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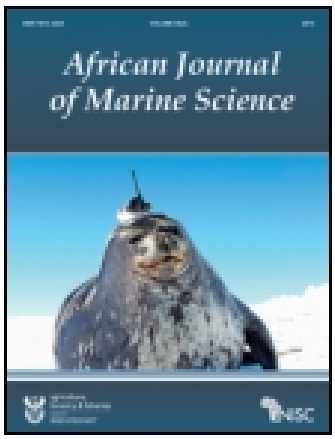

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Short Communication

Identification of the marine diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae) as a source of the toxin domoic acid in Algoa Bay, South Africa

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A unialgal culture of a *Pseudo-nitzschia* species dominant in the plankton of Algoa Bay in the spring of 2012 was established by isolation of clonal chains of cells. Identification of the species as *Pseudo-nitzschia multiseries* was based on frustule morphometrics provided by light and scanning electron microscopy, and confirmed by phylogenetic analysis of the LSU rDNA gene. Cultures were shown to produce domoic acid (DA) as measured by ELISA and LC/MS-MS methods, and levels of cellular DA were ~0.1 pg cell^{-1}. Although it is recognised as a cosmopolitan species, these observations provide the first account of this toxic diatom in the coastal waters of South Africa.

Keywords: ELISA, LC/MS-MS, phylogeny

Introduction

Pseudo-nitzschia is a globally distributed diatom genus that includes species that produce the neurotoxin domoic acid (DA), the cause of amnesic shellfish poisoning (ASP) (Lelong et al. 2012). Among 37 described *Pseudo-nitzschia* species, 14 have been shown to contain toxigenic members, although toxin production within a species tends to be inconsistent. Species of this genus are particularly common members of the coastal phytoplankton communities of eastern boundary upwelling systems (Trainer et al. 2010, 2012), with some efficient DA producers exhibiting a wide distribution (Hasle 2002). Consequently, *Pseudo-nitzschia* species are often found within phytoplankton communities in the Benguela Current upwelling system on the west coast of southern Africa (Pitcher and Calder 2000). In this system, DA has been measured in seawater samples containing *Pseudo-nitzschia* cells, although the toxigenic species have yet to be identified (Fawcett et al. 2007; Hubbart et al. 2012).

Our study undertakes identification of a *Pseudo-nitzschia* species in Algoa Bay, an open, relatively shallow (<70 m), eastward-facing bay located on the south-east coast of South Africa. Following its dominance in the plankton in the spring of 2012, our study further reports on the cell toxin content of this taxon and the consequent threat it poses to shellfish culture in Algoa Bay.

Material and methods

A single station (St1: 33°56.71′ S, 25°37.87′ E) located on an oyster farm in the western sector of Algoa Bay was sampled on three consecutive days, 2–4 October 2012. On 3 October 2012 a north-east transect was sampled, comprising a further six stations at intervals of one nautical mile (St2: 33°56.14′ S, 25°38.90′ E; St3: 33°55.61′ S, 25°39.92′ E; St4: 33°55.02′ S, 25°40.89′ E; St5: 33°54.47′ S, 25°41.96′ E; St6: 33°53.85′ S, 25°42.92′ E; St7: 33°53.38′ S, 25°43.98′ E).

Environmental and phytoplankton sampling

An SBE-19 Seacat CTD and WETLabs fluorometer (WETStar) were used to profile the water column at each station. Water samples collected from discrete depths by NIO bottles were subjected to nutrient analysis and fluorometric measurements of extracted chlorophyll *a*. NO_3^- was analysed according to Nydahl (1976), and PO_4^{3-} and SiO_4^{4-} according to Grasshoff et al. (1983) scaled down to 5 ml samples. Chlorophyll *a* analysis followed Parsons et al. (1984) and the data were used to calibrate *in situ* fluorescence profiles. Phytoplankton samples from the NIO bottles were fixed in buffered formalin (0.3% final concentration) and counted by the inverted microscope Utermöhl method (Hasle 1978).

Culture and identification of Pseudo-nitzschia

A unialgal culture of the *Pseudo-nitzschia* species dominant in Algoa Bay on 3 October 2012 was established from isolation of clonal chains of cells. The culture was maintained in F/2 medium at 16 °C on a light:dark cycle of 12:12 h.

For light microscopy, live cultures were examined with a Zeiss AXIO Observer.A1 inverted microscope and

photographed with an attached AxioCam ERc 5s camera. Scanning electron microscopy (SEM) was used to determine frustule morphometrics for identification of *Pseudo-nitzschia* species. For SEM, 10 ml samples of culture were acid-cleaned by addition of 1 ml of 10% HCl, 2 ml of 30% H_2SO_4, and 10 ml of a saturated aqueous solution of $KMnO_4$ for 24 h. Samples were cleared by addition of 10 ml of a saturated aqueous solution of oxalic acid prior to rinsing several times with distilled water. Samples were then dried onto Nucleopore filters adhered to SEM stubs, coated with carbon and viewed with an FEI NOVA Nano 230 scanning electron microscope with a field emission gun.

Sequence analysis

Subsamples of the *Pseudo-nitzschia* culture established in this study were fixed in absolute ethanol. Prior to genomic DNA extraction, fixed cells were concentrated by centrifugation and rinsed twice for 5 min in double-distilled water. Genomic DNA was extracted with the QIAamp® DNA micro kit (Qiagen) following the manufacturer's instructions. A fragment of the LSU rDNA was amplified with the universal primers D1R (Lenaers et al. 1989) and D3Ca (Scholin et al. 1994) according to conditions described by Lundholm et al. (2002). Purified PCR products were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems) and ABI3730xl genetic analyser (Applied Biosystems) according to the sequencer manufacturer's instructions. Both forward and reverse primers (D3Ca and D1R respectively) were utilised for cycle sequencing. Each sequence was edited and assembled by CLC Main Workbench v. 6.8.4 (CLC bio, a Qiagen company) and homology searches were carried out with the BLASTN algorithm provided by the NCBI.

The LSU rDNA sequence of the *Pseudo-nitzschia* species isolated from Algoa Bay was aligned with sequences from 50 known *Pseudo-nitzschia* taxa and one outgroup diatom species (*Cylindrotheca closterium*) downloaded from GenBank. Alignments were exported into Phylip format for construction of maximum likelihood (ML) trees using PHYML v. 3.1 (Guindon and Gascuel 2003). The Tamura-Nei substitution (HKY85) model and gamma parameters were used for construction of the ML tree. Gamma parameter values were estimated by the PHYML software. The reliability of the inferred phylogenetic tree was assessed by the bootstrap test with 1 000 bootstrap resamplings. Tree files were viewed and edited via Mega v. 6.0 (Tamura et al. 2013).

Domoic acid analysis

Particulate domoic acid was determined from cultures of *P. multiseries* after filtration (50 ml) upon Whatman GF/F filters (nominal pore size 0.7 µm). Samples were assayed by a quantitative enzyme-linked immunosorbent assay (ELISA, Biosense Laboratories – ASP test kit) and analysed by liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS). For the ELISA, DA concentrations were determined according to Kleivdal et al. (2007) using a 10-point calibration curve derived from certified DA standard (NRC CRM-DA-e). The LC/MS-MS determination of particulate domoic acid concentrations followed the multitoxin method of Krock et al. (2008).

Results

Environmental parameters and composition of the microplankton community

Surface waters along the transect of 3 October 2012 showed a small range in temperature (18.05–18.26 °C) with the warmest waters at the inner stations (Figure 1a). A weak thermocline was present inshore at around 8 m depth, increasing to around 16 m depth at the offshore stations. Nutrient concentrations were generally low. Surface NO_3^- concentrations were ≤0.10 mmol m^{-3} but increased with depth to a maximum of 1.27 mmol m^{-3}. Concentrations of PO_4^{3-} were also low (0.11–0.66 mmol m^{-3}), whereas SiO_4^{4-} concentrations were much higher (2.95–21.15 mmol m^{-3}). Chlorophyll *a* concentrations ranged from 0.2–4.0 mg m^{-3}, with higher concentrations near the thermocline, particularly at the inner stations (Figure 1b).

Enumeration of the microplankton (defined here as size range 10–200 µm) showed a diatom-dominated community with cell concentrations ranging from 197–905 cells ml^{-1} (Figure 1c). Community composition remained somewhat consistent along the transect with diatoms contributing between 81% and 98% to the total cell count. *Pseudo-nitzschia* species were typically the most abundant diatom, contributing between 21% and 51% to the total diatom cell count. Cell concentrations of *Pseudo-nitzschia* species ranged from 53 cells ml^{-1} (St5) to 307 cells ml^{-1} (St3). Other common diatoms (>20 cells ml^{-1}) included species of the genera *Bacteriastrum*, *Cerataulina*, *Chaetoceros*, *Coscinodiscus*, *Detonula*, *Eucampia*, *Guinardia*, *Lauderia*, *Leptocylidrus*, *Schroederella* and *Thalassiosira*. The only other microplankton to exceed 20 cells ml^{-1} included dinoflagellates of the genera *Peridinium* and *Prorocentrum*.

Pseudo-nitzschia *identification by microscopy*

The dominant *Pseudo-nitzschia* species observed in Algoa Bay on 3 October 2012 was *P. multiseries* (Figure 2). This identification was based on the linear to lanceolate shape of cells; the measures of the apical (68–140 µm) and transapical (3.4–6 µm) axes of the cells; the number (14–16) of transapical striae and fibulae within 10 µm; the number of rows of poroids (3) within valve striae; and the number of poroids (5/6) within 1 µm. Apparent variation in poroid size, notably in Figure 2d and 2e, is attributed to internal and external views of the valves.

LSU phylogeny of Pseudo-nitzschia multiseries

A BLAST search of the GenBank database revealed that the LSU rDNA sequence (752 bp) of the Algoa Bay isolate showed high similarity (99%) to a number of *P. multiseries* isolates (GenBank accession numbers AF417655, U41389, AF440770, KF006834). The phylogenetic analysis confirmed the above findings. The Algoa Bay isolate formed a monophyletic group together with *P. multiseries* isolates from Japan, Australia and the USA (Figure 3). This clade was supported by a high bootstrap value (91%) with the ML analysis.

Toxin content of Pseudo-nitzschia multiseries

Cultures of *P. multiseries* isolated from Algoa Bay were shown to produce DA. The toxin content of two subcultures,

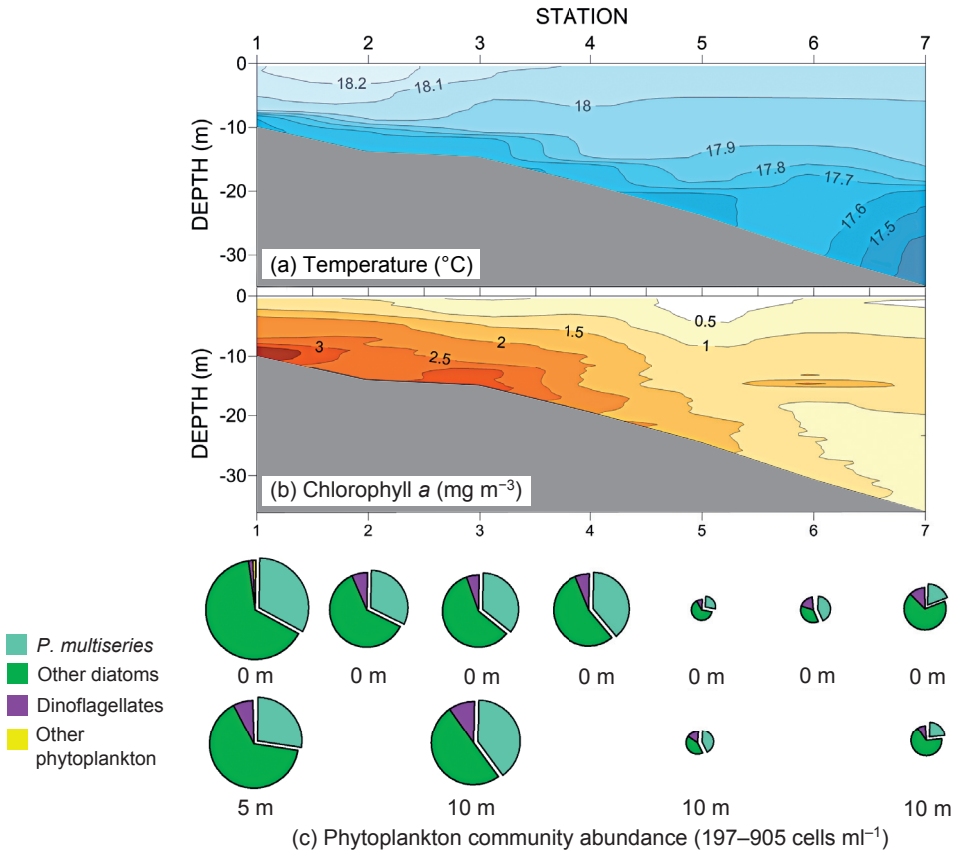

Figure 1: A 6-nautical-mile transect in the western sector of Algoa Bay on 3 October 2012 showing (a) temperature, (b) chlorophyll *a*, and (c) multiple pie diagrams depicting the abundance of the phytoplankton community (abundance proportional to pie-diagram diameter) and community composition at 0, 5 or 10 m depth

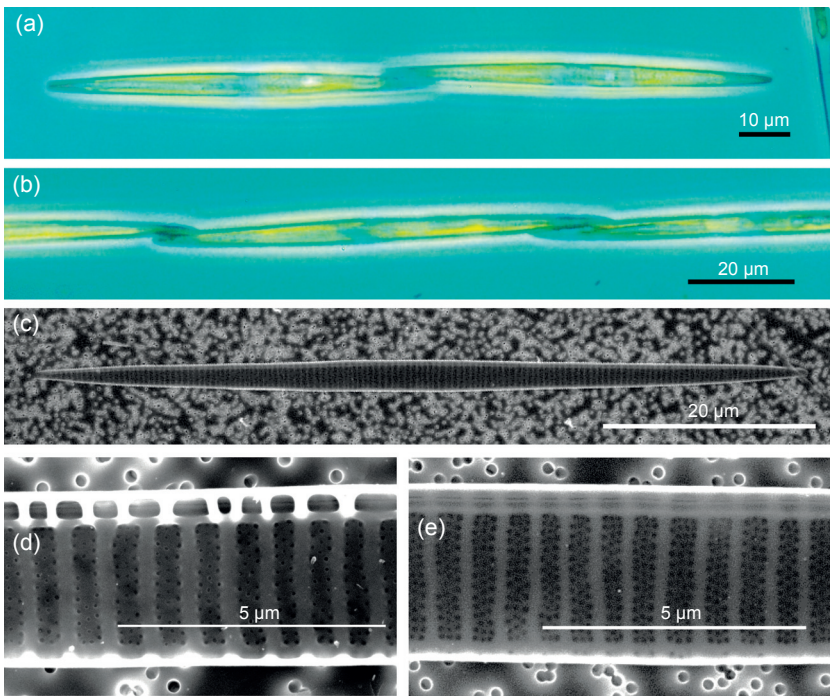

Figure 2: (a, b) Light micrographs providing girdle view of a *Pseudo-nitzchia multiseries* chain in culture showing cells attached by their overlapping apices; scanning electron micrographs of (c) whole valve and (d, e) the central regions of valves showing the poroid striae and fibulae

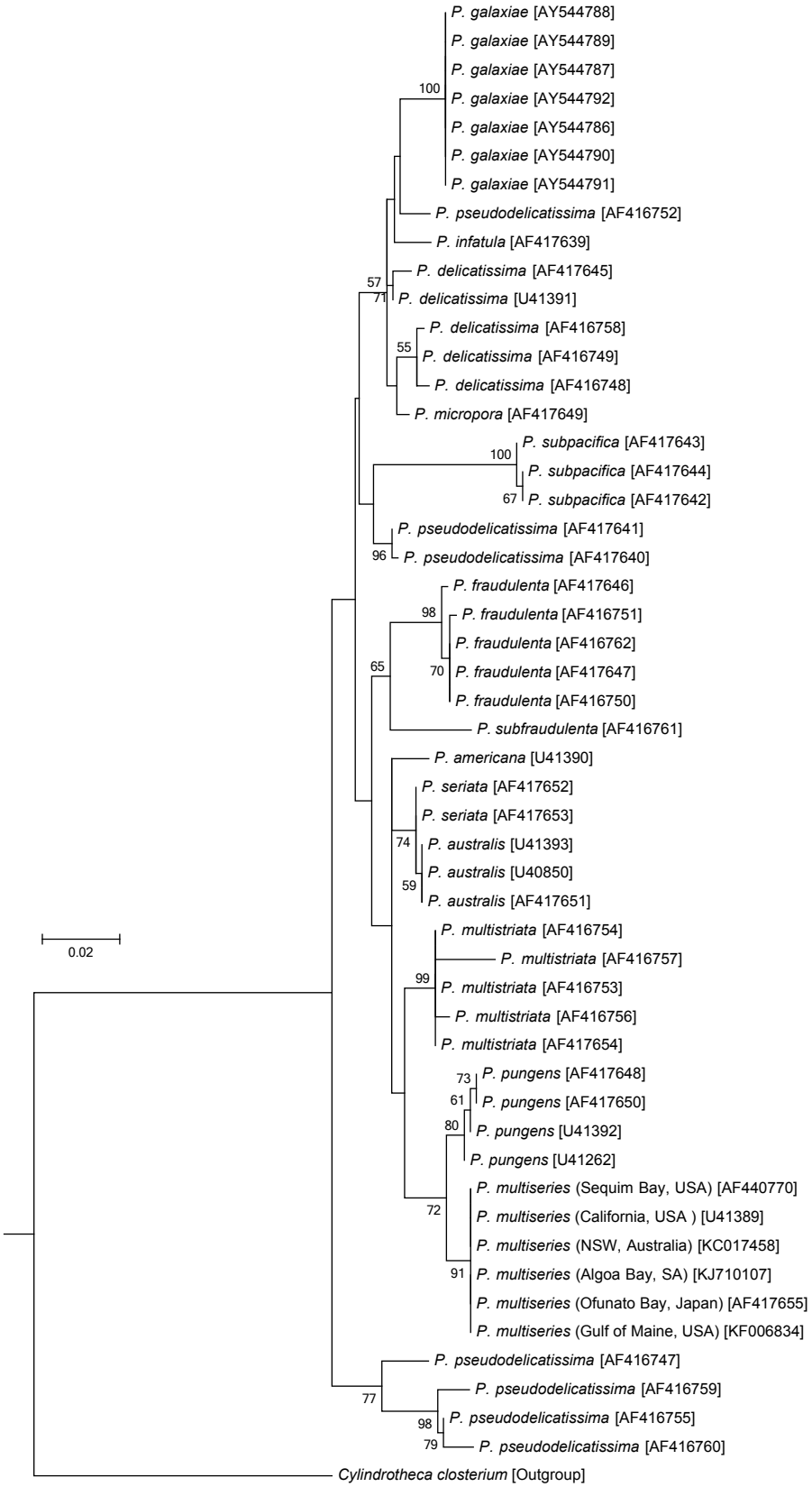

Figure 3: Maximum-likelihood tree inferred from the D1–D3 hypervariable domains of the LSU rDNA of *Pseudo-nitzschia* collected from Algoa Bay in South Africa, 50 taxa in the genus *Pseudo-nitzschia* and one outgroup species (*Cylindrotheca closterium*). The tree with the highest log likelihood (–2 539.82303) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches. Numbers next to the specific names correspond to the accession numbers for the LSU rDNA sequences. There were a total of 700 positions in the final dataset. The bar indicates 2 base substitutions per 100 nucleotides

Table 1: Culture toxin content expressed as particulate DA and per cell as determined by ELISA and LC/MS-MS

P. multiseries	Culture 1		Culture 2	
Cell concentration	47 239 cells ml^{-1}		48 716 cells ml^{-1}	
ELISA toxins	3.61 µg l^{-1}	0.076 pg cell^{-1}	4.76 µg l^{-1}	0.098 pg cell^{-1}
LC/MS-MS toxins	4.08 µg l^{-1}	0.086 pg cell^{-1}	4.18 µg l^{-1}	0.086 pg cell^{-1}

grown and harvested under the same conditions, appeared similar, by ELISA and LC/MS-MS methods (Table 1). Particulate DA concentrations in these cultures ranged from 3.61 to 4.76 µg l^{-1} and resulting cellular levels of DA ranged from 0.076 to 0.098 pg DA cell^{-1}.

Discussion

Although *P. multiseries* is recognised as cosmopolitan (Hasle 2002), its identification in Algoa Bay provides the first account of this species in the coastal waters of South Africa. Historically, the world's first recorded event of amnesic shellfish poisoning (ASP) in 1987, caused by consumption of blue mussels *Mytilus edulis*, was traced to a bloom of *P. multiseries* off Prince Edward Island, Canada (Bates et al. 1989). Identified at that time as *Nitzschia pungens* f. *multiseries* it is now known as *P. multiseries* following the reinstallation of the genus *Pseudo-nitzschia* by Hasle (1994) and the raising of the rank of *Pseudo-nitzschia pungens* f. *multiseries* from form to species by Hasle (1995). The two forms were distinguished by the structure of the valve face striae, with f. *multiseries* bearing 3–4 rows of small poroids compared to the 2 rows of larger poroids of the striae of f. *pungens*. Raising *Pseudo-nitzschia pungens* f. *multiseries* in rank to species was based on these morphological features and also on physiological and genetic features (Hasle 1995).

The impacts of *Pseudo-nitzschia* and its toxin have been considered by Trainer et al. (2010) to be especially problematic in upwelling systems, particularly in the California Current system where *P. multiseries* and *P. australis* have been identified as posing the greatest risk (Trainer et al. 2012). Here the impacts of DA are realised almost annually through direct toxicity of shellfish and through the effects on the health of marine life (Trainer et al. 2012). Although both Fawcett et al. (2007) and Hubbart et al. (2012) have shown the presence of particulate DA linked to the occurrence of *Pseudo-nitzschia* in the southern Benguela upwelling system, the absence of any recorded impact of DA on the South African coast is somewhat contrary to the observations of Trainer et al. (2010) of significant impact within the California Current system.

Compared to the west coast, the risk posed by harmful algae is significantly reduced on the south-east coast of South Africa (Pitcher and Calder 2000), with past reports of toxin-producing algae confined to a single account of diarrhetic shellfish poisoning (DSP) (Pitcher et al. 1993). However, the dominance of *P. multiseries* in Algoa Bay during the spring of 2012 suggests that it may be an important component of the phytoplankton of this region and could therefore pose a significant threat. The presence of *P. multiseries* in the bay in October, when winds are typically strongest and temperatures moderately low (Schumann et al. 2005), fits the ecological profile of *Pseudo-nitzschia* occurring during periods of mixing (Trainer et al. 2012).

Assessment of the risk posed by *Pseudo-nitzschia* is complicated by variability in cellular toxicity. The cell toxin quotas derived from the cultures of *P. multiseries* isolated from Algoa Bay (0.08–0.10 pg DA cell^{-1}) are somewhat lower than those determined from samples collected on the west coast of South Africa (0.17 pg DA cell^{-1} [Fawcett et al. 2007]; 0.21 pg DA cel^{l-1} [Hubbart et al. 2012]), but fall within the range reported for coastal assemblages and often attributed to variable nutrient regimes (Trainer et al. 2012). Although particulate DA levels in these cultures were considered sufficiently high to trigger trophic transfer, the relatively low concentrations of *Pseudo-nitzschia* observed in Algoa Bay are unlikely to contribute to particulate DA concentrations that would cause bivalve toxicity to exceed regulatory levels.

Owing to the potential impacts of DA on marine organisms and humans, further studies are required to better describe *Pseudo-nitzschia* diversity, population dynamics and toxigenicity on the coast of South Africa. Further to these studies, the uptake of DA by locally harvested or cultured shellfish needs to be investigated to better assess the potential impact on these operations.

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