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Do toxic *Pseudo-nitzschia* species pose a threat to aquaculture in the southern Benguela eastern boundary upwelling system?

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ABSTRACT

The productive but highly exposed coastline of the southern Benguela eastern boundary upwelling system offers limited natural environment for aquaculture. Saldanha Bay located on the west coast of South Africa is one of the few embayments on the coastline that provides a productive and relatively sheltered environment suitable for the cultivation of shellfish. Consequently, bivalve culture in South Africa is centered in Saldanha Bay and is presently targeted for expansion. *Pseudo-nitzschia* blooms including toxin-producing species are shown to contribute significantly to the phytoplankton of Saldanha Bay specifically in spring and summer. Their dominance at this time of the year, when upwelling is strongest, fits the ecological profile of *Pseudo-nitzschia* occurring during periods of high turbulence and nutrients. Multiple *Pseudo-nitzschia* blooms were sampled under varying environmental conditions and the strength of the relationship between *Pseudo-nitzschia* cell abundance and particulate domoic acid (pDA) content, reflecting bloom toxicity, varied greatly. This variability is the result of the combined influence of species and strain composition of the *Pseudo-nitzschia* assemblage and the effect of environmental conditions on toxin production. Elevated levels of pDA were associated with higher concentrations of cells of the *P. seriata* complex differentiated by frustule width (>3 μm). *P. australis* was identified as a toxin-producing species and a prominent member of the *P. seriata* complex. Low DA levels in shellfish in Saldanha Bay are considered a function of low cellular domoic acid (cDA). Silicate limitation has emerged as an important factor inducing DA production in *Pseudo-nitzschia* species. The high ratio of silicate to nitrate in Saldanha Bay provides a plausible explanation for the low toxin content of *Pseudo-nitzschia* blooms in the bay and the consequent low risk posed by these blooms to the aquaculture sector.

1. Introduction

The southern Benguela upwelling system located on the west coast of South Africa is subject to a high incidence of harmful algal blooms (HABs), which have caused fish mortalities, shellfish toxicity and related human health concerns for many decades (Pitcher and Louw, 2020). The most common impacts of HABs along the west coast are usually attributed to dinoflagellate blooms and include the poisoning syndromes known as paralytic shellfish poisoning (PSP) caused primarily by *Alexandrium catenella* and diarrhetic shellfish poisoning (DSP) attributed to *Dinophysis* species. Blooms of *Protoceratium reticulatum*, a known producer of yessotoxins (YTXs), are also common on the west coast. Although the toxin risk remains uncertain, regulation of YTXs has been the cause of the longest closure of shellfish harvesting in Saldanha Bay, a key aquaculture site in the southern Benguela (Pitcher et al., 2019; Pitcher and Louw, 2020).

Recently, toxigenic blooms of the marine pennate diatom genus *Pseudo-nitzschia* have been considered an emerging threat to the shellfish aquaculture industry, a risk to human health and a potential cause of marine faunal morbidities and mortalities in the Benguela region, as in other coastal upwelling systems. In humans and marine vertebrates, including seabirds, the neurotoxic tricarboxylic amino acid known as domoic acid (DA) is the cause of amnesic shellfish poisoning (ASP) via food chain accumulation or direct feeding upon plankton blooms. In the California Current system the negative impacts of DA are realized almost annually through trophic transfer of the toxin. Apart from the direct effects on the toxicity of shellfish resulting in harvest closures, these toxins also impact the health of marine life, including sea lions, sea

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otters and birds (McCabe et al., 2016).

Species of *Pseudo-nitzschia* tend to be common in coastal phytoplankton communities of eastern boundary upwelling systems, but their impacts vary notably (Trainer et al., 2010). Globally, among the 52 described species that comprise the genus *Pseudo-nitzschia*, 26 are known to produce DA (Bates et al., 2018). The increasing threat to human health posed by *Pseudo-nitzschia* species is at least partially attributable to the global distribution of this diatom. A recent barcoding study found this genus to account for 4.4% of the total diatom ribotype sequences worldwide and the highest number of operational taxonomic units amongst the pennate diatoms (Malviya et al., 2016).

The first identifications of species from the southern Benguela now assigned to *Pseudo-nitzschia* and known to produce DA were from unpublished observations made by Hasle (1972, 2002). These species included one of the most potent DA producers *P. australis* and its presence on the west coast of South Africa was verified by isolation from a bloom off Lambert's Bay in 2001 (Marangoni et al., 2001). In 2006 a study in the same area for the first time confirmed DA in phytoplankton concentrates collected from a bloom co-dominated by *Pseudo-nitzschia* species (Fawcett et al., 2007). Both this study and a subsequent field investigation off Lambert's Bay in 2007 (Hubbart et al., 2012) demonstrated a close correspondence between particulate DA levels and *Pseudo-nitzschia* cell abundance in the plankton. Unfortunately the dominant *Pseudo-nitzschia* species of these blooms were not established in either of these studies. In 2012 *Pseudo-nitzschia multiseries* was identified for the first time in the coastal waters of South Africa from water samples collected in Algoa Bay on the south coast (Pitcher et al., 2014). Cultures of *P. multiseries* established from Algoa Bay were also shown to produce DA. Furthermore, recent work off Namibia in the northern Benguela has identified several potentially toxigenic *Pseudo-nitzschia* species some of which have coincided with fish and bird mortalities (Gai et al., 2018; Louw et al., 2018). Among these, DA production has been confirmed in *P. australis* and *P. plurisecta* (Gai et al., 2018).

In the southern Benguela no ASP events linked to *Pseudo-nitzschia* blooms have been reported, and DA levels found in shellfish have yet to exceed harvestable limits. Furthermore, there is no record of impact of DA on marine life, despite regular observations of *Pseudo-nitzschia* species in this region (Pitcher and Calder, 2000; Pitcher and Louw, 2020). Nevertheless, the risk posed by ASP, to shellfish aquaculture development in the southern Benguela remains poorly evaluated, and justifies further assessment at key aquaculture sites.

Saldanha Bay is a large embayment on the west coast of South Africa that connects to a shallow lagoon in the south and to the southern Benguela upwelling system in the west. The bay is one of a very few sites on the South African coast that provides a productive and relatively sheltered environment suitable for the cultivation of shellfish. As a result, in situ bivalve culture in South Africa is centered in Saldanha Bay where the mussel *Mytilus galloprovincialis* and oyster *Crassostrea gigas* have been successfully farmed since the mid-1980s (Hecht and Britz, 1992). The development of the aquaculture sector within Saldanha Bay is perceived by Government to provide a solution to unemployment and poverty in the region and is now targeted for expansion (Olivier et al., 2013). Consequently Government has recently established an Aquaculture Development Zone (ADZ) in Saldanha Bay to expedite expansion of shellfish and finfish farming operations within the bay and to promote investor confidence in the sector.

This study reports on an investigation into the threat posed by toxin-producing *Pseudo-nitzschia* species to shellfish culture in Saldanha Bay. Initiated in 2012, this 3-year study undertook to establish the frequency of occurrence of *Pseudo-nitzschia* within the bay, to identify the dominant species, to establish the prevalence of DA production by these species and to assess the uptake of DA by cultured shellfish.

2. Methods

2.1. Sampling of the bay

The study site in Saldanha Bay was located on an oyster farm (33°01.748′ S, 18°00.888′ E) with a water column depth of approximately 13 m. The study spanned two periods of sampling: (1) 17 January 2012 – 23 January 2013, and (2) 27 February 2013 – 13 January 2015. The first sampling period comprised 7 short field studies during which samples were typically collected on 2 days, whereas the second sampling period comprised monthly field studies at which time samples were collected on a single day (Table 1).

A Turner Designs C3 Submersible Fluorometer (incorporating a temperature sensor) and a Starman-mini temperature recorder were moored for the duration of the study at 4 m and 10 m depth respectively, providing measures of in situ chlorophyll *a* (Chl *a*) and temperature at 10-min intervals. These instruments were serviced during each field study and the data uploaded. On each sampling day, water was collected by NIO bottle from the discrete depths of 1, 3 and 6 m during the first period and from 1, 3, 6 and 9 m during the second period. These water samples were subsampled for enumeration of *Pseudo-nitzschia* cells, for determination of Chl *a* and for measurement of particulate DA (pDA) levels. For the enumeration of *Pseudo-nitzschia* cells a subsample of 200 ml was fixed in buffered formalin. For measurements of Chl *a* and pDA, subsamples of 100 and 200 ml respectively were filtered through Whatman GFF glass fiber filters (nominal pore size 0.7 μm) and frozen (−80 °C) prior to analysis.

In addition to the samples collected during the field studies, surface water samples of 200 ml were collected daily from the oyster farm (all farm working days) and fixed in buffered formalin (1.5%) for enumeration of *Pseudo-nitzschia* cells. Also, during the first sampling period, samples of 30 mussels (*Mytilus galloprovincialis*) were collected from the oyster farm each month for the analysis of DA. These samples were frozen and stored at −20 °C prior to analysis.

2.2. Determination of Chl a

Chl *a* samples were analyzed by the fluorometric method, corrected for phaeopigments, as detailed by Parsons et al., (1984). Discrete water samples of 100 ml were filtered onto Whatman GF/F filters and analyzed fluorometrically using a Turner Designs 10-AU Fluorometer after extraction in cold 90% acetone. Extracted Chl *a* values were used to calibrate the in situ fluorescence measurements of the Turner Designs C3 Submersible Fluorometer (through application of a regression equation: Chl $a = 0.0061$ x measure of fluorescence; $r^2=0.78$, $n = 36$).

2.3. Enumeration of Pseudo-nitzschia cells

Pseudo-nitzschia cells from the field samples were provisionally identified and counted with a Zeiss AXIO Observer.A1 microscope by the

Table 1
Field study dates during the periods: (1) 17 January 2012 – 23 January 2013, and (2) 27 February 2013 – 13 January 2015.

Sample Period	Sample Dates
Period (1): 17 Jan 2012 – 23 Jan 2013	17 and 19 January 2012; 13 and 15 March 2012; 15 and 17 May 2012; 2 and 4 July 2012; 11 and 13 September 2012; 20 November 2012; 19, 21 and 23 January 2013
Period (2): 27 Feb 2013 – 13 Jan 2015	27 February 2013; 4 April 2013; 8 May 2013; 6 June 2013; 10 July 2013; 14 August 2013; 17 September 2013; 9 October 2013; 6 November 2013; 4 December 2013; 8 January 2014; 12 February 2014; 19 March 2014; 24 April 2014; 13 May 2014; 25 June 2014; 23 July 2014; 5 September 2014; 14 October 2014; 18 November 2014; 13 January 2015

Utermöhl inverted microscope method (Hasle, 1978). Owing to the difficulty of differentiating *Pseudo-nitzschia* species by light microscopy, cells were operationally assigned, based on the width of their frustules, to either the *P. seriata* (>3 μm) or *P. delicatissima* (<3 μm) complexes.

2.4. Critical taxonomic identification of Pseudo-nitzschia species

A unialgal culture of the *Pseudo-nitzschia* species dominant in Saldanha Bay on 20 November 2012 was established from the isolation of clonal chains of cells. The culture was maintained in f/2 growth medium (Guillard, 1975) at 16 °C on a light:dark cycle of 12:12 h.

2.4.1. Identification by microscopy

This culture was examined by light microscopy with an Olympus IX50 microscope and cells were photographed with an attached AxioCam ERc 5 s camera. Frustule morphometrics for identification of *Pseudo-nitzschia* species were determined by scanning electron microscopy (SEM). Cultured specimens (10 ml samples) 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 (x3) with distilled water following centrifugation (3000 x g for 10 min) and discarding the supernatant. 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 equipped with a field emission gun. Morphometric data were determined by measuring 20 individual cells from the culture.

2.4.2. Ribosomal DNA sequencing and phylogenetic assignment

For rDNA sequence analysis, subsamples of *Pseudo-nitzschia* culture were fixed in absolute ethanol. Fixed cells were concentrated by centrifugation (8000 x g for 30 s) and rinsed twice for 5 min in double-distilled water. Genomic DNA was extracted with the QIAamp® DNA micro kit (Qiagen, Cat. No. 56,304) 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 v3.1 cycle sequencing kit (Applied Biosystems, Cat. No. 4,337,455) and ABI3730xl genetic analyzer (Thermo Fisher Scientific, California, USA) 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 (http://clcbio.com/) and homology searches were carried out with the BLASTN algorithm provided by the NCBI. The nucleotide sequences were deposited in GenBank database as accession number MT682519.

The LSU rDNA sequence of the *Pseudo-nitzschia* species isolated from Saldanha 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 optimal model for ML analysis was found using Smart Model Selection (SMS v1.8.4) in PhyML (Lefort et al., 2017). The significantly most optimal model of nucleotide substitution was the general time-reversible model incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + G + I). The proportion of invariable sites was estimated (0.523) and on the remaining sites a gamma distribution with a shape of 0.630 with four categories was applied. PhyML bootstrap trees were constructed using the same parameters as the individual trees. 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 version X (Kumar et al., 2018).

2.5. Determination of domoic acid

For the first period of sampling (2012–13) pDA was analyzed by liquid chromatography with mass spectrometry (LC–MS/MS). For the second sampling period (2013–15) pDA was assayed with an enzyme-linked immunosorbent assay (ELISA). Both LC-MS/MS and the ELISA were employed to determine DA cell quotas of the *Pseudo-nitzschia* culture established on 20 November 2012. Assay of shellfish samples for DA was undertaken only during the first period of sampling by ELISA.

Estimates of cellular DA (cDA) were calculated from pDA levels and the abundances of *Pseudo-nitzschia* cells by: (1) dividing the pDA from filtered plankton concentrates by the corresponding concentration of *Pseudo-nitzschia* cells (mean concentrations were calculated for each day from values at each sample depth), and (2) application of a linear regression to pDA as a function of toxigenic *Pseudo-nitzschia* cell concentrations for each day sampled.

2.5.1. Liquid chromatography with mass spectrometry (LC–MS/MS)

Analysis of water samples for pDA by LC-MS/MS followed the multitoxin method of Krock et al. (2008). In brief, filters were homogenized with 1 ml methanol and lyzing Matrix D (Thermo Savant, Illkirch, France) by two reciprocal shaking cycles for 45 s each at 6.5 m s^{-1} in a FastPrep instrument (Thermo Savant). The homogenates were centrifuged for 15 min at 16 100 x g in a mini-centrifuge (5415 R, Eppendorf, Hamburg, Germany) and supernatants were passed through 0.45 μm centrifugal filters (Millipore Ultrafree, Eschborn, Germany). The filtrates were analyzed for lipophilic toxins and DA by LC-MS/MS (ABISciex 4000 Q Trap triple-quadrupole mass spectrometer equipped with a Turbo V ion source coupled to an Agilent 1100 LC liquid chromatograph) on a C8-phase analytical column (Hypersil BDS, 50 2 mm, 3 μm, 120 Å, Phenomenex, Aschaffenburg, Germany). The chromatographic and mass spectrometric parameters are detailed in Krock et al. (2008). Samples were calibrated against an external standard solution of DA with a concentration of 31 pg μl^{-1}. The certified DA standard (NRC CRM-DA-e) was obtained from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council, Halifax, Canada.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

DA in filtered plankton samples (particulate DA) was lysed from cells by sonication and extracted in 10% methanol. DA from mussel flesh (pooled samples of 20 mussels) was extracted in 50% methanol following shucking and homogenization of whole soft tissues. Assay of DA by ELISA (Biosense Laboratories – ASP test kit, Bergen Norway) followed centrifugation of extracted samples as detailed in the methods of Kleivdal et al., (2007). The assay is in a direct competition format, where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to anti-DA antibodies free in the solution. The assay was calibrated using a 10-point calibration curve derived from a certified DA standard (NRC CRM-DA-e, Institute for Marine Biosciences, NRC, Halifax, Canada). According to the manufacturer, the calibrated range of the assay is approximately 10 to 300 pg ml^{-1} of DA. The working range for DA in shellfish is 0.01–250 mg kg^{-1}.

3. Results

3.1. Bay temperatures and Chl a

The temperature time-series (January 2012 – January 2015) based on measurements at 4 and 10 m showed a clear seasonal trend (Fig. 1a, b; Table 2). The spring transition to the upwelling season was demarcated by increasing surface water temperatures and declining bottom water temperatures. Stratification, as evident from increasing differences in surface and bottom water temperatures, reached a peak in summer. At this time surface waters exceeded 20 °C on occasions, while bottom water temperatures sometimes declined to below 10 °C. Vertical

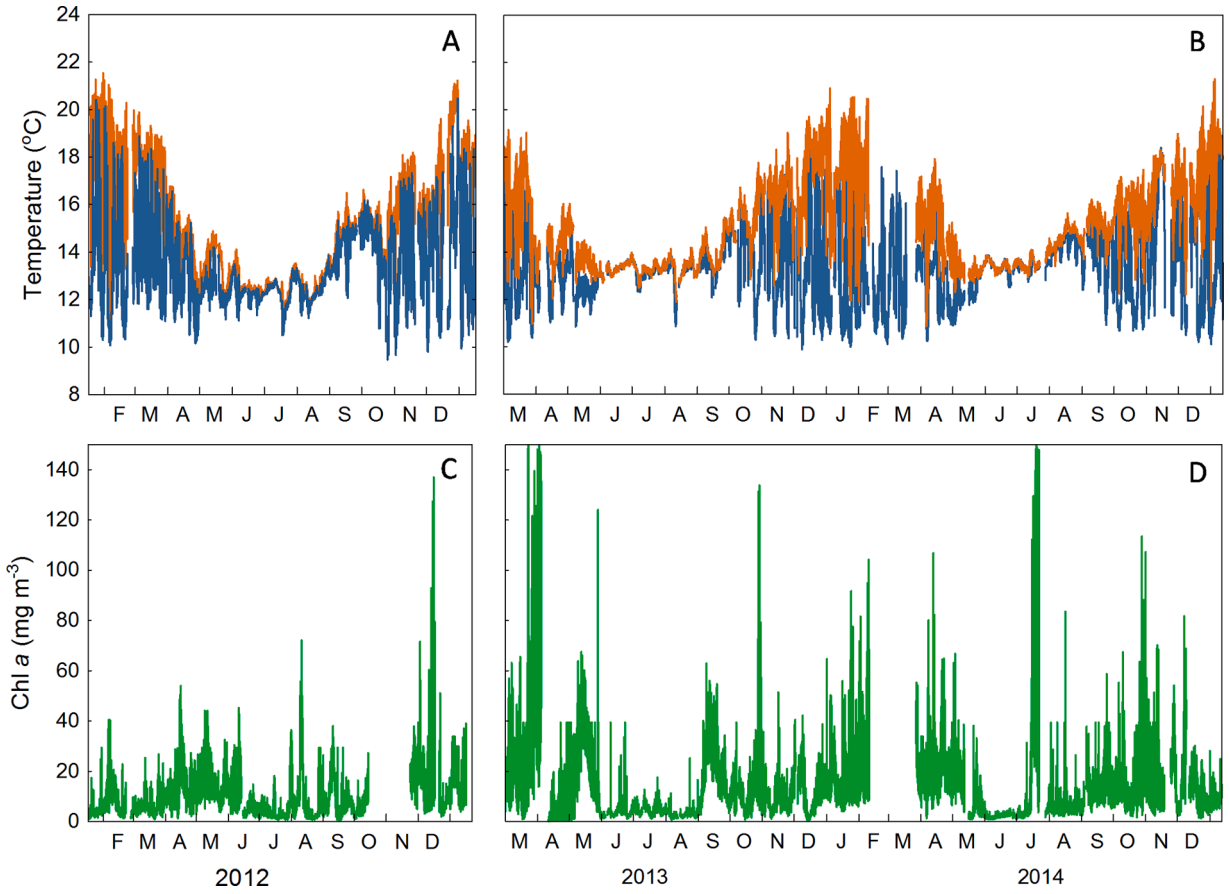

Fig. 1. A time-series at 10 min intervals of temperature at 4 m (orange) and 10 m (blue) (a, b) and of Chl a at 4 m (c, d) for the periods 17 January 2012 – 16 January 2013 and 1 March 2013 – 12 January 2015 respectively.

Table 2
Summary of mean seasonal temperatures at 4 and 10 m depth and Chl *a* concentrations at 4 m depth for the period January 2012 to January 2015.

	Spring	Summer	Autumn	Winter
Temperature (°C) 4 m	15.55 N = 36,323 SD=1.19 Range 12.24–19.04	17.79 N = 27,020 SD=1.70 Range 10.47–21.53	14.82 N = 33,210 SD=1.76 Range 10.88–19.88	13.25 N = 37,277 SD=0.73 Range 11.22–15.64
Temperature (°C) 10 m	13.85 N = 36,323 SD=1.51 Range 9.48–18.40	13.36 N = 29,121 SD=2.32 Range 9.81–20.47	12.88 n = 35,717 SD=1.37 Range 10.16–18.88	13.07 N = 37,227 SD=0.73 Range 10.5–15.05
Chlorophyll *a* (mg m^{-3}) 4 m	12.77 N = 31,160 SD=9.53 Range 0.46–221.87	12.62 N = 24,776 SD=11.13 Range 0.62–143.23	15.79 N = 33,257 SD=19.44 Range 0.13–394.84	6.61 N = 37,275 SD=15.58 Range 0.63–293.26

displacement of the thermocline forced by the coastal upwelling-downwelling cycle, and by local wind-driven mixing that causes the upper layer to deepen through entrainment of cold bottom water resulted in sharp temperature fluctuations through the water column during the course of the upwelling season. A decline in upwelling strength in autumn was associated with weakened stratification and the end of the upwelling season was demarcated by the transition to a well-mixed isothermal water column of around 13 °C in winter.

Seasonality in the Chl *a* time-series was less evident (Fig. 1c, d; Table 2), partly owing to the measurement of Chl *a* only from a single depth (4 m) in a highly stratified environment, typically characterised by a sharply defined subsurface Chl *a* maximum. Notable variability was evident at the event scale driven in all likelihood by the upwelling-downwelling cycle and corresponding vertical displacement of the thermocline and subsurface Chl *a* maximum. Several peaks exceeding 100 mg m^{-3} characterised the Chl *a* time-series with mean concentrations highest in autumn (15.79 mg m^{-3}) before dropping to a mean low in winter (6.61 mg m^{-3}; Table 2).

3.2. *Pseudo-nitzschia species and particulate and cellular DA*

Pseudo-nitzschia species were shown to be an important component of the phytoplankton of Saldanha Bay, specifically during spring and summer. Only during winter were *Pseudo-nitzschia* cell concentrations consistently low. The time series of *Pseudo-nitzschia* cell abundance in the surface waters of the oyster farm showed elevated concentrations (> 0.5 × 10^6 cells l^{-1}) during the spring and summer of the 2012–13 upwelling season (Fig. 2). A bloom of *Pseudo-nitzschia* (of the *P. seriata* [>3 μm] complex; >1 × 10^6 cells l^{-1}) was prominent from 12 November to 13 December 2012 (with a maximum concentration of 3.81 × 10^6 cells l^{-1} on 4 December 2012). The 2013–14 upwelling season was again characterised by an increase in *Pseudo-nitzschia* cell concentrations in surface waters in spring persisting though summer into autumn. The initial spring bloom was dominated by the *P. delicatissima* (<3 μm) complex (maximum concentration 3.63 × 10^6 cells l^{-1} on 17 September 2013) with cells of the *P. seriata* (>3 μm) complex more prominent later in the season (maximum concentration 3.03 × 10^6 cells l^{-1} on 21 November 2013). Surface concentrations of *Pseudo-nitzschia* cells were markedly lower in the spring and summer of the 2014–15 upwelling season (maximum concentration of 0.84 × 10^6 cells l^{-1} of the *P. delicatissima* (<3 μm) complex on 3 November 2014).

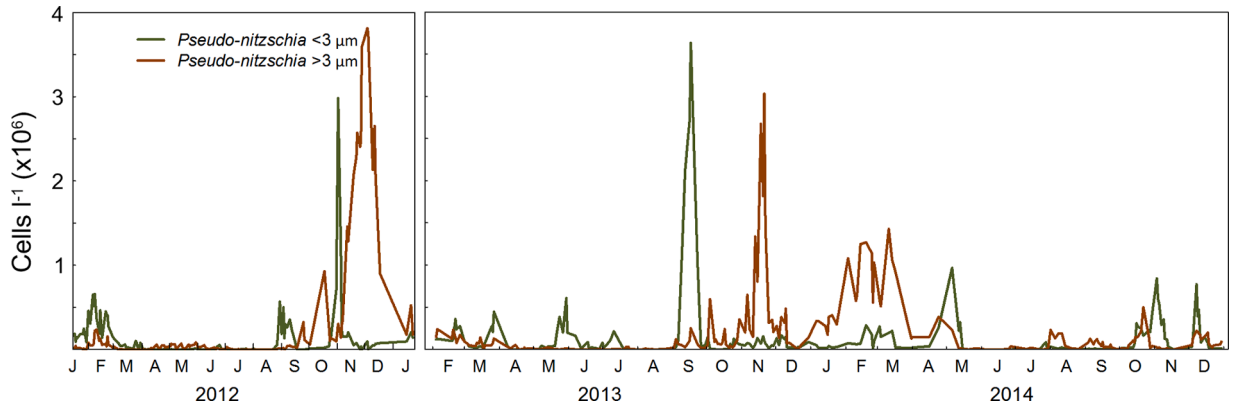

Fig. 2. *Pseudo-nitzschia* cell abundance in Saldanha Bay surface waters at approximately daily intervals from 17 January 2012 – 16 January 2013 and from 25 January 2013 – 13 January 2015. Cells were assigned to either the *P. seriata* (>3 µm) or *P. delicatissima* (<3 µm) complexes as determined by frustule width.

Pseudo-nitzschia cell concentrations determined from discrete depths through the water column (1, 3 and 6 m) during the first period of sampling were invariably highest at 6 m depth where a maximum of 6.18×10^6 cells l^{-1} was recorded on 20 November 2012 (Fig. 3a). The lowest concentration of 5.8×10^3 cells l^{-1} during this period was recorded in the surface waters during winter (2 July 2012). For the second sampling period elevated cell concentrations were observed during most of the 2013–14 upwelling season with the highest concentrations in spring (maximum of 6.52×10^6 cells l^{-1} at 3 m on 17 September 2013) (Fig. 3b). The upwelling season of 2014–15 was characterised by moderate cell concentrations of *Pseudo-nitzschia* with a subsurface maximum of 3.35×10^6 cells l^{-1} at 9 m depth on 13 January 2015. The lowest *Pseudo-nitzschia* concentrations were again recorded in winter with cells undetected in surface waters on 14 August 2013 (1, 3 m) and again on 23 July 2014 (3 m). No discernible trend was evident in the relative contributions of the *P. seriata* (>3 µm) and *P. delicatissima* (<3 µm) complexes to the total *Pseudo-nitzschia* concentrations.

Particulate DA did not necessarily reflect *Pseudo-nitzschia* cell abundance. For the first sampling period the only discrete samples in which pDA was not detected were collected in winter (at 1 and 6 m on 2 July 2012; Fig. 3c). The maximum pDA level during this period was measured in summer at 6 m on 19 January 2012. For the second period

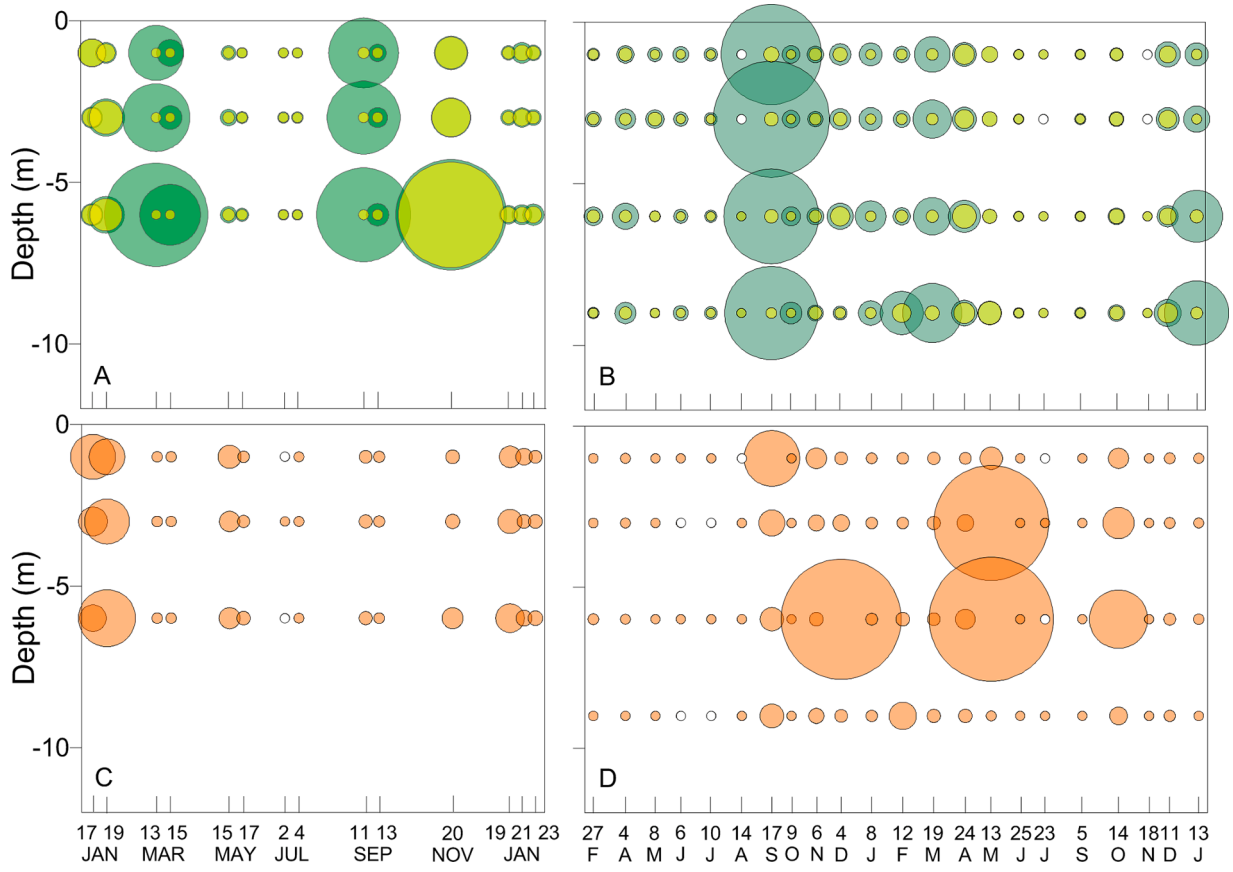

Fig. 3. *Pseudo-nitzschia* abundance (a, b) and particulate domoic acid (c, d) measured at 0, 3 and 6 m at approximately bimonthly intervals from 17 January 2012 – 16 January 2013 and at 0, 3, 6 and 9 m at approximately monthly intervals from 1 March 2013 – 12 January 2015. Green bubbles (a, b) represent total *Pseudo-nitzschia* cell abundance (bubble size depicts range from 0 – 6.52×10^6 cells l^{-1}) and superimposed yellow bubbles represent abundance of the *P. seriata* (> 3 µm) complex (bubble size depicts range from 0 – 5.88×10^6 cells l^{-1}). Orange bubbles (c, d) represent pDA (bubble size depicts range from 0 – 5 µg l^{-1}).

of sampling, pDA was again undetected in some winter samples (at 3 and 9 m on 6 June and 10 July 2013, at 1 m on 14 August 2013, and at 1 and 6 m on 23 July 2014; Fig. 3d). The maximum pDA level was recorded on 13 May 2014 again at 6 m depth.

Seasonal variation in *Pseudo-nitzschia* cell abundance and pDA is portrayed through presentation of mean values for spring, summer, autumn and winter at the depths of 1, 3, 6 and 9 m (Table 3). Clearly evident are the low cell concentrations and toxin levels in winter. Notable increases through the water column are associated with the onset of spring and the transition to conditions of upwelling. *Pseudo-nitzschia* concentrations were generally highest in spring with an average concentration of 2.1 × 10^6 cells l^{-1} at 6 m depth. In summer and autumn vertical gradients in both *Pseudo-nitzschia* and pDA intensified with increasingly stratified conditions resulting in a subsurface maximum pDA level of 0.87 μg l^{-1} in summer at 6 m depth.

Plots of pDA versus *Pseudo-nitzschia* show cell abundance to be a poor general predictor of particulate toxin (Fig. 4a, b). However, closer examination reveals that the relationship between *Pseudo-nitzschia* abundance and pDA tended to be specific for each field study. Generally, high pDA levels were associated with the presence of cells of the *Pseudo-nitzschia seriata* complex (>3 μm) rather than those of the *P. delicatissima* complex (<3 μm). However, cell concentrations of the *Pseudo-nitzschia seriata* complex also failed to serve as a general predictor of pDA as some field studies were characterised by low pDA despite the presence of high concentrations of the *Pseudo-nitzschia seriata* complex.

The relationships between *Pseudo-nitzschia* cell abundance and pDA varied markedly during the first period of sampling (Fig. 4a). On both 17 and 19 January 2012 pDA (0.76–2.08 μg l^{-1}) clearly corresponded to *Pseudo-nitzschia* abundance and *Pseudo-nitzschia* assemblages were clearly dominated by the *P. seriata* complex (0.55–1.47 × 10^6 cells l^{-1}) with far fewer cells of the *P. delicatissima* complex (0.06–0.26 × 10^6 cells l^{-1}). In contrast pDA on 13 and 15 March 2012 was particularly low (<0.06 μg L-^{1}) despite generally high concentrations of *Pseudo-nitzschia* (0.90–5.73 × 10^6 cells l^{-1}). However, these *Pseudo-nitzschia* assemblages were overwhelmingly dominated by cells of the *P. delicatissima* complex, with low concentrations of cells of the *P. seriata* complex (< 0.04 × 10^6 cells l^{-1}). Moderate pDA values on 15 May 2012 (0.48–0.60 μg l^{-1}) were notably higher than those on the 17 May 2012 (0.10–0.19 μg l^{-1}) and again corresponded to conspicuously higher concentrations of the *P. seriata* complex on the 15 May (0.1–0.18 × 10^6 cells l^{-1}) compared to the 17 May (0.03–0.09 × 10^6 cells l^{-1}). In July 2012 both *Pseudo-nitzschia* concentrations and pDA levels were very low. In September 2012, as in March, pDA was low (<0.17 μg l^{-1}) despite high cell concentrations of *Pseudo-nitzschia* spp. However, as in March the *Pseudo-nitzschia* assemblage was dominated by cells of the *P. delicatissima* complex with much lower concentrations of cells of the *P. seriata* complex (<0.12 × 10^6 cells l^{-1}). In November 2012, pDA again clearly corresponded to *Pseudo-nitzschia* abundance although pDA levels were only moderately elevated (0.2–0.51 μg l^{-1}) despite high concentrations of cells of the *P. seriata* complex (1.38–5.88 × 10^6 cells l^{-1}). In January 2013 moderately elevated levels of pDA particularly on 19 January (0.53–0.84 μgl^{-1}) were associated with moderate concentrations of cells from the *P. seriata* complex (0.18–0.39 × 10^6 cells l^{-1}) although the relationship between cells and pDA was less obvious on the 21 and 23 January 2013.

During the second sampling period the relationships between *Pseudo-nitzschia* abundance and particulate DA again varied between field studies (Fig. 4b). For the sampling days depicting elevated pDA and/or *Pseudo-nitzschia* cell abundances the following observations are noteworthy. Some of the highest pDA levels (4.83 μg l^{-1} at 6 m on 4 December 2013 and 4.61 and 5.00 μg l^{-1} at 3 m and 9 m on 13 May 2014) were characterized by moderate *Pseudo-nitzschia* cell concentrations dominated by the *P. seriata* complex (0.15–0.61 × 10^6 cells l^{-1}). Elevated pDA levels were also recorded on 14 October 2014 at 6 m depth (2.14 μg l^{-1}) where cell concentrations of the *P. seriata* complex were significantly higher (0.34 × 10^6 cells l^{-1}) compared to the other sampling depths. The highest *Pseudo-nitzschia* concentrations (5.12–6.52 × 10^6 cells l^{-1}) were recorded on 17 September 2013 and dominated by cells of the *P. delicatissima* complex. Corresponding toxin levels were generally low (<0.74 μg l^{-1}) except at 1 m depth where a markedly higher pDA (2.02 μg l^{-1}) level corresponded to a distinctly higher concentration of cells of the *P. seriata* complex (0.36 × 10^6 cells l^{-1}).

Estimates of cellular DA (cDA) calculated from pDA levels and *Pseudo-nitzschia* cell abundances ranged widely from 0.001 - 7.89 pg cell^{-1} (Table 4). All estimates >1 pg DA cell^{-1} were obtained from samples dominated by the *P. seriata* complex.

3.3. Identification and toxin content of cultured Pseudo-nitzschia australis

Cultured cells of *Pseudo-nitzschia* isolated from the plankton of Saldanha Bay on 20 November 2012 were identified by light- and scanning electron microscopy as *P. australis* (Fig. 5). The shape of cells was linear to lanceolate with rounded tips. Measurements (n = 20) of the apical axis ranged from 62 to 75 μm and transapical measurements from 6.0–7.4 μm. Two rows of poroids were located within valve striae and the number of transapical striae and fibulae within 10 μm ranged from 14 to 16.

Species identification of the culture was confirmed by sequencing the LSU rDNA and phylogenetic analysis (Fig. 6; Table 5). A BLAST search of the GenBank database revealed that the gene sequence (778 bp) showed high similarity (>99%) to a number of *Pseudo-nitzschia australis* isolates

Table 3
Summary of mean seasonal concentrations of *Pseudo-nitzschia* cell abundance and pDA sampled at the discrete depths of 0, 3, 6 and 9 m for the period January 2012 to January 2015.

		Mean *Pseudo-nitzschia* (cells l^{-1} x 10^6)	Mean pDA (μg l^{-1})
Spring [Sept, Oct, Nov]	0 m	1.389 (n = 9; sd=1.940; range 0–5.553)	0.381 (n = 9; sd=0.64; range 0.003–2.024)
	3 m	1.578 (n = 9; sd=2.224; range 0–6.524)	0.275 (n = 9; sd=0.35; range 0.003–0.964)
	6 m	2.100 (n = 9; sd=2.588; range 0.005–6.175)	0.411 (n = 9; sd=0.69; range 0.001–2.137)
	9 m	1.140 (n-6; sd=1.970; range 0.013–5.124)	0.213 (n = 6; sd=0.26; range 0.001–0.638)
Summer [Dec, Jan, Feb]	0 m	0.655 (n = 11; sd=0.301; range 0.183–1.161)	0.379 (n = 11; sd=0.51; range 0.018–1.551)
	3 m	0.732 (n = 11; sd=0.392; range 0.369–1.710)	0.372 (n = 11; sd=0.47; range 0.019–1.553)
	6 m	1.010 (n = 11; sd=0.637; range 0.489–2.597)	0.871 (n = 11; sd=1.44; range 0.049–4.833)
	9 m	1.300 (n = 6; sd=1.218; range 0.120–3.350)	0.190 (n = 6, sd=0.296; range 0.011–0.786)
Autumn [Mar, Apr, May]	0 m	0.880 (n = 9; sd=0.960; range 0.081–2.794)	0.186 (n = 9; sd=0.23; range 0.004–0.604)
	3 m	1.030 (n = 9; sd=1.057; range 0.164–3.565)	0.652 (n = 9; sd=1.49; range 0.008–4.609)
	6 m	1.560 (n = 9; sd=1.837; range 0.096–5.725)	0.719 (n = 9; sd=1.62; range 0.001–5.000)
	9 m	1.130 (n = 9; sd=1.130; range 0.031–3.040)	0.080 (n = 5; sd=0.09; range 0.002–0.182)
Winter [Jun, Jul, Aug]	0 m	0.117 (n = 7; sd=0.145; range 0–0.117)	0.005 (n = 7; sd=0.01; range 0–0.028)
	3 m	0.118 (n = 7; sd=0.110; range 0–0.292)	0.008 (n = 7; sd=0.01; range 0–0.034)
	6 m	0.100 (n = 7; sd=0.100; range 0.003–0.288)	0.006 (n = 7; sd=0.01; range 0–0.025)
	9 m	0.125 (n-5; sd=0.147; range 0.125–0.345)	0.001 (n = 5; sd=0.001; range 0–0.003)

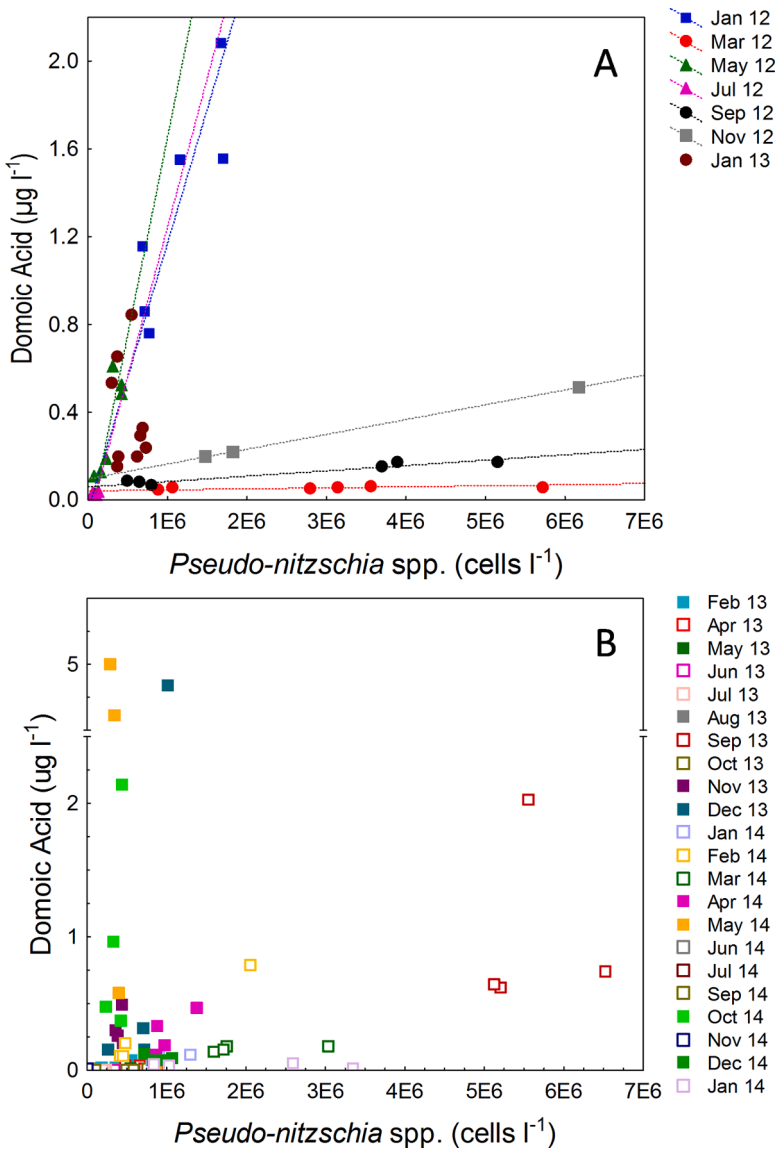

Fig. 4. Particulate domoic acid versus *Pseudo-nitzschia* cell abundances for the periods (a) 17 January 2012 – 16 January 2013 and (b) 1 March 2013 – 12 January 2015.

(KX290871, AF417651, and KX290869). The phylogenetic analysis confirmed the sequence comparison similarities, with the Saldanha Bay isolate clustering in a clade with other *P. australis*. Strains of *P. seriata* formed a sister group with *P. australis*, and this relationship was moderately supported by bootstrap values (78%) in the ML analysis.

Two clonal cultures of *P. australis* established in November 2012 were harvested in September 2013 during late exponential/early stationary growth phase for replicate determination of pDA and cDA by LC-MS/MS and ELISA (Table 6). DA was detected in both cultures and the two alternative methods showed good correspondence. However, cDA levels were remarkably different between the two cultures despite having been isolated from the same water sample and maintained under the same conditions. LC-MS/MS analyses provided marginally higher estimates for pDA than those measured by ELISA; calculated cDA as measured by LC-MS/MS for the two cultures were 2.49 and 0.011 pg cell^{-1}.

3.4. DA levels in shellfish

Low DA levels were detected in mussels (*M. galloprovincialis*) suspended at 1 m depth on the oyster farm and harvested monthly from February 2012 – January 2013. Values above the DA detection limit were found during most months, except for April, August and December (Fig. 7). The maximum value of 0.27 mg DA kg^{-1} shellfish recorded in spring (September) was well below the regulatory limit of 20 mg DA kg^{-1} shellfish.

4. Discussion

4.1. Upwelling and blooms of Pseudo-nitzschia

Saldanha Bay exhibits strong hydrographic variability at the event (6–8 days) and seasonal scales as is evident from this study (Fig. 1a, b) and previous studies (Monteiro and Largier, 1999; Smith and Pitcher, 2015). Winter mixing and isothermal conditions transition in spring to a two-layered stratified system, driven by solar warming of surface waters and the intermittent penetration of cold, nutrient-rich bottom water into the bay following upwelling on the shelf (Monteiro and Largier, 1999). Conversely, local winds cause vertical mixing that entrains nutrient-rich bottom waters into the surface layer and redistributes phytoplankton populations within the water column (Monteiro and Largier, 1999; Pitcher and Calder, 1998; Pitcher et al., 2015). Combined, these

Table 4
Day-specific estimates of cellular DA (cDA) calculated directly from pDA and *Pseudo-nitzschia* cell abundances (column 1) versus application of a linear regression to pDA as a function of toxigenic cell abundance (column 2; – negative correlation).

DATE	(1) Mean cDA (pg cell^{-1})	(2) cDA (pg cell^{-1})
17 Jan 2012	1.162	1.773; $p = 0.150$; $r^2 = 0.946$
19 Jan 2012	1.272	0.647; $p = 0.403$; $r^2 = 0.650$
13 Mar 2012	0.015	0.001; $p = 0.803$; $r^2 = 0.093$
15 Mar 2012	0.040	0.003; $p = 0.458$; $r^2 = 0.567$
15 May 2012	1.419	–
17 May 2012	0.956	0.547; $p = 0.216$; $r^2 = 0.889$
02 Jul 2012	0.024	0.135; $p = 0.343$; $r^2 = 0.736$
04 Jul 2012	0.315	0.092; $p = 0.476$; $r^2 = 0.537$
11 Sep 2012	0.039	0.008; $p = 0.599$; $r^2 = 0.347$
13 Sep 2012	0.124	–
20 Nov 2012	0.111	0.068; $p = 0.009$; $r^2 = 0.999$
19 Jan 2013	1.660	1.182; $p = 0.080$; $r^2 = 0.984$
21 Jan 2013	0.407	1.768; $p = 0.147$; $r^2 = 0.948$
23 Jan 2013	0.409	0.176; $p = 0.332$; $r^2 = 0.752$
27 Feb 2013	0.093	0.118; $p = 0.114$; $r^2 = 0.784$
04 Apr 2013	0.037	–
08 May 2013	0.026	0.013; $p = 0.055$; $r^2 = 0.893$
06 Jun 2013	0.002	0.005; $p = 0.750$; $r^2 = 0.063$
10 Jul 2013	0.007	–
14 Aug 2013	2.118	1.135; $p = 0.515$; $r^2 = 0.236$
17 Sep 2013	0.180	0.025; $p = 0.977$; $r^2 = 0.000$
09 Oct 2013	0.005	0.012; $p = 0.345$; $r^2 = 0.429$
06 Nov 2013	0.752	0.211; $p = 0.923$; $r^2 = 0.006$
04 Dec 2013	1.483	5.573; $p = 0.260$; $r^2 = 0.548$
08 Jan 2014	0.098	0.068; $p = 0.230$; $r^2 = 0.593$
12 Feb 2014	0.315	0.406; $p = 0.006$; $r^2 = 0.988$
19 Mar 2014	0.083	0.017; $p = 0.385$; $r^2 = 0.378$
24 Apr 2014	0.256	0.513; $p = 0.219$; $r^2 = 0.610$
13 May 2014	7.891	–
25 Jun 2014	0.055	–
23Jul 2014	0.162	–
05 Sep 2014	0.021	–
14 Oct 2014	2.618	4.217; $p = 0.516$; $r^2 = 0.234$
18 Nov 2014	0.508	–
11 Dec 2014	0.101	–
13 Jan 2014	0.025	–

processes result in high bay productivity that manifests in high Chl *a* levels that also exhibit considerable variability at the event scale (Fig. 1c, d). Mean Chl *a* levels were lowest in winter and highest in autumn, although only marginally higher than the mean values of spring and summer (Table 2). The mean Chl *a* levels of 9.52 mg m^{-3} (SD 8.59) and 12.97 mg m^{-3} (SD 17.40) as measured during phases 1 and 2 of this study are similar to the value of 12.1 mg m^{-3} (SD 10.15) reported by Smith and Pitcher (2015) for a similar time-series from the same locality in 2010–2012.

Pseudo-nitzschia species contributed significantly to phytoplankton abundance in Saldanha Bay at various times of the year. Although consistently low in winter, elevated *Pseudo-nitzschia* cell concentrations were regularly observed at other times of the year, but specifically in spring and summer. *Pseudo-nitzschia* was particularly prevalent during November and December 2012 at which time Chl *a* exceeded 100 mg m^{-3}. The bloom clearly corresponded to a period of cooling of both surface and bottom waters, indicative of coastal upwelling and local mixing leading to nutrient enrichment of the water column. The dominance of *Pseudo-nitzschia* at this time of the year, when upwelling is strongest, fits the ecological profile of *Pseudo-nitzschia* occurring during periods of high turbulence and nutrients (Fawcett et al., 2007; Trainer et al., 2012). The ability of *Pseudo-nitzschia* to utilize high NO_3^- concentrations during periods of upwelling has been confirmed by regional studies of nutrient uptake kinetics (Seeyave et al., 2009). Conversely, these studies also showed the ability of *Pseudo-nitzschia* to switch to NH_4^+ as its main source of nitrogen during periods of biomass accumulation and rapid depletion of NO_3^- following periods of upwelling (Seeyave et al., 2009). These findings are in agreement with those on the west coast of the USA in that elevated *Pseudo-nitzschia* cell abundances co-occur with upwelling conditions in many of the known bloom "hot spots" (Smith et al., 2017; 2018). Furthermore, monthly averages and maxima of DA in shellfish tissue along the entire coast of California over the past 15 years indicate that DA occurs in all seasons, but the highest averages and maximal tissue contents tend to occur in spring and the lowest values in winter (Smith et al., 2018).

Saldanha Bay is characterised by the development of a sometimes intense and sharply defined subsurface Chl *a* maximum, particularly during periods of enhanced stratification (Pitcher and Calder, 1998; Pitcher et al., 2015). Observations during this current study showed *Pseudo-nitzschia* to be associated with these biomass maxima. Even during spring average *Pseudo-nitzschia* cell concentrations were highest at 6 m depth and the vertical gradients of both *Pseudo-nitzschia* and DA were shown to intensify in summer and autumn with increasingly stratified conditions resulting in a subsurface DA maximum at 6 m depth. The association of *Pseudo-nitzschia* populations with subsurface chlorophyll maxima and thin layers has been documented on the California coast and elsewhere (e.g., Timmerman et al., 2014). Relaxation of upwelling is likely to be associated with population descent into the subsurface layer in the region of the thermocline and associated nitracline, as is observed in Monterey Bay (Bowers et al., 2018). In these subsurface populations high levels of DA are associated with nutrient limitation, mostly by macronutrients, including silicate and phosphate (Smith et al., 2018; Timmerman et al., 2014). Therefore, the conditions related to increases in *Pseudo-nitzschia* cell abundance can differ from the conditions related to DA production. Entrainment of subsurface layers of *Pseudo-nitzschia* into surface waters and the simultaneous enrichment of surface waters following mixing induced by local winds is likely to provide a mechanism to sustain blooms.

4.2. *Pseudo-nitzschia diversity and pDA*

Understanding the ecological dynamics of *Pseudo-nitzschia* blooms and the associated production of DA by these blooms has generally been restricted by the lack of species resolution. This is specifically true for the southern Benguela where *P. australis* is currently the only species identified within the upwelling system (Marangoni et al., 2001). The first studies to demonstrate the production of DA by *Pseudo-nitzschia* blooms in the southern Benguela (Fawcett et al., 2007; Hubbart et al., 2012) did not attempt to identify the causative species. Both studies nevertheless showed time series data depicting a close correspondence between pDA and *Pseudo-nitzschia* abundance. The first time-series (Fawcett et al., 2007) from 7 to 23 March 2006 showed daily *Pseudo-nitzschia* concentrations ranging from 0.31–15.67 × 10^6 cells l^{-1}, pDA from 0.47–2.99 μg l^{-1} and estimates of cDA from 0.10–1.29 pg DA cell^{-1}. The later time-series (Hubbart et al., 2012) obtained from the same locality from 20 March – 11 April 2007 showed daily *Pseudo-nitzschia* concentrations ranging from 0 to 1.23 × 10^6 cells l^{-1}, pDA from 0.0–0.46 μg l^{-1} and estimates of cDA from 0.0–0.72 pg DA cell^{-1}. Both studies are assumed to have sampled discrete *Pseudo-nitzschia* blooms and positive correlations were observed between *Pseudo-nitzschia* cell abundance and pDA. The application of a linear regression to pDA as a function of cell abundance provided similar estimates of cDA for each study (0.17 pg DA cell^{-1}, r^2=0.66, p=<0.0001, Fawcett et al., 2007; 0.21 pg DA cell^{-1}, r^2=0.63, p=<0.0001, Hubbart et al., 2012).

Although the plots of all pDA versus *Pseudo-nitzschia* data show cell abundance to be a poor predictor of particulate toxin (Fig. 4a, b), closer inspection reveals that the relationship between *Pseudo-nitzschia* abundance and pDA tends to be specific for each field study. These varying relationships likely reflect changes in the dominant *Pseudo-nitzschia* species present during any particular field study, as well as the suite of environmental factors influencing toxin production at that time. Unfortunately, as in the current study presented herein, calculations of cDA are compromised by the fact that the composition of toxic versus non-toxic *Pseudo-nitzschia* species and/or variation among intraspecific

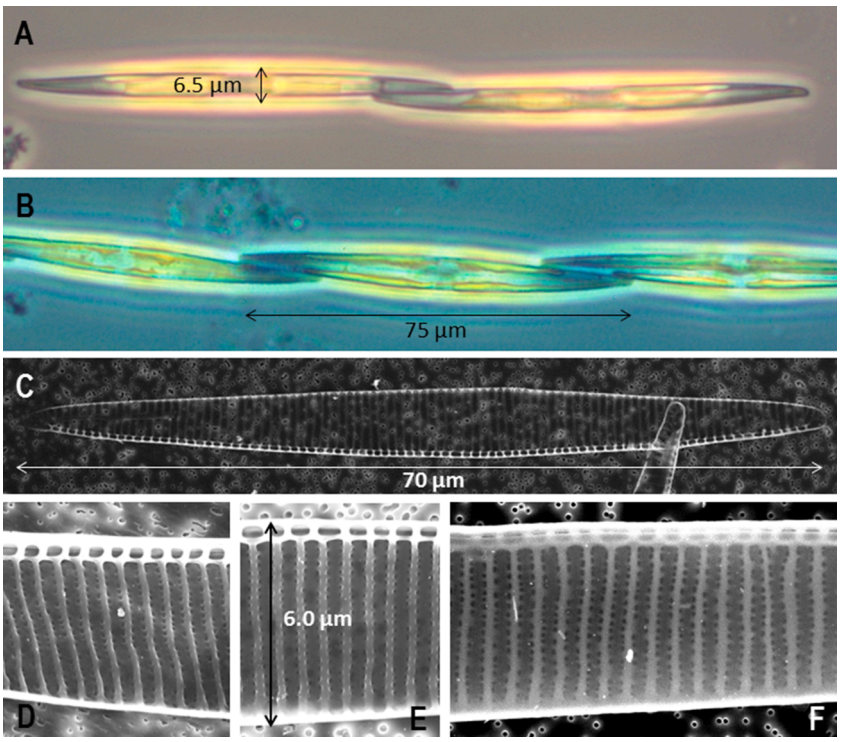

Fig. 5. Light micrographs in girdle view of a *Pseudo-nitzschia australis* cell chain from culture showing cells attached by their overlapping apices (A,B); scanning electron micrographs of a whole valve (C) and the central regions of valves showing the poroid striae and fibulae (D,E,F).

genetic variants in any particular sample is not usually known. Calculations of cDA, which ranged from 0.001 - 7.89 pg cell^{-1} in this study, are likely to be underestimates of maximum potential toxigenicity. In any case, all estimates >1 pg DA cell^{-1} were obtained from samples dominated by cells of the *P. seriata* complex suggesting that they were the primary proximal source of the toxin.

The data reported from Saldanha Bay as part of this study span a much longer period than either previous studies (Fawcett et al., 2007; Hubbart et al., 2012). This current study is therefore assumed to have sampled multiple *Pseudo-nitzschia* blooms under wide-ranging environmental conditions. Consequently the strength of the relationship between *Pseudo-nitzschia* cell abundance and pDA, reflecting bloom toxin content, varied greatly (Fig. 4; Table 4). Even for sampling events that showed strong correlations, the absolute value of the relationship between *Pseudo-nitzschia* abundance and pDA between these sampling events varied considerably (e.g., 1.77 pg DA cell^{-1}, r^2=0.95, $p = 0.15$ on 17 January 2012; 0.07 pg DA cell^{-1}, r^2=0.99, $p = 0.009$ on 20 November 2012).

This variability in cellular DA content is considered the result of a combined influence of species and strain composition of the *Pseudo-nitzschia* assemblage and the effect of environmental conditions on toxin production and release from cells. Other observations of correlates *of Pseudo-nitzschia* species composition and toxin content have been made, e.g., on the San Pedro Shelf region of the Southern California Bight (Smith et al., 2018) and in the northern Gulf of Mexico (MacIntyre et al., 2011). These studies demonstrate the need to understand not only the conditions that favour *Pseudo-nitzschia* blooms but more specifically those that control assemblage composition and further promote toxin production. Environmental cues controlling bloom formation and toxin production are complex and involve a suite of physical, chemical and biological factors. Multiple environmental stressors may stimulate toxin production and cDA may therefore vary by several orders of magnitude both among and within toxigenic species. Other factors influencing toxin production include the presence of herbivorous copepods that have been shown to induce DA production in *Pseudo-nitzschia* (Hardardottir et al., 2019). Finally, it must be acknowledged that determination of pDA and subsequent calculations of cDA are typically based upon filtered and/or centrifuged cells, whereby leakage and excretion of DA from intact or broken cells are not taken into account.

The present study assigned *Pseudo-nitzschia* cells, based on their frustule width, to either the *P. seriata* or *P. delicatissima* complexes. From these data it is clearly evident that elevated pDA was associated with cells of the *P. seriata* complex thereby implicating species of this complex as the likely primary source of DA. Similarly, documented DA events during blooms of *Pseudo-nitzschia* species in the southern California region have been attributed to members of the *P. seriata* size class (Seubert et al., 2013). One such species, dominant in the plankton of Saldanha Bay on 20 November 2012, was identified as *P. australis* following successful isolation and culture of the species. Toxin production by *P. australis* in Saldanha Bay was confirmed by analysis of these cultures for DA. Strain-specificity in DA production as observed in these cultures is well documented (Bates et al., 2018) and serves to demonstrate the role of different strains in determining bloom toxin content. The identification of *P. australis* as a bloom-forming, toxigenic species in Saldanha Bay is in line with findings in other eastern boundary upwelling systems where *P. australis* has been identified as a primary source of DA and is regularly implicated in toxic events (e.g., California Current upwelling system, Bowers et al., 2018; Smith et al., 2018; Trainer et al., 2012; Iberian–Canary Current upwelling system, Torres Palenzuela et al., 2019; and the upwelling system of the Humboldt Current, Álvarez et al., 2009). Similarly, *P. australis* has been shown to be a dominant species in the northern Benguela, specifically inshore, where it has also been shown to produce DA (Gai et al., 2018; Louw et al., 2018).

4.3. *Pseudo-nitzschia bloom impacts*

Pseudo-nitzschia species are found in all oceans of the world and bivalve shellfish remain the most likely vector to cause ASP in humans. Therefore, traditional food products such as mussels, oysters, scallops and clams are the primary source of DA contamination. However,

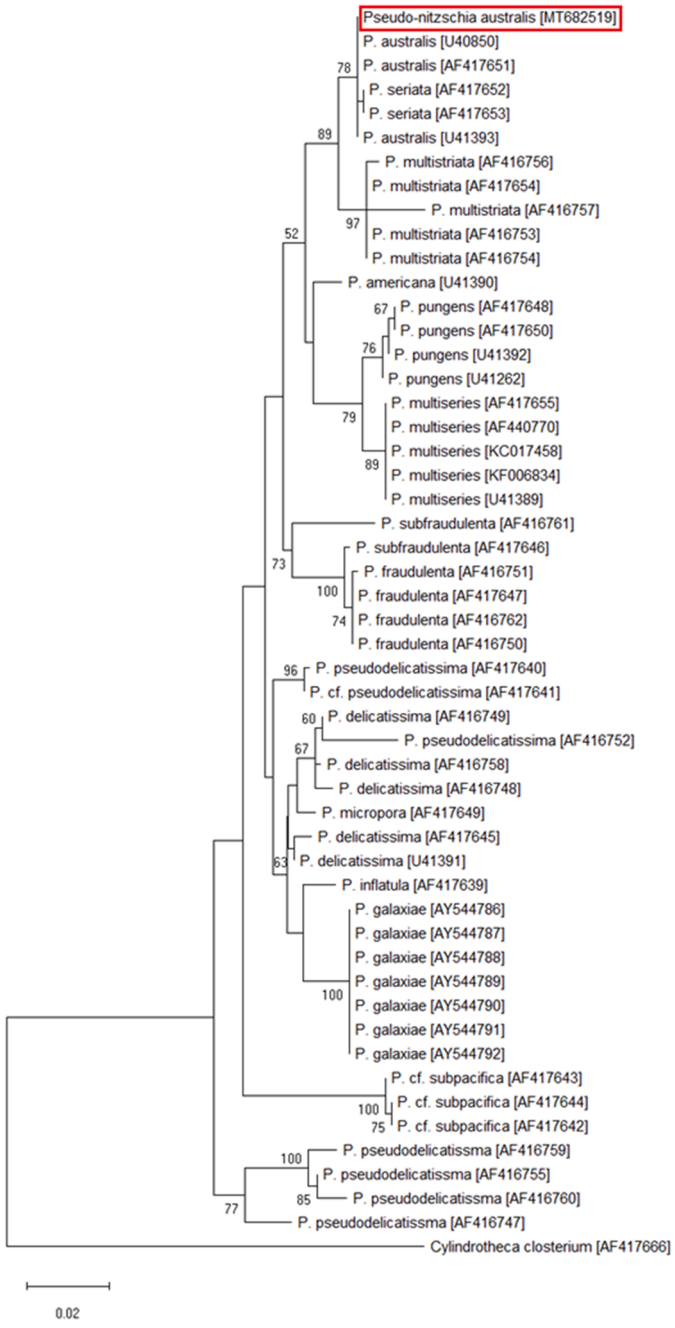

Fig. 6. Maximum-likelihood tree inferred from the D1-D3 hypervariable domains of the LSU rDNA of a cultured *Pseudo-nitzschia* isolate from Saldanha Bay, South Africa, 50 taxa in the genus *Pseudo-nitzschia* and one outgroup species (*Cylindrotheca closterium*). Numbers next to the specific names correspond to the accession numbers for the LSU rDNA sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. There were a total of 809 positions in the final dataset. The bar indicates 2 base substitutions per 100 nucleotides.

trophic transfer of DA has been shown to be extensive with reports of DA in fish species, in many species of mammals and also in birds; all indicative of the pervasive spreading of the toxin as it enters the food-web and impacts ecosystems (Bates et al., 2018). In eastern boundary upwelling systems the impacts of *Pseudo-nitzschia* and its toxin have been particularly evident in the California Current system (Trainer et al., 2012), none more so than during 2015–16 with the largest recorded outbreak of the neurotoxin along the North American west coast attributed to an unprecedented bloom of *P. australis* (McCabe et al.,

Table 5
Strains used for the large-subunit (LSU) rDNA phylogenetic analysis.

Taxon	Clone Designation	Collection location	Reference	GenBank Accession Number
Pseudo-nitzschia australis	SB	Saldanha Bay, South Africa	This study	MT682519
P. australis	ØM1	Aveiro, Portugal	Lundholm et al., 2002	AF417651
P. australis	CV17	Santa Cruz, California	Scholin et al., 1994	U40850
P. australis	CV18	Monterey Bay, California	Scholin et al., 1994	U41393
P. seriata	Nissum 3	Nissum Bredning, Denmark	Lundholm et al., 2002	AF417652
P. seriata	Lynaes 8	Lynaes, Isefjord, Denmark	Lundholm et al., 2002	AF417653
P. multistriata	SZN-B29	Gulf of Naples, Italy	Orsini et al., 2002	AF416754
P. multistriata	SZN-B32	Gulf of Naples, Italy	Orsini et al., 2002	AF416757
P. multistriata	SZN-B27	Gulf of Naples, Italy	Orsini et al., 2002	AF416753
P. multistriata	SZN-B31	Gulf of Naples, Italy	Orsini et al., 2002	AF416756
P. multistriata	SZN-B29	Gulf of Naples, Italy	Orsini et al., 2002	AF417654
P. americana	CV2	Santa Cruz, California	Scholin et al., 1994	U41390
P. pungens	P-24	Costa Nova, Portugal	Lundholm et al., 2002	AF417648
P. pungens	KBH2	Khan Hoa Bay, Vietnam	Lundholm et al., 2002	AF417650
P. pungens	CV5	Santa Cruz, California	Scholin et al., 1994	U41392
P. pungens	CV4	Monterey Bay, California	Scholin et al., 1994	U41262
P. multiseries	CLN-20	Gulf of Maine, North-western Atlantic	Fernandes et al., 2014	KF006834
P. multiseries	NWFSC-005	Sequim Bay, Washington State Island	Stehr et al., 2002	AF440770
P. multiseries	CV19	Santa Cruz, California	Scholin et al., 1994	U41389
P. multiseries	COOG	Coogee Beach, NSW	Ajani et al., 2013	KC017458
P. multiseries	OFPm984	Ofunato Bay, Japan	Lundholm et al., 2002	AF417655
P. galaxiae	SZN-B58	Gulf of Naples, Italy	Cerino et al., 2005	AY544788
P. galaxiae	SZN-B57	Gulf of Naples, Italy	Cerino et al., 2005	AY544789
P. galaxiae	SZN-P1	Gulf of Naples, Italy	Cerino et al., 2005	AY544787
P. galaxiae	SZN-P5	Gulf of Naples, Italy	Cerino et al., 2005	AY544792
P. galaxiae	SZN-B54	Gulf of Naples, Italy	Cerino et al., 2005	AY544786
P. galaxiae	SZN-B56	Gulf of Naples, Italy	Cerino et al., 2005	AY544790
P. galaxiae	SZN-B55	Gulf of Naples, Italy	Cerino et al., 2005	AY544791
P. inflatula	No7	Phuket, Thailand	Lundholm et al., 2002	AF417639
P. delicatissima	1001 2b	Kattegat, Denmark	Lundholm et al., 2002	AF417645
P. delicatissima	CV3	Santa Cruz, California	Scholin et al., 1994	U41391

(*continued on next page*)

Table 5 (*continued*)

Taxon	Clone Designation	Collection location	Reference	GenBank Accession Number
P. delicatissima	SZN-B19	Gulf of Naples, Italy	Orsini et al., 2002	AF416749
P. delicatissima	SZN-B33	Gulf of Naples, Italy	Orsini et al., 2002	AF416758
P. delicatissima	SZN-B18	Gulf of Naples, Italy	Orsini et al., 2002	AF416748
P. pseudodelicatissima	SZN-B26	Gulf of Naples, Italy	Orsini et al., 2002	AF416752
P. pseudodelicatissima	P-11	Gafahna, Portugal	Lundholm et al., 2002	AF417640
P. cf. *pseudodelicatissima*	Hobart5	Hobart, Tasmania, Australia	Lundholm et al., 2002	AF417641
P. pseudodelicatissima	SZN-B35	Gulf of Naples, Italy	Orsini et al., 2002	AF416760
P. pseudodelicatissima	SZN-B30	Gulf of Naples, Italy	Orsini et al., 2002	AF416755
P. pseudodelicatissima	SZN-B34	Gulf of Naples, Italy	Orsini et al., 2002	AF416759
P. pseudodelicatissima	SZN-B17	Gulf of Naples, Italy	Orsini et al., 2002	AF416747
P. micropora	VPB-B3	Van Phong Bay, Vietnam	Lundholm et al., 2002	AF417649
P. fraudulenta	Limens1	Limens, Spain	Lundholm et al., 2002	AF417647
P. fraudulenta	SZN-B21	Gulf of Naples, Italy	Orsini et al., 2002	AF416750
P. fraudulenta	SZN-B22	Gulf of Naples, Italy	Orsini et al., 2002	AF416751
P. fraudulenta	SZN-B40	Gulf of Naples, Italy	Orsini et al., 2002	AF416762
P. subfraudulenta	rensubfrau	Chinhae Bay, Korea	Lundholm et al., 2002	AF417646
P. subfraudulenta	SZN-B39	Gulf of Naples, Italy	Orsini et al., 2002	AF416761
P. cf. *subpacifica*	RdA8	Ria de Arosa, Spain	Lundholm et al., 2002	AF417642
P. cf. *subpacifica*	P28	Costa Nova, Portugal	Lundholm et al., 2002	AF417643
P. cf. *subpacifica*	Zhenbo7B	Port Shelter, Hong Kong	Lundholm et al., 2002	AF417644
Cylindrotheca closterium	K-520	Kattegat, Denmark	Lundholm et al., 2002	AF417666

Table 6
Culture DA toxin content expressed as pDA and cDA as determined by ELISA and LC/MS-MS.

P. australis	Culture 1 (7.56×10^4 cells ml^{-1})		Culture 2 (1.10×10^5 cells ml^{-1})	
	pDA	cDA	pDA	cDA
ELISA	169.15 μg l^{-1}	2.24 pg cell^{-1}	0.92 μg l^{-1}	0.009 pg cell^{-1}
LC-MS/MS	188.26 μgl^{-1}	2.49 pg cell^{-1}	1.16 μg l^{-1}	0.011 pg cell^{-1}

2016). During this event elevated toxins were measured in numerous stranded marine mammals and were also the cause of geographically extensive and prolonged closures of razor clam, rock crab, and Dungeness crab fisheries.

Observations in the southern Benguela upwelling system are somewhat contrary to those in the California Current system in that DA has yet to be associated with any harmful impact despite the known occurrence of toxin-producing *Pseudo-nitzschia* blooms within the region (Fawcett et al., 2007; Hubbart et al., 2012).

The low risk posed by *Pseudo-nitzschia* to seafood safety and to the aquaculture sector of the region is again evident in the findings of this study of the low DA contamination of farmed mussels in Saldanha Bay. Confirmation of the low risk posed by *Pseudo-nitzschia* in Saldanha Bay is provided in the records of the *South African Molluscan Shellfish Monitoring and Control Programme,* in that monthly sampling of shellfish in the bay since 2005 has failed to register a single DA level above the regulatory limit of 20 mg DA kg^{-1} shellfish.

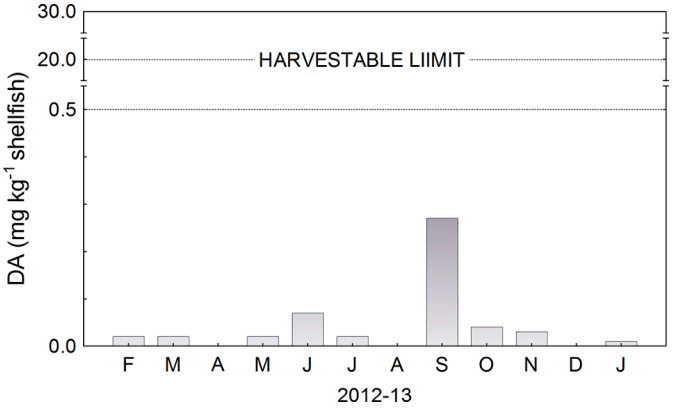

Fig. 7. DA levels as determined by a quantitative ELISA in the cultured mussel *Mytilus galloprovincialis* sampled monthly from Saldanha Bay from February 2012 – January 2013.

The detection by an ELISA of only traces of DA in Saldanha Bay mussels was not entirely unforeseen. Previously Hubbart et al. (2012) were unable to detect DA in shellfish off Lambert's Bay, using LC–MS/MS, during a toxin-producing bloom of *Pseudo-nitzschia.* Similar observations have been reported elsewhere. For example, Torres Palenzuela et al. (2018) reported pDA up to 2.51 μg l^{-1} in 2007 and up to 1.18 μg l^{-1} in 2009 in the Rias de Vigo and Pontevedra, in Galicia, Spain, while DA levels in mussels remained below regulatory levels. The accumulation of toxins in bivalves is dependent on the concentration, the time of exposure and the toxicity of cells in suspension, and on the balance between the mechanisms regulating toxin uptake and elimination in various tissue compartments. The presence in Saldanha Bay of high concentrations (>1 × 10^6 cells l^{-1}) of toxic *Pseudo-nitzschia* for extended periods would suggest that the low toxin levels in shellfish were a function of low cellular toxicity.

Levels of cDA in *Pseudo-nitzschia* are known to vary widely. The maximum cDA recorded in Saldanha Bay of 5.0 pg cell^{-1}, and the maxima of 1.29 and 0.72 pg cell^{-1} reported by Fawcett et al. (2007) and Hubbart et al. (2012) from Lambert's Bay are well below maxima reported elsewhere. Examples, from the California Current system, of significantly higher estimates of cDA during blooms of *P. australis,* include reported maxima of 75 pg cell^{-1} in Monterey Bay in 1998 (Scholin et al., 2000), 78 pg cell^{-1} in Morro Bay on the Central Californian coast in 1998 (Trainer et al., 2000), 43 pg cell^{-1} off San Diego in Southern California in 2004 (Busse et al., 2006), and of 117 pg cell^{-1} in the Southern California Bight in 2004 (Schnetzer et al., 2007). More specifically, during the ecologically and economically disruptive *Pseudo-nitzschia* bloom affecting much of the northeast Pacific margin in 2015–16, cDA in Monterey Bay was regularly found to exceed 100 pg cell^{-1} (Ryan et al., 2017).

Several environmental factors have been shown to be important in the initiation of DA production by *Pseudo-nitzschia* species none more so than limiting macronutrients. Silicate limitation in particular has emerged as an important factor inducing DA production in several studies (Lelong et al., 2012). Many of these studies have been laboratory based; but more recently the role of nutrient ratios in determining bloom toxicity has been clearly demonstrated in the field. As an example, a study of the population dynamics of different *Pseudo-nitzschia* species, along with pDA, in the Bay of Seine (2012–13) showed that high pDA coincided with the presence of *P. australis* and with silicate limitation (Si:N <1), while nitrate concentrations were still replete (Thorel et al., 2017). Similarly, the high toxicity of the *Pseudo-nitzschia* bloom off the North American west coast in 2015–16 was attributed to anomalous

nutrient stoichiometry with severe depletion of silicate relative to nitrate (Ryan et al., 2017).

Understanding the observations of low cellular toxicity made during this study and in previous studies in the southern Benguela may therefore require consideration of nutrient conditions. In the southern Benguela, source water silicate concentrations range from 5 to 15 mmol m^{-3} (Chapman and Shannon, 1985) and waters upwelling onto the shelf have a silicate to nitrate ratio of ~1 (Andrews and Hutchings, 1980; Olivieri, 1983). However, in areas where the shelf tends to broaden silicate concentrations are greatly enhanced after passage across the shelf. Consequently, in areas such as St Helena Bay (located adjacent to Saldanha Bay and incorporating Lambert's Bay) silicate concentrations often exceed 50 mmol m^{-3} and the silicate to nitrate ratio is >>1 (Bailey and Chapman, 1991; Pitcher and Probyn, 2017). Low cellular toxicity in *Pseudo-nitzschia* blooms in Saldanha Bay may therefore be due to the fact that silicate is seldom limiting. The apparent low risk posed by *Pseudo-nitzschia* blooms to the aquaculture sector of Saldanha Bay may be at least partially attributed to the nutrient content and ratios of upwelling waters in the region.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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