Version of Record: https://www.sciencedirect.com/science/article/pii/S1568988318300581
Manuscript_852d3293262e74c80290f84eb7a900a8

Morphology and toxicity of *Pseudo-nitzschia* species in the northern Benguela Upwelling System.

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Abstract

The Benguela upwelling system, considered the world's most productive marine ecosystem, has a long record of potentially toxic diatoms belonging to the genus *Pseudo-nitzschia*. Species of *Pseudo-nitzschia* were reported as early as 1936 from the northern Benguela upwelling system (nBUS). For the current study, long-term phytoplankton monitoring data (2004-2011) for the Namibian coast were analysed to examine inshore and offshore temporal distribution of *Pseudo-nitzschia* species, their diversity and ultrastructure. The potentially toxigenic *P. pungens* and *P. australis* were the dominant inshore species, whereas offshore *Pseudo-nitzschia* showed a higher diversity that also included potentially toxic species. During a warming event, a community shift from *P. pungens* and *P. australis* dominance to *P. fraudulenta* and *P. multiseries* was documented in the central nBUS.

A case study of a toxic event (August 2004) revealed that *P. australis* and *P. pungens* were present at multiple inshore and offshore stations, coincident with fish (pilchard) and bird mortalities reported from the central part of Namibia. Toxin analyses (LC-MS/MS) of samples collected from June to August 2004 revealed the presence of particulate domoic acid (DA) in seawater at multiple stations (maximum ~180 ng DA/L) in the > 0.45 µm size-fraction, as well as detectable DA (0.12 µg DA/g) in the gut of one of two pilchard samples tested. These findings indicate that DA may have been associated with the fish and bird mortalities reported from this event in the nBUS. However, the co-occurrence of very high biomass phytoplankton blooms suggests that other explanations may be possible.

Key words

35 *Pseudo-nitzschia* species, northern Benguela upwelling system, amnesic shellfish
36 poisoning, domoic acid, toxicity, fish mortality, bird mortality

37

1. Introduction

Many potentially toxic phytoplankton species have been reported in the northern Benguela upwelling system (nBUS), both historically (Kollmer, 1963; Pieterse and Van der Post, 1967; Kruger, 1980; Vavilova, 1990) and more recently (Hansen et al., 2014; Louw et al., 2017). In the nBUS, *Pseudo-nitzschia* spp. are among the taxa reported regularly; nevertheless, the species have never been identified nor has the potential risk for toxic blooms been evaluated.

The *Pseudo-nitzschia* species is a marine diatom genus with which are distributed throughout the world's oceans (Trainer et al. 2012). The distribution and growth of *Pseudo-nitzschia* species can be influenced by various environmental factors, including salinity (Thessen et al., 2005), nutrient concentrations (Caroppo et al., 2005, Louw et al., 2017), pH (Lundholm et al., 2004), photoperiod (Fehling et al., 2005, 2006) and wind (Louw et al., 2017). In addition, coastal upwelling events, mesoscale eddies and anthropogenic inputs of nutrients from river discharge and land runoff have been implicated as possible causes for *Pseudo-nitzschia* blooms (Parsons et al., 2002; Kudela et al., 2005; Anderson et al., 2008; Trainer et al., 2009).

Presently, 49 *Pseudo-nitzschia* species are known, of which 26 have been demonstrated to produce the potent neurotoxin, domoic acid (DA) (Lundholm, 2017). Domoic acid is responsible for outbreaks of amnesic shellfish poisoning (ASP) in humans and DA poisoning (DAP) in sea birds, marine mammals and other wildlife (Trainer et al., 2012). This DA poisoning has resulted in large-scale mortality in marine animal populations including sea lions and seabirds (Bejarano et al. 2008, McCabe et al., 2016, Nash et al., 2017). Harvesting closures in certain regions as a consequence of DA contamination

have caused significant economic losses. Laboratory and field studies have demonstrated that depletion of silicate and/or phosphate (Pan et al., 1996a,b; Fehling, 2004), nutrient ratios (e.g. low Si:N ratios; Ryan et al., 2017) (Schnetzer et al., 2007, Heisler et al., 2008), the form of available nitrogen (Howard et al., 2007; Cochlan et al., 2008; Thessen et al., 2009), trace metal concentrations (iron deficiency and copper surplus) (Rue and Bruland, 2001; Wells et al., 2005; Silver et al., 2010), bacterial associations (Bates, 1998; Kaczmarska et al., 2005; Sison-Mangus et al., 2016), pH and partial CO_2 concentration (Lundholm et al., 2004, Tatters et al., 2012) and presence of grazers (Harðardóttir et al., 2015, Tammilehto et al., 2015) may affect toxin production.

The primary aims of this study were to provide a detailed account of the diversity of *Pseudo-nitzschia* species in the nBUS and evaluate the relationship between their abundance and the occurrence of bloom events, using a long-term phytoplankton time series database. Species morphology, abundance and diversity over a long time series are discussed, with special emphasis on toxicity during a 2004 bloom event that was associated with marine wildlife mortalities. Findings are considered in the context of suggesting improvements to Namibia's existing phytoplankton monitoring programme.

2. Materials and methods

2.1. Study area

Along the Namibian coast on the southwestern part of Africa, the northwards flowing cold, nutrient rich upwelling system is the driving force for this productive marine ecosystem. The large Benguela current marine ecosystem includes the coasts of South Africa, Namibia and southern Angola. The southern Benguela upwelling system (sBUS) and the

northern Benguela upwelling system (nBUS) are divided by the strong Lüderitz upwelling cell, driven by south-easterly winds (Shillington, 1998). The study area comprises a 23˚S transect in the nBUS, situated on the downside of the Lüderitz upwelling cell (Fig. 1). This transect covers the entire shelf, from the coastline to the shelf edge with sampling stations 2, 5, 10, 20, 30, 40, 50, 60 and 70 nautical miles (nm) off the coast, and although it has been sampled regularly since 2001 (Louw et al., 2016, 2017), only samples from 2004 and later, which included transmission electron microscopy (TEM) examination, were considered for the current analysis. Stations with high *Pseudo-nitzschia* spp. concentrations were chosen for this study, beginning with samples collected in 2004 when a fish mortality event was recorded. In addition, stations with high *Pseudo-nitzschia* spp. abundance from other transects (24°S, 22°S, 21°S, 20°S 19°S and 18°S) covering the entire nBUS (Fig. 1) were sampled during this 2004 fish mortality event. Station numbering includes information on latitude and offshore distance in nautical miles (e.g. 23010 represents a station at 23°S latitude and 10 nm from shore).

2.2. Sampling, sample preparation and measurements

2.2.1. Chlorophyll-*a*

Seawater for chlorophyll-a analyses was obtained at multiple depths using 5 L Niskin bottles mounted on a standard rosette sampler. For the purpose of this paper, only results obtained for surface samples (0-2 m) were considered. A subsample of seawater (200 mL) was filtered through a 25 mm diameter Whatman GF/F filter and filters were kept at -20 °C until transferred to the laboratory at the National Marine Information and Research Centre (NatMIRC) in Swakopmund, Namibia. In the laboratory, chlorophyll-a was extracted into 90% acetone over a 24 h period. Samples were analysed with a Turner

106 Designs Model 10 Fluorometer (San Jose, CA, USA) previously blanked with 90%
107 acetone. The fluorometer was calibrated with a chlorophyll-a reference standard (Sigma-
108 Aldrich, USA) following the protocol of Welschmeyer (1994). Final chlorophyll-a
109 concentrations were given as mg m^{-3} (additional details in Louw et al. (2016)).

110 **2.2.2. *Pseudo-nitzschia* species counting**

111 Net samples for qualitative analyses were collected with a 20 µm-mesh phytoplankton
112 net lowered to 30 meters and hauled to the surface. Samples were pre-examined under
113 a compound microscope for *Pseudo-nitzschia* spp. and fixed with 40% formaldehyde
114 (Throndsen, 1978). At the same time, water samples for quantitative analyses were
115 collected with Niskin bottles and a subsample of ~200 mL was preserved for cell counts
116 by adding 5 mL of 40% formaldehyde (Throndsen, 1978). Cells were counted with a Zeiss
117 Axiovert 200 inverted light microscope using the Utermöhl method (Utermöhl, 1958). Prior
118 to counting, the formalin-preserved samples were settled in a 25 mL chamber for 24 h.
119 The concentrations of *Pseudo-nitzschia* spp., as well as other taxa, were calculated using
120 the equation given by Utermöhl (1958).

121 ***2.2.3. Pseudo-nitzschia species identification***

122 Phytoplankton samples collected from 2004-2011 during different annual period's (i.e.
123 austral summer/autumn and upwelling period (August-November)) with a high abundance
124 of *Pseudo-nitzschia* spp. were selected for further analyses using differential interference
125 contrast (DIC) microscopy and electron microscopy (EM) to identify the taxa present.
126 Samples were cleaned of organic material following the protocol of Lundholm et al.
127 (2002). For TEM, drops of the cleaned material were placed on Formvar-coated copper

grids, dried, and examined with a JEOL JEM-1010 electron microscope. Morphometric measurements were performed on TEM images. The valve width and the density of interstriae, fibulae and poroids were measured in the middle of the valve. In addition, the following characteristics were determined for identification to species level: number of rows of poroids in each stria, the pattern of hymen perforations of each poroid, the valve shape, and the shape of valve ends.

2.2.4. Toxins analyses

Water samples (500 mL) were collected onboard research vessels, filtered through a 0.45 µm GF/F filter and the filtrate stored at -20 °C until analysed for domoic acid (DA). Filters containing phytoplankton material were placed in 5 mL of 10% methanol, ground and sonicated to disrupt the filter and cells, filtered (0.45 µm) to remove particulates, and stored frozen (-20 °C) until analysed.

Dead washed-up fish (i.e. pilchard (*Sardinops sagax*), mullet (*Liza richardsonii)*) as well as live shellfish (clams) samples were collected in the vicinity of Swakopmund and Walvis Bay, respectively. Material was weighed and mixed with a known volume of 50% methanol (1:4 wt.:vol.), homogenized, centrifuged (3000 x *g*) and filtered through a GF/F filter (0.45 µm) to remove particulates and stored at -20 °C until analysed.

All sample extracts (phytoplankton, fish and shellfish) were analysed for the presence of DA by tandem mass spectrometry preceded by liquid chromatographic separation (LC-MS/MS) employing reversed phase chromatography, using an Agilent 1100 HPLC coupled to an ABI-SCIEX API-4000 triple quadrupole mass spectrometer in ESI+ mode. All reagents used for LC-MS/MS were analytical grade or higher. Chromatography was

150 performed on a Phenomenex Luna C18 column (5 µm, 150x2 mm). Mobile phase
151 consisted of water and acetonitrile in a binary system, with 0.1% formic acid as an
152 additive. The elution gradient was as follows: 2 min 95% water, linear gradient to 60%
153 water at 16 min, 95% water at 17 min, held for 5 min, then returned to initial conditions at
154 23 min and held for 5 min before the next injection. Positive identification was based on
155 presence of the DA parent ion (312 *m/z*) as well as its associated fragment ion peaks
156 (266 *m/z*, 161 *m/z*; Quilliam, 1996). Retention time and calibration for DA quantification
157 were based on the DACS-1C Certified Reference Material obtained from the Institute for
158 Marine Biosciences, NRC Canada (Halifax, NS, Canada). The limit of quantification
159 (LOQ; signal:noise (S:N) ratio >10) for this method was approximately 0.4 ng DA mL^{-1} in
160 the sample extract. The limit of detection (LOD; S:N ~5) was estimated at ~0.14 ng DA
161 mL^{-1}.

162 **3. Results**

163 Sporadic monitoring of nBus phytoplankton was initiated during the 1970s and more than
164 500 taxa have now been identified at inshore and offshore stations (Kruger, 1980;
165 NatMIRC unpubl. data). Regular sampling began in 2001 and extraction of *Pseudo-*
166 *nitzschia* spp. information from this database revealed regularly occurring *Pseudo-*
167 *nitzschia* bloom events, including some with extremely high cell concentrations and one
168 associated with a fish kill in 2004 (Louw et al., 2017). Moreover, *Pseudo-nitzschia* spp.
169 numerically dominated the phytoplankton community at certain stations, representing a
170 significant component of the food available for secondary producers and thus being an
171 integral and vital part of the regional food web.

3.1. Morphology of *Pseudo nitzschia* species in nBUS

Based on the morphological data, the following species were identified in the nBUS: *P. americana, P. australis, P.* cf*. dolorosa, P. fraudulenta, P.* cf*. heimii, P. kodamae, P.* cf*. kodamae, P. lineola, P. multiseries, P. pungens* and *P.* cf*. turgidula* (Table 1). In addition, a potential new species was observed and reported as *P.* sp. NamA (see section 3.2). The species diversity found during the July 2004 toxic event is discussed separately (see section 3.3).

Description of species

***Pseudo-nitzschia americana* (Hasle) Fryxell (Plate 1A and 1B)**

The observed morphology of *P. americana* was completely in agreement with the emended description (Plate 1A and 1B, Table 2) (Lundholm et al., 2002). This species was only reported from a single inshore sample at a low concentration (Fig. 2). The distribution of *P. americana* comprises temperate to tropical waters around the world including South Africa as well as NW Africa (Lundholm et al., 2002; Lelong et al., 2012; Teng et al. 2013; Bresnan et al., 2015).

***Pseudo-nitzschia australis* Frenguelli (Plate 1C and 1D)**

Morphologically, the cells were in agreement with previous descriptions (Table 2, Plate 1C and 1D) (Hasle, 1965). The species, *P. australis* occurred at several inshore and offshore nBUS stations, often comprising up to 96% of the *Pseudo-nitzschia* species present (Fig. 2). This globally distributed specie is found in temperate and sub-tropical regions (Hasle, 2002). It is a toxic specie known to produce high amounts of DA in the

laboratory as well as in the field (Fritz et al., 1992; Trainer et al., 2008; Lelong et al., 2012). It has been identified as the cause of toxic blooms in many parts of the world, including those associated with mammal mortalities off the US west coast (Trainer et al., 2012; Guiry and Guiry, 2017; McCabe et al., 2016; Nash et al., 2017) and it has also been reported from the sBUS (Marangoni et al., 2001).

***Pseudo-nitzschia* cf*. dolorosa* Lundholm et Moestrup (Plate 1E and 1F)**

Specimens looking similar to the original description of *P. dolorosa* (Lundholm et al., 2006) but differing with regard to valve width, and fibula and stria density were referred to *P.* cf. *dolorosa.* The valve width (2.9-3.5 µm) was greater than for *P. dolorosa* (reported up to 3.0 µm in Lundholm et al., 2006 and Teng et al., 2013). Similar to *P. dolorosa*, the observed specimens (Plate 1E and 1F) had lanceolate valves with a central nodule present. The densities of fibulae and striae were 16-20 and 28-30 in 10 µm, respectively, in the Namibian specimens and hence lower than in *P. dolorosa* (18-22 and 30-36, respectively in 10 µm). The poroid density was 3-8, which overlaps with the 5-8 poroids in 1 µm for *P. dolorosa* (Lundholm et al., 2006). This species was reported from inshore and offshore stations during 2006 and 2007 (Fig. 2).

***Pseudo-nitzschia fraudulenta* (Cleve) Hasle (Plate 2G & 2H)**

The morphological characteristics of *P. fraudulenta* (Plate 2G and 2H) in the nBUS agreed with the description of the species (Hasle, 1965). This taxon was detected regularly at inshore and offshore stations in low concentrations. In 2011, it was the dominant (96%) *Pseudo-nitzschia* species during a warm water intrusion bloom event in May (Fig. 2). The species *P. fraudulenta* is widely distributed in temperate and

subtropical waters, and strains have been found to produce DA (Lelong et al., 2012; Tatters et al., 2012).

***Pseudo-nitzschia* cf. *heimii* Manguin (Plate 2I)**

Specimens of *P.* cf. *heimii* were observed to have lanceolate valves and broadly rounded valve ends (Plate 2I). The valve length was 60-70 µm and the valve width 3-4 µm. A central nodule was present and the valve possessed 16-20 fibulae and 28-30 striae in 10 µm. The striae contained two rows of poroids, which had a density of 5-10 poroids in 1 µm. The observed specimens differed from *P. heimii* in having a narrower valve width and a higher density of striae compared to the description by Hasle and Syvertsen (1996) (5-6 µm width and 26-28 striae in 10 µm, respectively). The species were found in offshore stations in 2005 and 2006 (Fig. 2), and also observed by Guannel et al. (2015) offshore of the Namibian and southern Angolan coasts.

***Pseudo-nitzschia kodamae* S.T.Teng, H.C.Lim, C.P.Leaw and P.T.Lim (Plate 2J and 2K)**

The valve morphology of *P. kodamae* (Plate 2J and 2K) was in agreement with the original description (Teng et al., 2014). Other cells observed were morphologically similar, but had a valve width of ~1.7 µm (compared to 2.1-3.3 µm in *P. kodamae*) (Teng et al., 2014) and were therefore identified as *P.* cf. *kodamae*. Both were observed in low quantities at offshore stations (Fig. 2). Strains of *P. kodamae* from Malaysia have been found to produce DA (Teng et al., 2014). The present record is to our knowledge the first after the original description of strains from Malaysia.

***Pseudo-nitzschia lineola* (Cleve) Hasle (Plate 3L and 3M)**

All observations of *P. lineola* (Plate 3L and 3M) corresponded well with previous descriptions by Hasle and Syvertsen (1996) and Lundholm et al. (2012). *It* was observed on two occasions at offshore stations (Fig. 2). This species is globally distributed and has been recorded in both coastal and oceanic waters; it is presently reported to be non-toxic (Lundholm et al., 2012).

***Pseudo-nitzschia multiseries* (Hasle) Hasle (Plate 3N and 3O)**

The morphology of *P. multiseries* specimens agreed with previous descriptions (Hasle 1995) (Plate 3N and 3O; Table 1). The *P. multiseries* species appeared together with *P. pungens* during the nBUS warm water event in 2011 (Fig. 2). The *P. multiseries* is a globally distributed species known to produce high amounts of toxin and it has been involved in highly toxic bloom events (e.g. Bates et al., 1989). It has been reported previously from South Africa (Pitcher et al., 2014).

***Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle (Plate 3P and 3Q)**

Cells of *P. pungens* found in the nBUS were linear in valve view (Plate 3P and 3Q) with a valve width of 2.8-3.5 µm. The densities of fibulae and interstriae were 11-13 and 10-13 µm in 10 µm, respectively, and each stria comprised two and sometimes three rows of poroids, with 3-5 poroids in 1 µm. The morphological characters agreed best with the description of *P. pungens* var*. cingulata* (Villac and Fryxell, 1998). The species was observed inshore as well as offshore during several sampling periods (Fig. 2). It was one of the most frequently observed and abundant inshore species in the nBUS together with *P. australis*. In 2007, it was also the only *Pseudo-nitzschia* species found at the

inshore station off Walvis Bay. *The species* is globally distributed and commonly recorded. It has been reported previously to produce DA (e.g. Fernandes et al., 2014).

***Pseudo-nitzschia* cf. *turgidula* (Hustedt) Hasle (Plate 4R and 4S)**

Cells of *P.* cf. *turgidula* were characterized by lanceolate valves (Plate 4R and 4S) with a width of about 3.8 µm. The numbers of fibulae and interstriae were 18 and 24 in 10 µm, respectively. Each stria had 2 rows of poroids and 8 poroids in 1 µm. A central nodule could be distinguished in the middle of the frustule. For *P. turgidula,* Hasle (1965) reported that the valve width was 2.5-3.5 µm, the lanceolate valve was characterised by being expanded in the middle, and densities of fibulae and striae were 13-18 and 23-28 in 10 µm, and poroids 7-9 in 1 µm. The characters observed agreed with the description of Hasle (1965) except for a wider valve and the lack of a central expansion of the valve. The taxon was therefore named as *P.* cf. *turgidula.* The species was only observed at offshore stations during 2006 (Fig. 2).

***Pseudo-nitzschia* sp. *NamA* (Plate 4T and 4U)**

The *Pseudo-nitzschia* sp. *NamA* was an abundant and yet unidentified species (Plate 4T and 4U) with lanceolate and broad valves, showing a width of approximately 7.5 µm and a length of about 53 µm. A central nodule was present and fibulae and striae were found at a density of 15 and 31 in 10 µm, respectively. The striae comprised one row of poroids, and 4-5 poroids in 1 µm. This species was observed at an offshore station in 2006 (Fig. 2).

4. Discussion

279 ### 4.1. Abundance and diversity of *Pseudo-nitzschia* species inshore and
280 ### offshore in the nBUS

281 A number of *Pseudo-nitzschia* spp. have been identified inshore and offshore in the nBUS
282 as described in section 3.1. Annual increases and decreases in the abundance of
283 *Pseudo-nitzschia* spp. on the 23°S transect off Walvis Bay were reported by Louw et al.
284 (2017) to occur during May-July and August-November, respectively, based on data from
285 2004-2011. In the samples examined during the present study, high abundances of
286 *Pseudo-nitzschia* spp. were observed mostly inshore, whereas low levels occurred
287 offshore (Table 2). Inshore stations represented the Namibian pelagic fisheries region,
288 feeding grounds for birds as well as for seal colonies located close to the Namibian coast.
289 This area is also near Walvis Bay harbor, where there is intensive aquaculture activity.
290 *Pseudo-nitzschia* spp. found offshore can also be relevant to consider inshore, given the
291 potential intrusion of warmer oceanic water into coastal areas during periods of upwelling
292 relaxation.

293 An illustration of the diversity of the *Pseudo-nitzschia* spp. in the nBUS is given in Fig. 2.
294 Most of the *Pseudo-nitzschia* spp. found inshore, namely *P. australis, P. pungens, P.*
295 *fraudulenta* and *P. multiseries,* are known as toxigenic and these occurred in much higher
296 concentrations inshore than offshore. It is notable that the diversity of species inshore
297 was relatively low. After 2004, *P. australis* and *P. pungens* dominated inshore, making up
298 86-100% of the *Pseudo-nitzschia* spp., although which species was predominant varied
299 annually and seasonally (Fig. 2). Monthly changes in dominance between the two taxa
300 were observed, as shown in May versus June in 2005 and May versus July in 2007 (Fig.
301 2). The duration of the bloom over these months suggests that either *Pseudo-nitzschia*

bloom events can be long-lived with changing species composition, or two separate blooms occurred closely (in time and space) to each other. Low percentages of additional species (i.e. *P. americana, P. fraudulenta, P. multiseries* and *P.* cf. *dolorosa*) were observed inshore along with *P. australis* and *P. pungens*.

In May 2011, a completely different *Pseudo-nitzschia* community dominance was observed, with only *P. fraudulenta* (dominant, 96%) and *P. multiseries* present. Louw et al. (2017) showed that cooler summers were generally experienced in the nBUS over the inshore shelf region (i.e. median temperature was below or equal to the long-term annual median value),whereas 2011 was distinguished as a warmer year. Temperatures ranged high above the annual median and were associated with a Benguela Niño in 2011, which has the same characteristics as the El Niño that occurs in the Humboldt upwelling ecosystem. This suggests that warmer conditions accompanied by lower nutrient levels may favor the growth of certain *Pseudo-nitzschia* spp., here *P. fraudulenta* and *P. multiseries*. As the predominant winds in the nBUS decline (i.e. upwelling favorable wind), wind speed and direction become low and/or northerly, which increases the potential for offshore water intrusion closer to the coast. Moreover, the offshore environment may serve as a retentive region for development or regeneration of *Pseudo-nitzschia* spp. capable of seeding inshore waters where coastal eddies can provide more favorable nutrient levels (GEOHAB, 2005; Seegers et al., 2015) In the California current system, Kudela et al. (2004), found that weak upwelling, the transitional period between warm and cool water, as well as low macronutrients were conducive to development of *Pseudo-nitzschia* blooms.

The species diversity of the offshore *Pseudo-nitzschia* communities observed in September 2005 and March 2006 was considerably higher than any inshore stations (Fig. 2) comprising taxa not found inshore: *P. kodamae*, *P. lineola*, *P.* cf. *heimii*, *P.* cf. *turgidula*, *P.* sp. NamA. By comparison, during August 2004 and April 2008, *P. australis* and *P. pungens* were the dominant offshore species, although offshore concentrations were much lower than inshore (Table 2). In addition to the potentially toxic species observed inshore in the nBUS, *Pseudo-nitzschia* cf. *turgidula* and *P. kodamae* represented potential DA producers from the offshore region.

4.2. Case study of domoic acid found in the nBUS

At the end of July 2004, reports of dead fish and seals in the nBUS were communicated to the Namibian Ministry of Fisheries and Marine Resources, along with sightings of irregular behaviour of birds, suggestive of a DA poisoning event. Concurrently, an annual phytoplankton survey being conducted in the nBUS showed extremely high biomass based on measurements of chlorophyll-*a* concentration (Fig. 3) and suggested the presence of high density blooms. The *Pseudo-nitzschia* cell counts were, however, relatively low compared to annual highs, with values below the 200,000 cells/L action level for a *Pseudo-nitzschia* bloom event. These counts conducted for surface samples were below 120,000 cells/L (Fig. 3). Based on findings described in section 3.1 (Fig 2) and reanalyses of data by Marangoni (unpublished) (Table 3), nine *Pseudo-nitzschia* taxa (Table 1) were identified: *P. australis, P.* cf. *caciantha, P.* cf. *cuspidata, P. delicatissima, P.* cf. *dolorosa, P. fraudulenta, P. multiseries, P.* cf. *pseudodelicatissima* and *P. pungens.* Among these, *P.* cf. *caciantha, P.* cf. *cuspidata* and *P.* cf. *pseudodelicatissima* were recorded offshore, whereas *P. australis, P. fraudulenta, P. multiseries and P. pungens*

were present inshore. Some of these species were identified during the nBUS transects as described in section 3.2, whereas others were identified in the nBUS only during the toxic event, as indicated in Table 1. *Pseudo-nitzschia* taxa were also documented along the southern Angolan and Namibian coasts during a transoceanic survey conducted in 2007 (Guannel et al., 2015), but cells were generally not identified to species level. In the sBUS, *P. australis* and *P. multiseries* have previously been identified and documented (Pitcher et al., 2014; Marangoni et al., 2001).

The potentially toxic *Pseudo-nitzschia* spp. reported included: *P. australis, P.* cf. *caciantha, P. delicatissima, P. kodamae, P. pungens, P.* cf. *pseudodelicatissima, P. fraudulenta, P. multiseries* and *P.* cf. *cuspidata.* Some of the highest particulate DA concentrations measured in seawater samples collected during August 2004 were associated with the presence of *P. australis*, an undescribed species reported as "*P.* cf. *australis*" (with a central nodule) and *P. pungens*, with values as high as 118.7 ng/L at an offshore station (Table 3). The maximum particulate DA level observed at an inshore station (18°S) was ~182.2 ng/L and included *P.* cf. *pseudodelicatissima* (high abundance), *P.* cf. *caciantha, P. pungens* (few cells), *P. australis* (few cells), *P. "calliantha"* (but with 2 rows of poroids), and *P.* cf. *"australis"* (with a central nodule) (Table 3). Occasionally, only *P. pungens* was found in association with DA, but toxin levels were uniformly low (< 5 ng/L) when this was the case (Table 3). Toxic blooms of *P. australis* have previously been linked to numerous wildlife mortalities as well as harvesting closures of various wild and farmed shellfisheries as reviewed by Trainer et al. (2012). The spatial and temporal association of *P. australis* with the 2004 wildlife mortality event recorded off the Namibian coast is thus consistent with prior reports of toxic impacts

370 related to blooms of this widespread *Pseudo-nitzschia* species. The inshore areas were
371 dominated by *P. australis* and *P. pungens* (Tables 2, 3; Fig. 3).
372 Clam samples were collected in 2004 in the area surrounding Walvis Bay harbour where
373 most of Namibia's mariculture farms are located. These clams tested negative for DA
374 (Table 3), which indicated that the event did not have an effect inside the harbour. Dead
375 pilchards and mullets, both pelagic planktivorous fish generally inhabiting offshore waters,
376 were found along the beaches closer to Swakopmund, located north of Walvis Bay. Gut
377 contents of one of the dead pilchards tested positive for DA (0.12 µg g^{-1}; Table 3), a level
378 that is considered low in comparison to those measured in other planktivorous fish during
379 toxic *Pseudo-nitzschia* blooms in other regions (e.g. Scholin et al., 2000; Lefebvre et al.,
380 2002). The remaining pilchard and mullet samples tested for DA were negative (Table 3).
381 Further investigations showed high phytoplankton biomass in this northerly area (Fig. 3),
382 which suggests the possibility of clogged gills as an alternative or additional factor
383 involved in the mortalities of pilchards and mullets.

384 The BUS is also susceptible to the detrimental effects of low oxygen events and has in
385 the past experienced fish kills and rock lobster walkouts in the sBUS at Elands Bay
386 (Cockcroft et al, 2000) and in central Namibia (NatMIRC unpubl. data). One of the main
387 characteristics of eastern boundary upwelling systems is the high primary production due
388 to elevated concentrations of phosphorus and nitrogen, introduced into the upper water
389 column as also reported by Louw et al. (2016). This nutrient enrichment causes major
390 blooms during summer and early autumn. If not grazed effectively, these blooms collapse
391 and decompose, consuming much of the dissolved oxygen. The large area occupied by

392 this dense bloom might have impacted fish directly and in more severe cases caused fish-
393 kills due to excessively low oxygen conditions associated with bloom termination.

394 **5. Conclusions**

395 In this study, 16 *Pseudo-nitzschia* spp. were identified. In the eight-year time series 12
396 *Pseudo-nitzschia* spp., were identified, from which five were recognized as potential DA
397 producers, and of these, *P. australis* and *P. multiseries* are known to form highly toxic
398 blooms around the world (Lelong et al., 2012; Trainer et al., 2012). One potential new
399 *Pseudo-nitzschia* species was also recorded and other new species may have also been
400 present (e.g. offshore stations in 2005 and 2006; Marangoni data in Table 3), but a lack
401 of clear morphological data precluded any further identification. Four additional *Pseudo-*
402 *nitzschia* taxa were identified from the toxic event in July 2004, all being potentially
403 toxigenic species. Although the role of DA in the 2004 fish mortality event remains
404 inconclusive, it is evident that some of the *Pseudo-nitzschia* spp. in the nBUS did produce
405 DA, which has the potential to impact large mammals, birds or possibly humans.

406 The *Pseudo-nitzschia* concentrations seem to follow a similar pattern inside and outside
407 of Walvis Bay harbour, with higher levels occurring during the first part of the year
408 (NatMIRC unpubl. data). Low levels of DA are detected occasionally in oysters, as
409 reported in regular internal MFMR reports; however, these measurements are part of a
410 monitoring programme operating only during February and August by the mariculture sub-
411 division. Domoic acid producing *Pseudo-nitzschia* spp., *P. australis, P. pungens* and *P.*
412 *fraudulenta*, occur regularly as dominant taxa inshore and can easily be transported into
413 the bay. A concentration of 200,000 cells/L is generally accepted as the action level for

414 *Pseudo-nitzschia* spp. Reports from Danish and Canadian waters of *Pseudo-nitzschia*
415 concentrations of 50,000 and 62,000 cells/L, respectively, resulting in DA levels in
416 shellfish above the regulatory limit of 20 µg g^{-1} (Lundholm et al., 2005) illustrate that a
417 lower threshold (e.g. 50,000 cells/L) should be considered for potential DA contamination
418 of shellfish or wildlife impacts. This 50,000 cells/L value will apply only if large, toxigenic
419 *Pseudo-nitzschia* spp. are present. In the present study, all inshore samples analysed for
420 *Pseudo-nitzschia* diversity had cell densities above 50,000 cells/L (Table 2). Although
421 many factors can influence DA production, it would nonetheless be valuable for Namibia
422 to establish a baseline annual toxin monitoring program that would provide data on
423 *Pseudo-nitzschia* and DA levels to the mariculture industry and inform policy makers to
424 improve food safety.

425 Most areas along the Namibian coastline are inaccessible for monitoring. Therefore, the
426 impacts of toxic or even non-toxic, high-biomass bloom events are often not recorded,
427 except in the central and some southern parts of Namibia. Since some of the species
428 observed during this study could not be identified, as was also the case for Guannel et
429 al. (2015), culturing of Namibian *Pseudo-nitzschia* spp. for identification and toxicity
430 testing is recommended. Moreover, investigations of toxicity trends related to the
431 distribution and abundance of *Pseudo-nitzschia* taxa in the nBUS are needed in order to
432 evaluate the potential for DA to impact the Namibia's coastal environment and economy
433 (e.g. contamination of fishery resources, toxin trophic transfer into pelagic and benthic
434 foodwebs, etc.).

435 **Acknowledgements**

We thank the Namibian Ministry of Fisheries and Marine Resources for its support, as well as all our colleagues involved in the data collection -particularly the staff of the Environment Subdivision. Sampling was done on the *RV Welwitschia* and captain and crew members from the Ministry of Fisheries and Marine Resources and scientific staff from the NOAA/NOS/NCCOS/Center for Coastal Environmental Health and Biomolecular Research (CCEHBR) (Charleston, SC, USA) are hereby also acknowledged. We acknowledge Claudio Marangoni from the University of Witwatersrand in Johannesburg for *Pseudo-nitzschia* species identification during the August 2004 event. The expertise of Dr. Zhihong Wang with the NOAA Marine Biotoxins Program, NOAA/NOS/NCCOS/CCEHBR is also acknowledged for LC-MS/MS analyses of domoic acid for samples collected during this event. The generous financial support from Margarita Fernandez-Tejedor from IRTA, Institut de Recerca i Tecnologia Agroalimentaries-Centre d'Aqüicultura is thankfully acknowledged. We would like to gratefully acknowledge the support provided by Bronwen Currie from NatMIRC for her role and contribution in preparation of samples for toxicity analysis.

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459 **FUNDING**

460 The research project GENUS (Geochemistry and Ecology of the Namibian Upwelling
461 System) funded by the German Federal Ministry for Education and Research (BMBF,
462 FKZ 03F0497). This project was also partially funded by the National Commission on
463 Research Science and Technology (NCRST) of Namibia.

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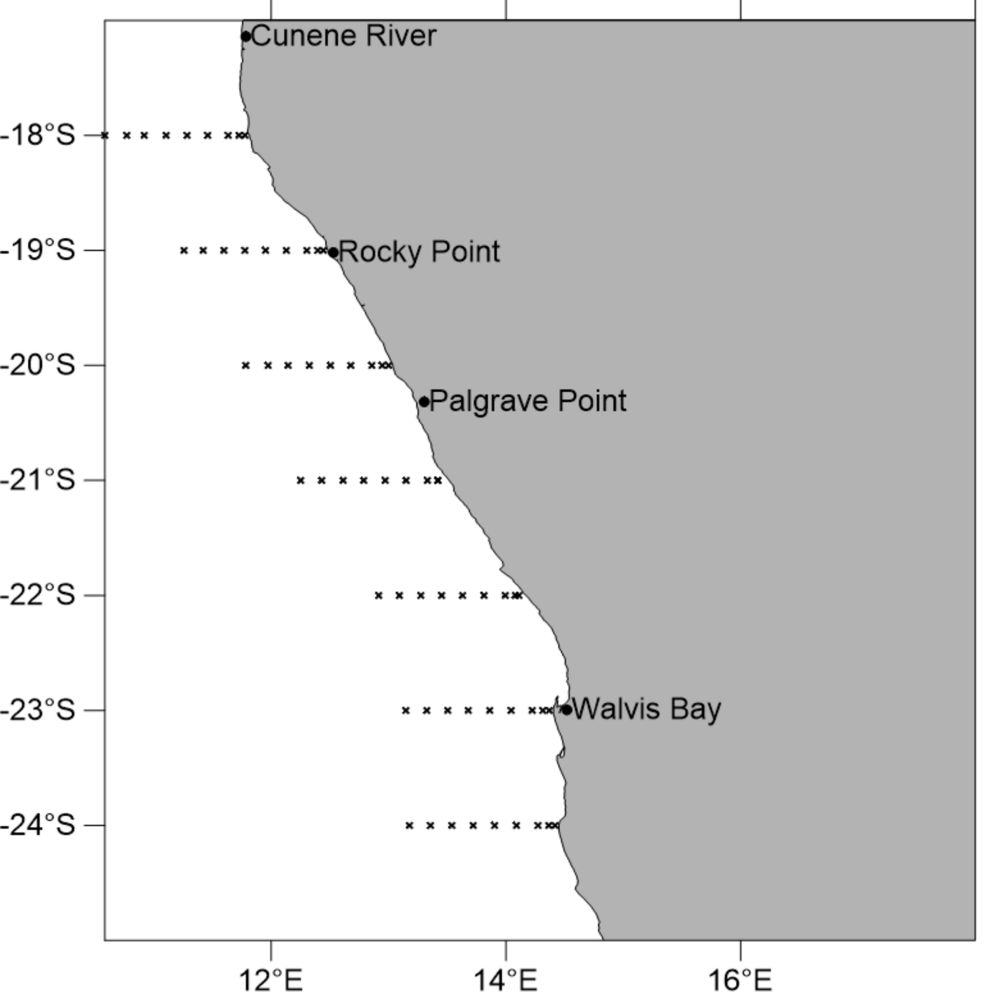
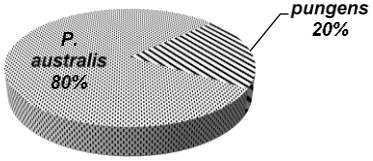

Cunene River
Rocky Point
Palgrave Point
Walvis Bay
-18°S
-19°S
-20°S
-21°S
-22°S
-23°S
-24°S
12°E
14°E
16°E

Inshore

Offshore

WW23010 May-2004



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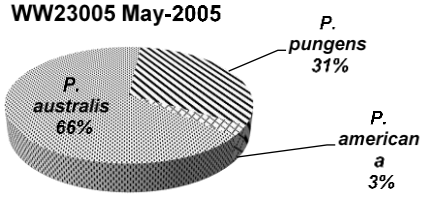

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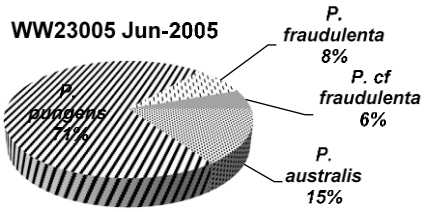

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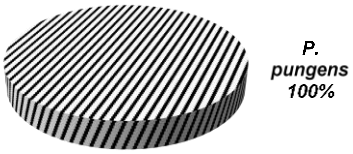

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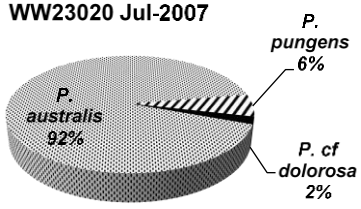

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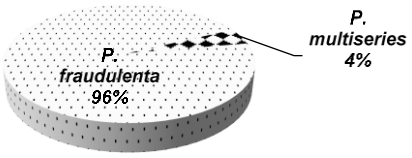

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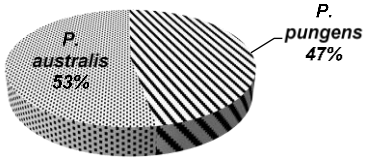

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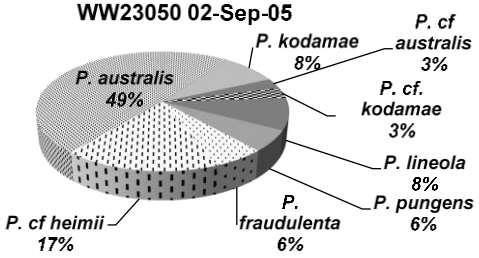

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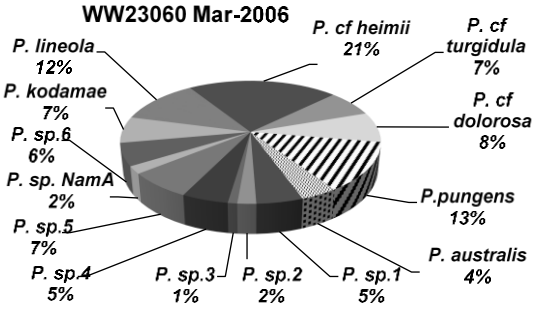

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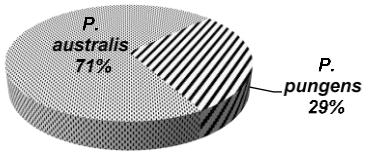

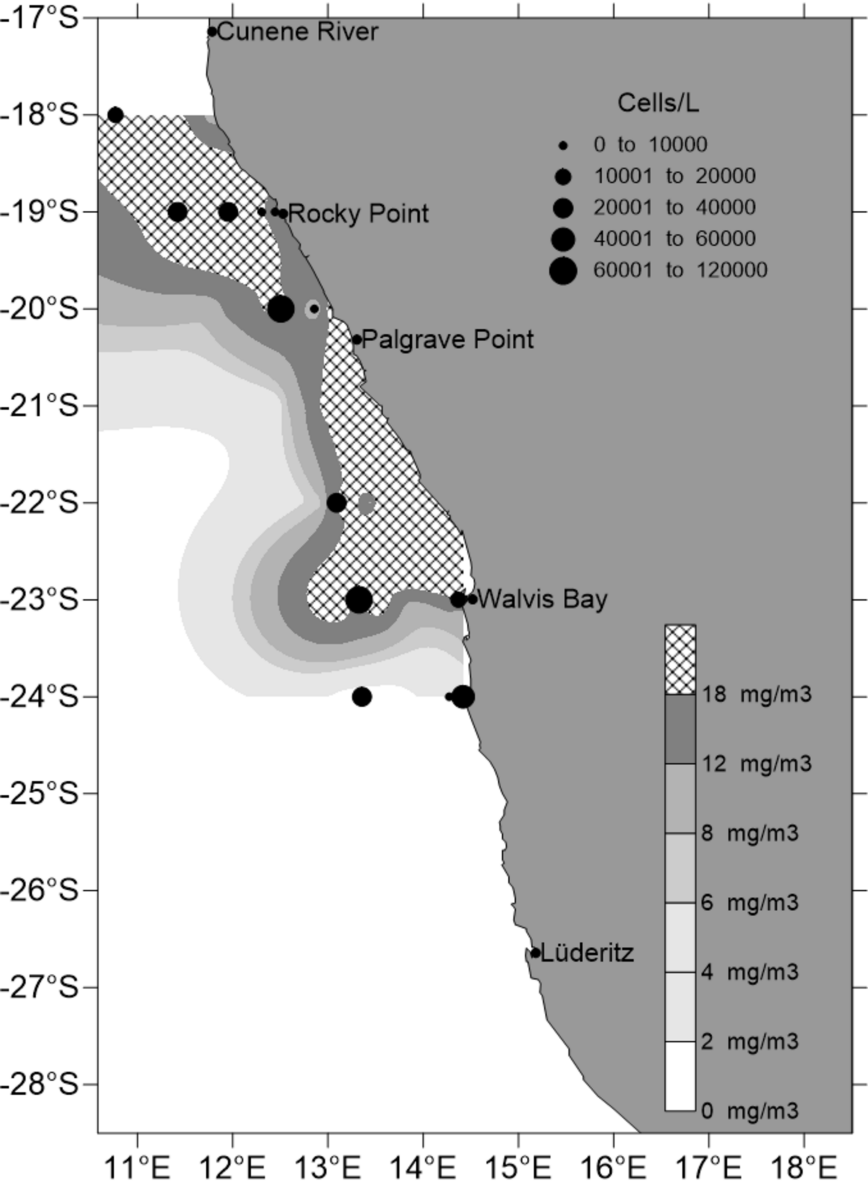
Cunene River
Rocky Point
Palgrave Point
Walvis Bay
Lüderitz
Cells/L
0 to 10000
10001 to 20000
20001 to 40000
40001 to 60000
60001 to 120000
18 mg/m3
12 mg/m3
8 mg/m3
6 mg/m3
4 mg/m3
2 mg/m3
0 mg/m3
17°S
18°S
19°S
20°S
21°S
22°S
23°S
24°S
25°S
26°S
27°S
28°S
11°E
12°E
13°E
14°E
15°E
16°E
17°E
18°E

Tables

	Taxa	Valve Shape	Fibula/ 10 µm	Striae/ 10 µm	Central nodule	Rows of Poroids	Poroides /µm	Sectors in Poroids	Apical-axis (µm)	Trans-apical axis (µm)
Min-Max	*P. americana*	Rectangular	20	30	No	2	9-10		21.8	3.5
Mean ±SD										
Min-Max	*P. australis**	Lanceolate/Asym.	12-17	12-18	No	2	4-6	X	87-110	6-8
Mean ±SD			13.8±1.4	14.5±1.7			4.6±0.6		96.4±8.4	6.3±0.9
Min-Max	*P.* cf *dolorosa*	Lanceolate	16-20	28-30	Yes	2	3-8		50-100	2.9-3.4
Mean ±SD			17.3±2.3	28.7±1.2			6.5±1.0		77.3±24.2	3.1±0.3
Min-Max	*P. delicatissima***	Lanceolate	16-21	27-42	Yes	2	6-13	1	50-70	1.5-3.6
Mean ±SD										
Min-Max	*P.* cf *caciantha***		16-19	29-32	Yes	1	3-4	(3-4)	52-65	1.9-3.5
Mean ±SD										
Min-Max	*P.* cf *cuspidata***		19-24	36-38	Yes	1	4-5	(2)	45-58	2.1-2.9
Mean ±SD										
Min-Max	*P. fraudulenta**	Lanceolate/Asym.	16-22	20-22	Yes	2-3	5-7			5.5-6.5
Mean ±SD			20.1±1.6	21.6±0.7		2.4±0.5	5.6±06		87.3±49	5.5±0.5
Min-Max	*P.* cf *heimii*	Lanceolate	16-20	28-30	Yes	2	5-10		60-70	3-4
Mean ±SD			18.0±2.8	29.0±1.4		2.0±0.0	7.5±2.1		77.8	3.5
Min-Max	*P. kodamae*	Linear			Yes					
Mean ±SD			15.0±1.7	26.0±2.8		1.0±0.0	4.5±0.6		83.6	2.4±0.3
Min-Max	*P.* cf *kodamae*	Linear	14-16	26		1	4-5			1.73
Mean ±SD			15.0±1.2							
Min-Max	*P. lineola*	Lanceolate	16	22-26	Yes	1	4-5	2-3	80-96	1.3-1.7
Mean ±SD			16.0±0.0	24.7±2.3		1.0±0.0	4.4±0.5		88.0±11.0	1.8±0.5
Min-Max	*P. multiseries**	Lanceolate	11-13	11-13	No	3-5	5-6		100-107	3.6-4.6
Mean ±SD			11.8±0.8	11.8±0.8		3.6±0.7	5.4±0.5		104.5±5.4	4.1±0.4
Min-Max	*P.* cf *pseudodelicatissima***		17-24	35-38	Yes	1	4-5	(2)	55-75	1.3-1.9
Mean ±SD										
Min-Max	*P. pungens**	Linear	11-13	10-13	No	2(3)	3-5			2.8-3.5
Mean ±SD			12.2±0.6	11.6±0.8		2.0±0.0	3.7±0.7			3.3±0.2
Min-Max	*P.* cf *turgidula*	Lanceolate	18	24	Yes	2	8		90.1	3.8
Mean ±SD										
Min-Max	*P. sp. NamA*		15	31	Yes	1	4-5	2-4	53.4	7.5
Mean ±SD										

* *Pseudo-nitzschia* species present during the 2004 event

** *Pseudo-nitzschia* species present during the 2004 event identified based on reanalyses of (Marangoni, unpublished)

***Asym. = Asymmetry

Date collected	Station	Distance offshore	Year	Position	Cells L^{-1}
05-May-04	WW23010	10 nm	2004	Inshore	93,484
05-Aug-04	WW23060	60 nm	2004	Offshore	118,230
12-May-05	WW23005	5 nm	2005	Inshore	3,346,173
30-Jun-05	WW23005	5 nm	2005	Inshore	861,976
02-Sep-05	WW23050	50 nm	2005	Offshore	17,528
03-Mar-06	WW23060	60 nm	2006	Offshore	9,280
07-May-07	WW23020	20 nm	2007	Inshore	106,200
10-Jul-07	WW23020	20 nm	2007	Inshore	1,499,179
13-Apr-08	WW23060	60 nm	2008	Offshore	4,124
17-May-11	WW23020	20 nm	2011	Inshore	13,416,312

Date	Stations	ng DA mL^{-1} (in extract)	DA concentration in tissue (µg g^{-1}) or in seawater (ng L^{-1}; particulate DA)	Sample type	Species
04 08 2004	WW24002	5.57	25.5	Phytoplankton	*P. australis, P. pungens*
04 08 2004	WW24005	2.38	13.0	Phytoplankton	*P. australis* (high abundance)
04 08 2004	WW24020	0.73	4.1	Phytoplankton	*P. pungens* (high abundance)
04 08 2004	WW24040	< LOQ	-	Phytoplankton	*P. australis, P. cf. pseudodelicatissima, P. cf. caciantha*
04 08 2004	WW24050	0.45	2.5	Phytoplankton	*P. australis, P. cf. pseudodelicatissima, P. cf. caciantha*
05 08 2004	WW23050		-	Phytoplankton	*P. australis, P. pungens*
05 08 2004	WW23060		-	Phytoplankton	*P. cf. cuspidata*
05 08 2004	WW23070	20.80	118.7	Phytoplankton	*P. cf. pseudodelicatissima, P. cf. caciantha, P. pungens* (few cells) , *P. australis* (few cells), P. "calliantha" (but with 2 rows of poroids)
06 08 2004	WW22050	3.41	19.1	Phytoplankton	*P. cf. pseudodelicatissima, P. cf. caciantha,* P. "australis" (but with a central nodule)
06 08 2004	WW22060	< LOD	-	Phytoplankton	*P. cf. pseudodelicatissima*
07 08 2004	WW21005	< LOD	-	Phytoplankton	(No Species Data)
08 08 2004	WW20030	0.47	2.6	Phytoplankton	*P. pungens*
10 08 2004	WW18020	10.42	57.8	Phytoplankton	*P. australis, P. pungens, P. cf. pseudodelicatissima*
10 08 2004	WW18010	32.94	182.2	Phytoplankton	*P. australis, P. pungens*
10 08 2004	WW18002	0.54	3.2	Phytoplankton	(No Species Data)
	NAB2	28.97	0.12	Pilchard (guts)	
	NAB6	<LOD	-	Mullet (guts)	
	NAB8	<LOQ	-	Pilchard (guts)	
	NAB9	< LOD	-	Clam (whole)	

LOD – 0.14 ng mL^{-1} in extract (S:N ratio ~5); LOQ – 0.4 ng mL^{-1} in extract (S:N ratio >10)