary phase, showed no consistent pattern with respect to N source or relationship to the prior exponential growth. An exception was for two strains of *P. multiseriis* that reached the highest toxin content at the highest growth rate. Based on results to date, it is clear that the N substrate affects DA production in addition to its direct effect on growth. Still, neither specific growth rate nor toxin content (in either exponential or stationary phase) can be consistently predicted as a function of N substrate due to species and strain variability in this genus.

The significance of dissolved organic N (DON) sources, other than urea, for the growth of *Pseudo-nitzschia* species is still unclear. Studies have demonstrated that *P. multiseriis* is able to grow equally well using glutamine or nitrate (Hillebrand and Sommer, 1996). The same species was suggested to use glutamine and glutamate as a dark survival strategy (Mengelt, 2005), and glutamine was used at a rate equivalent to that of  $\text{NH}_4^+$  by *P. australis*, based on short-term uptake kinetic experiments (Cochlan et al., 2008). See Table 5 for a summary of species-specificity for growth under different nitrogenous nutrients. Enrichment experiments conducted with natural assemblages from the Mediterranean Sea (Loureiro et al., 2009a) and cultures of *P. delicatissima* (Loureiro et al., 2009b), isolated from coastal Spain, showed that in low inorganic N environments, high molecular-weight dissolved organic matter enrichment can contribute positively to the growth of *P. delicatissima* and *Pseudo-nitzschia* assemblages; this has been suggested as the “missing” nitrogenous source for sustenance of *Pseudo-nitzschia* during periods of depleted reserves of inorganic N. The effects of DON on DA production have not been studied to date.

## 6. Ecology and nature of *Pseudo-nitzschia* harmfulness

### 6.1. Food web aspects and potential ecological disruptions

As a diatom, *Pseudo-nitzschia* is an important primary producer at the base of the food web. It is consumed directly by a wide variety of organisms, from heterotrophic dinoflagellates to planktivorous fish (Table 1). As a hydrophilic molecule, DA does not bioaccumulate. Instead, DA is concentrated in the digestive system by filter feeding, with little transfer to surrounding tissues and in most cases quickly eliminated from the body. The toxin is moved through the food chain during blooms when primary consumers with guts full of *Pseudo-nitzschia* are eaten by

**Table 5**  
Exponential growth rate ( $\mu$ ;  $d^{-1}$ ) of *Pseudo-nitzschia* species, isolated from North American waters, grown in N-replete batch cultures. Rates reported are the mean  $\pm$  one standard deviation (SD).

Species	N conc. ( $\mu$ M N)	PPFD <sup>a</sup> /L:D cycle	NO <sub>3</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	Urea	Origin of isolate	Reference
<i>P. calliantha</i>	88	150–200/14:10	0.55 $\pm$ 0.047	0.87 $\pm$ 0.154	0.42 $\pm$ 0.056	Choptank R., MD	Thessen et al. (2009)
<i>P. fraudulenta</i>	88	150–200/14:10	0.72 $\pm$ 0.071	0.71 $\pm$ 0.064	0.44 $\pm$ 0.028	Asilomar, CA	Thessen et al. (2009)
			0.75 $\pm$ 0.051	0.62 $\pm$ 0.038	1.02 $\pm$ 0.137	Assateague I., MD	
			0.84 $\pm$ 0.035	0.85 $\pm$ 0.058	0.58 $\pm$ 0.067	Assateague I., MD	
			0.73 $\pm$ 0.036	1.22 $\pm$ 0.177	0.55 $\pm$ 0.103	Assateague I., MD	
			0.75 $\pm$ 0.039	0.88 $\pm$ 0.048	0.58 $\pm$ 0.181	Assateague I., MD	
<i>P. multiseriis</i>	88	150–200/14:10	1.05 $\pm$ 0.033	1.16 $\pm$ 0.020	1.05 $\pm$ 0.030	Asilomar, CA	Thessen et al. (2009)
			0.65 $\pm$ 0.018	0.61 $\pm$ 0.054	0.68 $\pm$ 0.070	Eastern Canada	
			0.76 $\pm$ 0.104	0.76 $\pm$ 0.175	0.30 $\pm$ 0.100	Choptank R., MD	
			0.85 $\pm$ 0.028	0.83 $\pm$ 0.028	0.81 $\pm$ 0.071	Sequim Bay, WA	
			0.49 $\pm$ 0.063	No growth	–	Canada	
<i>P. australis</i>	50	100/12:12	0.54 $\pm$ 0.170	0.35 $\pm$ 0.021	0.56 $\pm$ 0.057 <sup>c</sup>	PEI <sup>b</sup> , Canada	Hillebrand and Sommer (1996)
			0.89 $\pm$ 0.077	0.93 $\pm$ 0.001	0.52 $\pm$ 0.099 <sup>d</sup>	Monterey Bay, CA	Howard et al. (2007)
<i>P. cuspidata</i>	40	120/24:0	0.88 $\pm$ 0.01	0.91 $\pm$ 0.02	0.84 $\pm$ 0.03	Offshore Washington State	Auro (2007) <sup>e</sup>
			0.55 $\pm$ 0.01	0.51 $\pm$ 0.02	0.65 $\pm$ 0.003		

<sup>a</sup> Photosynthetic photon flux density (PPFD) measured in  $\mu$ mol photons  $m^{-2} s^{-1}$ .

<sup>b</sup> Prince Edward Island

<sup>c</sup> Growth on glutamine =  $0.74 \pm 0.127 d^{-1}$ .

<sup>d</sup> Incorrect SD provided in Howard et al. (2007).

<sup>e</sup> Standard error reported for rates determined from replicate cultures ( $n=4$ ).

secondary consumers. DA is eventually depurated, but depuration rates can vary, from hours in the blue mussel (*M. edulis*), Mediterranean cockle (*Acanthocardia tuberculatum*) and Green-shell™ mussel (*Perma canaliculus*), to several days in the Mediterranean mussel (*M. galloprovincialis*) and eastern oyster (*Crassostrea virginica*) (Novaczek et al., 1992; Wohlgeschaffen et al., 1992; Mackenzie et al., 1993; Vale and Sampayo, 2002; Mafra et al., 2010a). Three bivalves that are very slow to depurate are the razor clam *Siliqua patula* (>86 days), the scallop *P. magellanicus* (>14 days) and the scallop *Pecten maximus* (~416 days) (Wohlgeschaffen et al., 1992, 1993; Douglas et al., 1997; Blanco et al., 2002).

DA may accumulate in organisms other than by filter feeding. Scavengers and deposit feeders have also been found to contain DA (Powell et al., 2002; Goldberg, 2003). Scavengers could become contaminated by eating DA-contaminated remains. Deposit feeders could become contaminated by consuming flocs of *Pseudo-nitzschia* that sink to the benthos at the end of a bloom (Goldberg, 2003). Some carnivores, like the swimming crab (*Polybius henslowii*), can contain high levels of DA but there have been no recorded incidents of poisoning in their predator, the yellow-legged gull (*Larus cachinnans*) that feeds on them almost exclusively (Álvarez, 1968; Munilla, 1997; Costa et al., 2003). Vertical flux of DA to the sediment appears to be a substantial source of toxin to the benthic food chain (Sekula-Wood et al., 2009, 2011) as DA is stable when adsorbed onto sediments (Burns and Ferry, 2007), long after *Pseudo-nitzschia* blooms have dissipated in the upper water column. There may be a limit in the number of trophic transfers over which DA can still be present at high enough concentrations to cause a toxic event. To date, all recorded ASP events have involved only three trophic levels: the diatom *Pseudo-nitzschia*, a bivalve or a planktivorous fish, and a human, marine mammal or marine bird. Interestingly, most cases of three-tiered trophic transfer have been recorded on the west coast of North America and Europe, and none were reported in the massive events in the southern Gulf of St. Lawrence in 1987 and 2002.

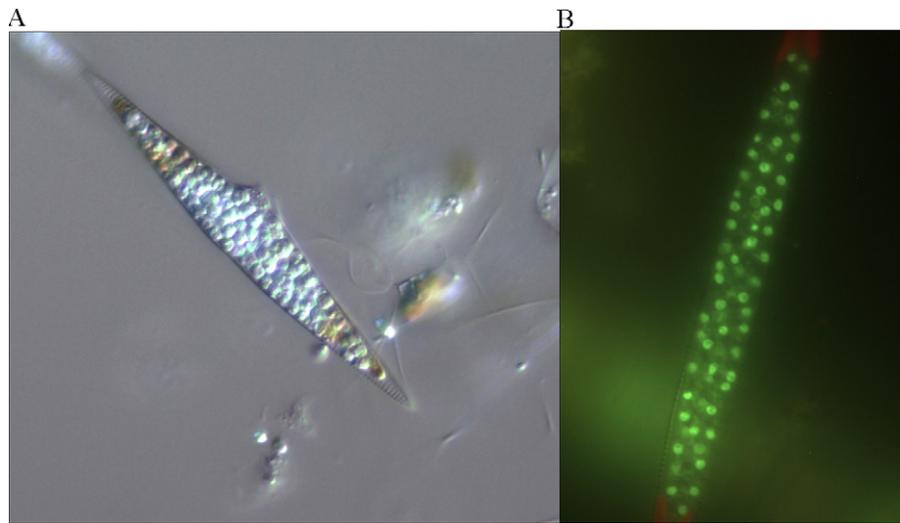
## 6.2. Ecological profile of *Pseudo-nitzschia* species

Blooms of *Pseudo-nitzschia* happen relatively frequently, in some regions seasonally, and in a wide variety of locations. In culture, *Pseudo-nitzschia* spp. can grow at salinities as low as 6 and as high as 48, and at temperatures as low as 5 °C and as high as 30 °C, with a broad range for optimum growth (Miller and

Kamykowski, 1986; Jackson et al., 1992; Lundholm et al., 1997; Cho et al., 2001; Thessen et al., 2005). However, different species in natural populations can demonstrate distinct correlations with environmental characteristics, which suggests seasonal succession of species or regional specificity (Fryxell et al., 1997). In the South China Sea, *P. pungens* peaks in April, May and June, *P. multistriata* is present only in spring and *P. sinica* and *P. subpacificca* are found in late fall and early winter (Qi et al., 1994). In addition, *P. pungens* in the colder (1–10 °C) waters of the North China Sea is present only in winter and spring, whereas *P. pungens* in the warmer (21–29 °C) East and South China Seas is present year round, revealing two ecotypes (Zou et al., 1993). Many species may coexist, but different growth and loss rates can lead to complex bloom dynamics and seasonal succession.

*Pseudo-nitzschia* blooms can be stimulated by nutrients from several sources, including upwelling or mixing events and riverine inputs. Both sources stimulate *Pseudo-nitzschia* blooms at concentrations of 8–22  $\mu$ M NO<sub>3</sub><sup>-</sup>, 2.4–35  $\mu$ M Si and 0.2–2  $\mu$ M PO<sub>4</sub><sup>3-</sup> (Dortch et al., 1997; Scholin et al., 2000; Trainer et al., 2000; Loureiro et al., 2005), but in different temperature and salinity regimes. *Pseudo-nitzschia* abundances and DA concentrations on the west coast of the U.S. are associated with low temperature, high salinity and high nutrient conditions typical of upwelling (Villac, 1996; Trainer et al., 2000, 2002). Similarly, upwelling regions off the coast of Portugal contain high numbers of *Pseudo-nitzschia* cells, which are used as upwelling indicators during spring and summer (Moita, 2001; Loureiro et al., 2005).

Riverine inputs have stimulated toxic *Pseudo-nitzschia* blooms in many regions and are characterized by lower salinities and higher temperatures than upwelling zones (Smith et al., 1990; Horner and Postel, 1993; Dortch et al., 1997; Trainer et al., 1998; Scholin et al., 2000; Spatharis et al., 2007; Kudela et al., 2008). A distinction between nutrients in upwelling and river plumes is that riverine inputs are likely the result of anthropogenic nutrient loading, including agricultural runoff and sewage. Sedimentological data show an increase in *Pseudo-nitzschia* abundance in the Mississippi River plume since 1950, suggesting a response to eutrophication (Parsons et al., 2002). However, in addition to an increase in nitrogen and phosphorus, nutrient ratios in Mississippi River water have also changed, i.e. the Si:N ratio has decreased (Turner and Rabalais, 1991), which favors *Pseudo-nitzschia* growth in culture (Sommer, 1994). Other river systems have also affected *Pseudo-nitzschia* abundances. When the mouth of the Yellow River



**Fig. 3.** Zoospores of a fungal parasite filling a *Pseudo-nitzschia* cell, from a natural sample collected in southern Hood Canal, WA, USA (Twanoh State Park), on 31 March 2007. (A) Note the exit pore, on the upper part of the valve surface, through which the zoospores will exit the cell. (B) Syber green stain fluorescent stain of the parasitic oomycetes. Photomicrographs courtesy of Karie Holtermann and E. Virginia Armbrust.

in China was artificially redirected in 1976, the location of the *Pseudo-nitzschia* bloom abruptly changed to follow the river plume (Zou et al., 1993). *Pseudo-nitzschia* abundance in the plume of the Yangtze River was positively correlated to  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  concentrations (Zou et al., 1993). In the South China Sea, *Pseudo-nitzschia* abundances responded to increased land runoff after rainfall (Qi et al., 1994) and in the Aegean Sea, Greece, a winter bloom of *P. calliantha* appeared to be induced by agricultural runoff shortly after a strong rainfall event (Spatharis et al., 2007). An analysis of *P. delicatissima* and *P. pseudodelicatissima* dynamics and environmental parameters in the Bay of Fundy, Canada, showed a strong relationship between  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations and the abundance of these species (Kaczmarek et al., 2007). These coastal studies show a response to riverine nutrients, changing nutrient ratios and eutrophication.

Much of the seasonal variability in *Pseudo-nitzschia* abundance can be explained by regular shifts in wind, irradiance, temperature and river flow. In the northern Gulf of Mexico, *Pseudo-nitzschia* abundance peaked in spring, corresponding to the average maximum in river flow, with another small peak in fall during wind events that mixed the stratified water column (Dortch et al., 1997). Many *Pseudo-nitzschia* blooms occur in the spring and fall, when irradiance is relatively low (Parsons et al., 1998; Mercado et al., 2005). In culture, *P. multiseriata* can outcompete other phytoplankton species at low irradiance with a short photoperiod (Sommer, 1994). However, low light may contribute to the demise of autumn blooms (Bates et al., 1998). Day length can affect sexual reproduction (Hiltz et al., 2000), growth rates, cell yield, toxin production and influence which species of *Pseudo-nitzschia* becomes dominant (Fehling et al., 2005).

Local meteorological phenomenon, such as winds and heavy rainfall events, can stimulate *Pseudo-nitzschia* blooms. Wind events can be especially important for transporting toxic blooms inland from upwelling sites offshore (Trainer et al., 2000, 2002) or providing mixing necessary to bring nutrients into the photic zone (Lund-Hansen and Vang, 2004). Heavy rainfall after a drought can cause a dramatic increase in toxic *Pseudo-nitzschia* abundances in the river outflow, such as in eastern Canada in 1987 (Bates et al., 1998).

Larger scale changes in weather, such as the El Niño Southern Oscillation (ENSO), can affect *Pseudo-nitzschia* abundances by controlling upwelling near the west coast of the U.S. During weak ENSO years, upwelling is high and therefore so are *Pseudo-nitzschia*

abundances (Fryxell et al., 1997). However, *Pseudo-nitzschia* can still bloom by taking advantage of other favorable events, such as increased runoff after rainfall, during strong ENSO years. Both 1991 and 1998, years with large toxic events on the west coast of the U.S., were strong ENSO years.

The decline of *Pseudo-nitzschia* blooms is less studied than its initiation. Parasitic fungi may play an important role in the demise of *Pseudo-nitzschia* blooms (Bates et al., 1998). Parasitic oomycetes and chytrids have infected *P. pungens* in eastern Prince Edward Island, Canada (Hanic et al., 2009). Additionally, fungal parasites have been observed in cells during bloom decline in coastal Washington, USA (Fig. 3; Horner et al., 1996) and an unexpected decrease in *P. multiseriata* abundance in the Skagerrak between 1991 and 1993 was suspected to be caused by parasitic fungi (Hasle et al., 1996). Viruses are known to infect marine diatoms (e.g. Nagasaki et al., 2005), but studies on viral infections in *Pseudo-nitzschia* are just beginning (Carlson et al., 2009). High pH, resulting from dense blooms, could also lead to bloom decline. Laboratory cultures of several *Pseudo-nitzschia* species could not continue exponential growth at pH from 8.7 to 9.3 (Lundholm et al., 2004). The exact mechanisms of *Pseudo-nitzschia* bloom decline are uncertain and could be caused by multiple factors.

### 6.3. Allelopathy

Allelopathic effects, i.e. inhibitory effects on the growth of competitors caused by production of certain allelopathic substances, are known from prymnesiophytes and dinoflagellates and this may contribute to their ability to form monospecific blooms (Arzul et al., 1993; Granéli and Johansson, 2003). *Pseudo-nitzschia* species are also known to form dense blooms ( $10^6$  to  $10^8$  cells  $l^{-1}$ ), which are often more or less monospecific, accounting for up to 99% of the total phytoplankton and sometimes lasting for a long time, up to two months (e.g. Bates et al., 1989; Martin et al., 1990; Fryxell et al., 1997; Gallacher et al., 2001). It is known that DA leaks, or is actively transported, out of the cells, resulting in high levels of the toxin in the surrounding medium (reviewed by Bates, 1998; Wells et al., 2005). It is therefore reasonable to speculate that this toxin could have an allelopathic effect. Mixed culture experiments, however, showed no allelopathic effects of toxic *P. multiseriata* on *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* or *Rhodomonas marina* (Lundholm et al., 2005b). Similarly, additions of DA alone to the water had no allelopathic effects on 11

different phytoplankton organisms (Windust, 1992; Lundholm et al., 2005b). Thus, there is still no evidence that allelopathic effects are likely to contribute to the formation of long-lasting *Pseudo-nitzschia* blooms, or serve as an explanation for the evolution of DA production.

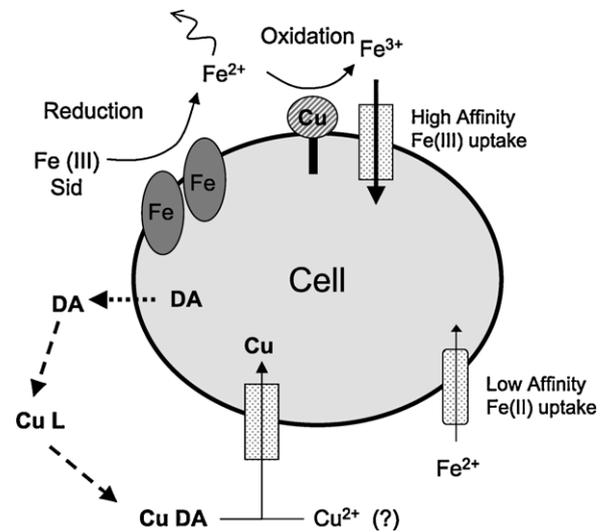
#### 6.4. The ecological role of DA production

The ecological position of *Pseudo-nitzschia* is in part related to its ability to produce DA. While most studies on DA production are focused on the harmful effects and gross levels of DA produced, the possible role of DA in the intracellular regulation of *Pseudo-nitzschia* physiology has also been considered. The molecular structure of DA evokes the possibility that this ecological toxin could serve as an iron or copper chelator for *Pseudo-nitzschia*. Significant findings by Rue and Bruland (2001) documented that the high stability constants of copper and iron to DA may provide an important ecological role as an iron-siderophore, keeping iron soluble and bioavailable in the environment for retrieval by *Pseudo-nitzschia*, or as a means of copper detoxification. Thus, they opened the idea that certain trace metals are critical to the ecological success of DA-producing species.

The physiological role of DA was further documented in Maldonado et al. (2002) and Wells et al. (2005). Maldonado et al. (2002) reported that DA production was inversely related to cellular growth rates when cells were limited by low iron or copper availabilities. While the cellular levels of DA increased, there was an additional increase in the levels of dissolved DA under metal stressed conditions; with up to 95% of the DA actively released into the environment. The presence of DA in the medium enhanced iron transport into low-iron grown cells. Further to this study, Wells et al. (2005) confirmed that DA is a functional component of the unusual high-affinity copper-regulated, iron acquisition system in *Pseudo-nitzschia* spp. They proposed that in the absence of an adequate copper supply, iron-limited natural populations of *Pseudo-nitzschia* will become increasingly toxic, and conversely, the production of DA will provide the *Pseudo-nitzschia* cells with a distinct ecological advantage. The cellular model of DA production, retention and release (Fig. 4; Wells et al., 2005) provides the backdrop for understanding the ecological dynamics of DA, where specific conditions are required to evoke the production of DA, the storage as particulate DA (pDA) and the release as dissolved DA (dDA).

It should be noted that the degree of iron stress experienced by the cells may be important in determining the response of *Pseudo-nitzschia* cells to iron. This may explain the contrary findings of the above studies and those of Bates et al. (2002), who reported that DA production decreased, rather than increased, as a result of depriving them of iron. The cells used by Maldonado et al. (2002) and Wells et al. (2005) were growing exponentially in semi-continuous cultures, and it could be argued that they were therefore less stressed by iron (“iron depleted”), whereas those used by Bates et al. (2002) were in stationary phase and therefore more highly stressed by iron (“iron limited”). With “iron limitation”, less iron is available for chlorophyll synthesis and nitrogen metabolism, two processes that are necessary for maintaining elevated levels of DA production (Bates et al., 2002). This would not be the case with “iron-depleted” cells, which might then have a greater ability to produce DA.

The relationship between DA production and the ecological mosaic of coastal waters has been considered (Trainer et al., 2002, 2009a,b, 2010; Marchetti et al., 2004; MacFadyen et al., 2008). Trainer et al. (2009a) summarized the findings from a 6-cruise, 4-year study at the Juan de Fuca eddy – a region off Washington State (USA) and British Columbia (Canada) that has a long history of DA production. They concluded that while *Pseudo-nitzschia* spp. at



**Fig. 4.** Conceptual model of the high affinity iron uptake system found in yeast, *S. cerevisiae*, comprising a membrane bound, iron-containing iron reductase, a multi-copper iron oxidase and high affinity Fe(III) transporter. In this sequence, organically bound Fe(III) is reduced, releasing Fe(II) from the complex. This Fe(II) may have a lifetime of minutes to hours depending on temperature, so before diffusion transports Fe(II) away from the cell it is oxidized enzymatically with the resulting Fe(III) being bound and transported into the cell. Copper uptake in this case is shown modulated by the release of DA. From Wells et al. (2005; Fig. 8 on p. 1915).

times exceeded  $1.3 \times 10^7$  cells  $l^{-1}$ , and pDA reached  $80 \text{ nmol } l^{-1}$ , there was no single unifying relationship between environmental conditions and either the presence of *Pseudo-nitzschia* or the level of DA. A similar conclusion was reached earlier by Marchetti et al. (2004). However, within one well-studied *P. cuspidata* bloom (September 2004), where cell concentrations reached  $6.1 \times 10^6$  cells  $l^{-1}$ , with pDA, dDA and cellular DA concentrations of  $43 \text{ nmol } l^{-1}$ ,  $4 \text{ nmol } l^{-1}$  and  $63 \text{ pg cell}^{-1}$ , respectively, DA levels did not correlate with macronutrient levels. Instead, stations where dissolved Fe concentrations were limiting ( $<0.5 \text{ nmol } l^{-1}$ ) had the highest *Pseudo-nitzschia* abundances and pDA and cellular DA values (Trainer et al., 2009b). These results provide evidence that an iron-regulated physiological model is present in natural communities. A study by Ribalet et al. (2010) verified the iron mosaic of some coastal waters and suggests a *Pseudo-nitzschia* “hotspot”, where iron-rich coastal waters meet the low-iron oceanic waters, creating a natural iron enrichment area. Presently, levels of DA in this region have not been documented.

While the physiological model linking DA production with the iron requirements of *Pseudo-nitzschia* cells has been proposed (Wells et al., 2005), numerous studies indicate that other environmental factors are also critical in establishing the level of DA in *Pseudo-nitzschia*. Key early studies (e.g. Pan et al., 1996a,b,c; reviewed by Bates et al., 2008), with a follow-up study by Fehling et al. (2004a), document a link between DA levels per *Pseudo-nitzschia* cell to both phosphorus- and silicate-limitation. Under these limitations, but particularly under silicate-limitation, levels of DA per *Pseudo-nitzschia* cell increased at early senescence stage, with little production during exponential and early-senescence stages of growth. Similarly, in two studies considering the relationship between pH/dissolved inorganic carbon and DA production, the highest levels of DA per cell were achieved at high pH, when the cell growth rate was negatively influenced (Lundholm et al., 2004; Trimbom et al., 2008). It is, however, not revealed whether it is the reduced cell growth, or the higher external pH resulting in potentially higher internal (intracellular) pH that is responsible for the higher production of DA. The effect of

high pH could be part of an explanation for why dense bloom are shown to produce high levels of DA.

The common link between these sets of studies is that DA production, *per se*, is not connected to the growth rate of the *Pseudo-nitzschia* cells, only the accumulation of DA in the cells. Under any of the limiting conditions mentioned above, the cells continue to produce DA just as the rate of division decreases. This metabolic mismatch elevates the level of DA per cell simply because the DA is not being “diluted” by partitioning the new DA into new cells. Thus, it is becoming clearer that DA levels do not correspond specifically to a set environmental condition but rather to the growth characteristics of DA-accumulating cells.

Doucette et al. (2008) examined the influence of salinity on both the growth rate and DA levels per cell in *P. multiseriis*. Under conditions of poor growth (10 psu), the isolate neither grew nor produced DA. At a salinity of 20 psu, cell growth rate was maximal (at  $\sim 0.9 \text{ d}^{-1}$ ) but DA levels were 3–7-fold lower than cells grown at either 30 or 40 psu. The authors reject the simple relationship between cell growth rate and DA production, concluding that the extra carbon that is needed to form osmolytes at 20 psu limits that carbon pool available to produce DA.

Ecological studies provide some corroborative evidence linking nutrient status and DA production. MacIntyre et al. (2011), using environmental correlations from phytoplankton communities in the Gulf of Mexico, found a strong correlation between cellular DA levels and low silicate, high salinity, high dissolved inorganic carbon and high light flux. They concluded, consistent with laboratory experiments, that DA accumulated in the cells when conditions for macronutrient uptake and growth were out of balance with photosynthetically driven carbon uptake (“photon pressure”).

### 6.5. Iron-fertilization studies and DA

The ecological role of DA and the role of iron have been considered concurrently in several of the mesoscale iron-fertilization studies. While it is generally considered that natural oceanic *Pseudo-nitzschia* populations do not produce DA, or only at very low levels (in the range of fg DA cell<sup>-1</sup> or pg DA l<sup>-1</sup>) (Marchetti et al., 2004, 2006, 2008), the mesoscale iron-fertilization experiments (cf. reviews by de Baar et al., 2005; Boyd et al., 2007), starting in the 1990s, have provided some opportunist information on both *Pseudo-nitzschia* competition and DA production. Trick et al. (2010) summarized that many of the iron-fertilization experiments, where low levels of iron are added to macronutrient-rich regions of the ocean (high-nitrate, low chlorophyll; HNLC) to assess the carbon-sequestering capacity of the ocean (de Baar et al., 2008), results in a stimulation of specific species of *Pseudo-nitzschia*. After fertilization of open-ocean HNLC waters with iron, its nutrient composition approaches that of coastal waters and results in a *Pseudo-nitzschia* bloom dynamics similar to that observed in coastal or upwelling regions. Silver et al. (2010), looking at *Pseudo-nitzschia* abundance, estimated that DA levels in the equatorial fertilization (IronEx II) and the Southern Ocean (SOFEx) studies reached 45 and 220 ng DA l<sup>-1</sup>, respectively.

Trick et al. (2010) presented results from *in situ* measurements and shipboard culture experiments demonstrating that the sparse oceanic *Pseudo-nitzschia* community at the HNLC Ocean Station PAPA (OSP, 50°N, 145°W) could produce on the order of  $\sim 200 \text{ pg DA l}^{-1}$  and retain that capacity upon iron and copper amendment. Their findings demonstrated that toxin production occurs with iron fertilization of HNLC waters, that the specific composition of commercial iron substrates is a critical parameter in the degree of toxin production, and that increasing the copper availability further enhances the cellular DA level (from 0.1 to 0.3 fg DA cell<sup>-1</sup>).

The experiments and oceanic observations presented in Silver et al. (2010) and Trick et al. (2010) strongly support the link between the physiological model for iron acquisition, the selective importance of DA and the recognition that total DA production potentially could reach ecologically harmful levels during large-scale iron-fertilization programs.

## 7. Monitoring and public health

In most coastal regions of the world, closures of shellfish harvesting based on monitoring for DA are reactionary. Shellfish are routinely tested for toxins and harvest closures are instated only when the regulatory threshold is exceeded. This system has succeeded in protecting human health, but has often led to conservative, coastwide closures of shellfish harvesting areas, which negatively impacts the shellfish industry and the economy. Sentinel shellfish, typically mussels in cages, may not always provide the best warning of DA events. Alternatives to mussels, including crustaceans that retain toxins (Powell et al., 2002), or solid phase adsorption toxin tracking (SPATT) technology (Lane et al., 2010) can provide a more effective “history” of DA in the phytoplankton assemblage or in shellfish.

A combination of more proactive approaches to monitoring that allow targeted closures have been demonstrated. In the case of eastern oysters (*Crassostrea virginica*) from Atlantic Canada, it has been demonstrated that blanket closures that prohibit the harvesting of all shellfish species may not be necessary, because these oysters accumulate little, or no, DA at the same location where other shellfish species are over the regulatory limit (Mafra et al., 2009a,b, 2010b). In another example, from the coast of Washington State, the Olympic Region Harmful Algal Bloom (ORHAB) monitoring partnership uses a simple combination of analytical techniques, which includes weekly determination of total *Pseudo-nitzschia* cells using light microscopy and levels of particulate DA in seawater, using antibody-based methods (B.-T. L. Eberhart, pers. comm.), to give an effective early warning of shellfish toxification events.

In order to sustain a monitoring program such as ORHAB, progressive integration of newer methods into the state management plans for HABs must occur. By rapidly assisting managers during toxic bloom events, ORHAB partners have effectively demonstrated to state legislators how integral the monitoring program is to effective and timely management of shellfish resources. Resulting legislation has instated a surcharge on shellfish license fees that will provide enough funding to sustain a state-run program, when the federally funded program ended in 2005.

Beach monitoring programs such as ORHAB are now being integrated with fine-scale sampling, using automated devices on moorings, to allow detailed determination of fluctuations in biological, physical and chemical parameters that influence HAB intensity. An example of a remote sensing technology is the automated molecular detection and quantification of *Pseudo-nitzschia* cells and DA, using the environmental sample processor (ESP; Scholin et al., 1999; Greenfield et al., 2006, 2008; Doucette et al., 2009).

Developing forecasting capability for the transport and impact of toxic *Pseudo-nitzschia* blooms will require sustained monitoring as well as additional efforts in the critical areas of basic research and model development. This early warning network for the detection and transport of toxic *Pseudo-nitzschia* blooms will ultimately use an integrated suite of sensors on satellites and stationary sensor platforms that measure ocean water properties, currents, *Pseudo-nitzschia* cell numbers and DA; using telemetry, these data will add real-time elements to shore-based lab testing. The suite of real-time data from moorings will also be used to

initialize, calibrate and validate physical and biological models and associated forecasts. Such models will allow monitoring programs and health departments to take preventive actions (e.g. increase monitoring efforts, close targeted shellfish beds, warn at-risk communities) to safeguard public health, local economies and fisheries. In addition, an integrated forecasting system will allow the proactive management of resources, e.g. by making possible the early opening of the recreational and subsistence clamming seasons or early warning to commercial crab and clam fishers who are impacted by DA-related closures. Together, these technologies will result in the most accurate forecasting bulletins possible, for the early warning of HAB events. One such bulletin for the Washington State coast is currently in its pilot stage (see <http://pnwhabs.org/pnwhabbulletin/index.html>).

## 8. Future needs

In the over two decades since the first deaths associated with the DA outbreak in Prince Edward Island, Canada, considerable progress has been achieved in all areas of the science of *Pseudo-nitzschia*. The rapid initial characterization of the toxin, and the clear establishment of the causative genus, have opened the door to extensive, hypothesis-driven research on factors that lead to toxic bloom development and ways to avoid future incidence of ASP. Researchers have challenged our scientific understanding at all levels – from the global ecology to the genome similarities among DA-producing strains. With all the progress achieved so far, the unique nature of *Pseudo-nitzschia* offers considerable potential for continued, intensive study. Uncertainty still abounds, however, and opportunity exists at all levels of research.

The complex nature of *Pseudo-nitzschia* taxonomy, based on physical size, shape and frustule ornamentation, phylogenetics and the breakthroughs made on elucidating the sexual reproduction of this diatom, provide a foundation for future studies on functional genome analysis, evolutionary genetic associations and molecular probe development. The advancement of the complex taxonomy is critical to the development of ecophysiological and toxicological studies.

The geographical mosaic of *Pseudo-nitzschia* presence and distribution has been considered in detail at some specific locations – primarily regions of upwelling – and coastal-open ocean interfaces. However, we now recognize the broader potential of toxigenic *Pseudo-nitzschia*, even in open-ocean environments and in non-traditional ocean zones susceptible to the influences of human activities. Are these areas where *Pseudo-nitzschia* can outcompete other species or are these transition zones where *Pseudo-nitzschia* thrives due to the lower competitive ability of other species to the dynamically changing environmental conditions? To achieve this understanding, more routine monitoring that allows characterization of the initiation and development of blooms, with the careful notation of conditions that correlate to a bloom or its toxicity, is essential. Accumulated knowledge often describes conditions present during a bloom, implying that these factors may also be instrumental in promoting the bloom. This is not necessarily the case, as it is likely that *Pseudo-nitzschia* may modify or condition the waters, allowing it establish an ecological advantage for growth. Challenging, persistent work is required, using a combination of modern molecular tools, to determine population structure. Careful microscopic observation of both live and preserved material is nevertheless essential to witness unique morphologies, including sexual stages (Fig. 2) and declining cell sizes.

The conditioning of seawater by established, growing populations of *Pseudo-nitzschia* has not received adequate attention, even with our increasing appreciation that DA or other metabolites could be involved in allelopathic, anti-grazing or sexual reproduction

activities. These processes may be fundamental in establishing the ecological complexity of *Pseudo-nitzschia* abundance or bloom establishment.

A better understanding of the currently uncertain role of DA production is fundamental to our understanding of the ecology of toxic and nontoxic species of *Pseudo-nitzschia*. While recognized as a compound transferred through the food web, resulting in mammal, bird and human health damage, alternative roles for DA may assist *Pseudo-nitzschia* in establishing itself in a unique or modified ecological space. Its documented chelation of iron and copper may enhance species-specific iron acquisition or may reduce free copper or exchange copper from established weak-copper ligands, e.g. produced by cyanobacteria. If DA does play a role in the acquisition of trace metals by *Pseudo-nitzschia*, then why are new DA closure events occurring in nearshore waters where trace metals should be in abundance? The answer may lie in the study of the bioavailability of these trace metals and their complexation by organic material in the coastal ocean. Alternatively, the ecological competitiveness of *Pseudo-nitzschia* may be enhanced if DA functions as dynamic anti-grazing infochemical – a biologically active metabolite, stored intracellularly then released extracellularly, when grazing levels increase. Uncertainties remain regarding DA production, storage and release, but this opens new options for studying its potential role as a cell “communication” molecule that could help to explain the ecological success of toxigenic *Pseudo-nitzschia* species. Interactions between bacteria (epibiotic and extracellular) and *Pseudo-nitzschia*, in relation to DA production (reviewed by Bates, 1998; Kaczmarek et al., 2005; Guannel et al., 2011) may also provide an advantage to this diatom. In contrast to the success of DA-producing species, one must also ask what comparable strategies may be used by those *Pseudo-nitzschia* species that do not produce this toxin, yet are still successful.

Does the key to understanding the nature of *Pseudo-nitzschia* harmfulness lie in an understanding of its versatility relative to other phytoplankton? *Pseudo-nitzschia* blooms occur in the most pristine (Juan de Fuca eddy region; Trainer et al., 2009a) waters, as well as in some of the most nutrient-impacted (Gulf of Mexico; Parsons et al., 2002), nutrient-variable (Monterey Bay, CA), or nutrient-poor oceanic (Trick et al., 2010; Silver et al., 2010) waters. Is *Pseudo-nitzschia* more versatile at adapting to variable environmental regimes, e.g. by effective use of macronutrients, scavenging potentially limiting trace elements, or by maintaining sustenance nutrients during bloom senescence, when its competitors are not? Evidence points to this possibility. *Pseudo-nitzschia* thrives under a variety of macronutrient conditions by effectively using nitrate, ammonium and urea as a sole nitrogen source. The link between nitrogen source and toxin production, where maximum toxin production is expressed by cells grown on the more complex source (urea) (Radan, 2008), illustrates the need to better understand the relationship between primary cell metabolism and secondary metabolism.

Confining regular sampling to the bloom formation phase, without a serious consideration of either bloom maintenance or demise, may restrict our ability to describe the overall ecology and significance of *Pseudo-nitzschia*. Careful microscopic observation of *Pseudo-nitzschia* cells during prolonged events will help determine the possible importance of parasites (Fig. 3) and/or viruses in bloom decline, and the significance of the momentary halt in vegetative cell proliferation while the cells undergo sexual reproduction. This, plus the use of molecular probes, may help to determine if toxigenicity may help protect the cells from microbial, fungal or viral attack. Perhaps the long, sustained bloom conditions provide both ecological and evolutionary histories, aiding in the redistribution of seed populations. Biochemical and molecular tools should continue to be developed to allow us to

monitor cell health and signaling pathways that may regulate toxicity.

The negative impacts of this species on human, marine mammal and bird health, highlight the intense social need for timely and critical *Pseudo-nitzschia* research. The complexity of the taxonomy, toxin production, toxin storage/release, bloom initiation/retention, and nutrient requirements places *Pseudo-nitzschia* as an ideal genus for intense scientific inquiry. Our accumulated knowledge of this genus opens further research opportunities, provides a means to critically evaluate the macro- and micro-physiology of phytoplankton, and offers a model system to assess the importance of an established toxin in ecotoxicological studies and as a possible info-chemical for affecting cellular, population and community dynamics.

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