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Morphological and toxicological studies of *Pseudo-nitzschia* species from the central coast of Peru

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Currently, there is little information on the genus *Pseudo-nitzschia* in Peruvian coastal waters available and some species have been misidentified. This is the first study of *Pseudo-nitzschia* species found in several blooms off the central coast of Peru. The cultures obtained and identified by scanning electron microscopy revealed the presence of *Pseudo-nitzschia subpacifica* and *P. pungens*, the first record of *P. subpacifica* in Peruvian coastal waters. Neither *P. subpacifica* nor *P. pungens* cultures contained domoic acid (DA) in detectable amounts using HPLC–MS/MS. Our results suggest that *Pseudo-nitzschia* species are common off the central coast of Peru. The detection of non-toxic strains of *Pseudo-nitzschia* does not necessarily mean that other populations or strains of this genus in Peru cannot produce DA. Research is needed to evaluate other strains from different locations along the Peruvian coast and to explore whether environmental factors or genetic variability affect the production of DA.

Keywords: *domoic acid, Humboldt Current, Peru,* Pseudo-nitzschia, Pseudo-nitzschia subpacifica, Pseudo-nitzschia pungens

Introduction

Pseudo-nitzschia H. Peragallo is a marine genus of diatoms which is a common component of marine phytoplankton assemblages (Hasle 2002). Currently, *Pseudo-nitzschia* comprises 46 species (Percopo et al. 2016), of which at least 22 have been shown to produce the neurotoxin domoic acid (DA), which is responsible for amnesic shellfish poisoning (ASP) (Lundholm 2016).

The first DA poisoning occurred in Prince Edward Island (PEI), Canada in 1987, where at least three people died and over 100 became ill due to the consumption of cultivated blue mussels (*Mytilus edulis*). The resultant shellfish poisoning was termed ASP, because one of the distinctive symptoms of acute exposure was short-term memory loss (Bates et al. 1989, Wright et al. 1989, Perl et al. 1990). Further research established that DA was produced by the planktonic diatom, *Nitzschia pungens f. multiseries* Hasle (now known as *Pseudo-nitzschia multiseries* (Hasle) Hasle) (Bates et al. 1989).

Since this event, blooms of potentially toxic *Pseudo-nitzschia* species have been frequently reported and are now known to be distributed worldwide (reviewed in Lelong et al. 2012, Trainer et al. 2012), prompting local and international agencies to adopt specific public health regulations and marine toxin monitoring programmes with respect to DA and ASP (Fernandes et al. 2014). The presence of DA is more persistent and has caused negative impacts, particularly in upwelling regions (Trainer et al. 2010, 2012).

The coastal upwelling system of Peru constitutes a large part of the Humboldt Current System and is considered one of the most productive regions in the world, fixing 3000–4000 mg C m^{-2} d^{-1} (Calienes et al. 1985, Graco et al. 2007). Due to this high productivity, the area is susceptible to harmful algal blooms (Pitcher & Pillar 2010, Trainer et al. 2010).

There is little information available on *Pseudo-nitzschia* in Peruvian waters and it is likely that previously reported species have been misidentified because species identification within *Pseudo-nitzschia* is notoriously difficult. The first reports based on electron microscopy reported the presence of *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle and *P. australis Frenguelli* (as *P. pseudoseriata* G.R. Hasle) (Hasle 1965, Trainer et al. 2012).

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Materials and methods

Biological samples

Phytoplankton samples were obtained periodically from July 2012 to February 2013 in three locations off the Peruvian central coast, Isla San Lorenzo (12°03′S, 77°13′O), Bahía de Pucusana (12°28′S, 76°47′O), and Bahía de Paracas (13°49′S, 76°17′O) (Fig. 1). The samples were collected using vertical net hauls (20 µm mesh), returned to the laboratory in 1 L glass bottles, chilled on ice (10°C), and protected from sunlight. These were used to establish cultures of the *Pseudo-nitzschia* species.

Culture methods

Single chains of *Pseudo-nitzschia* cells were micropipetted and transferred to multi-well culture plates filled with 2 mL of f/2 culture medium (35 psu) (Guillard 1975). The plates were maintained at 14°C in a 12:12-h light:dark

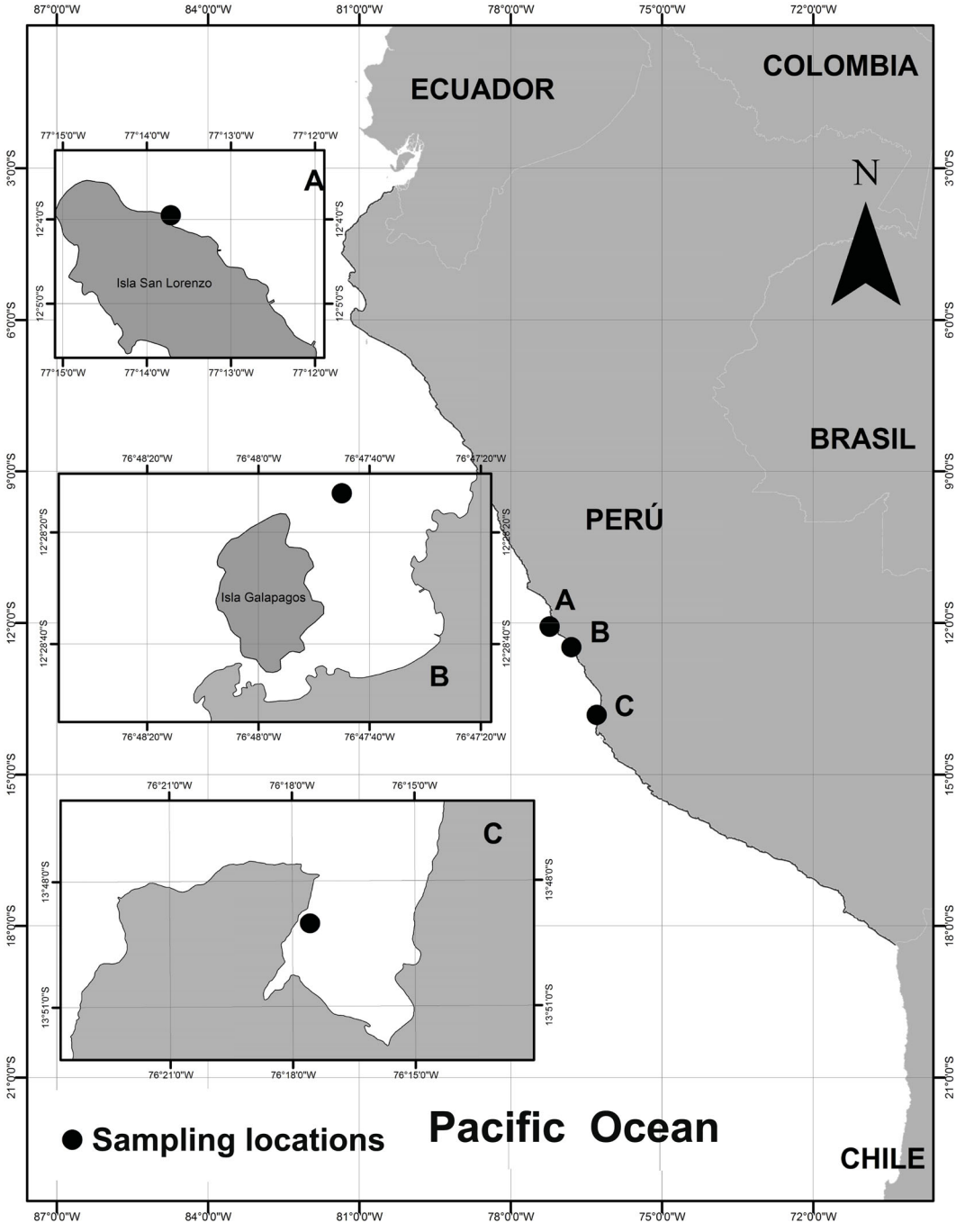


Fig. 1. Location of the sampling stations along the central coast of Peru.

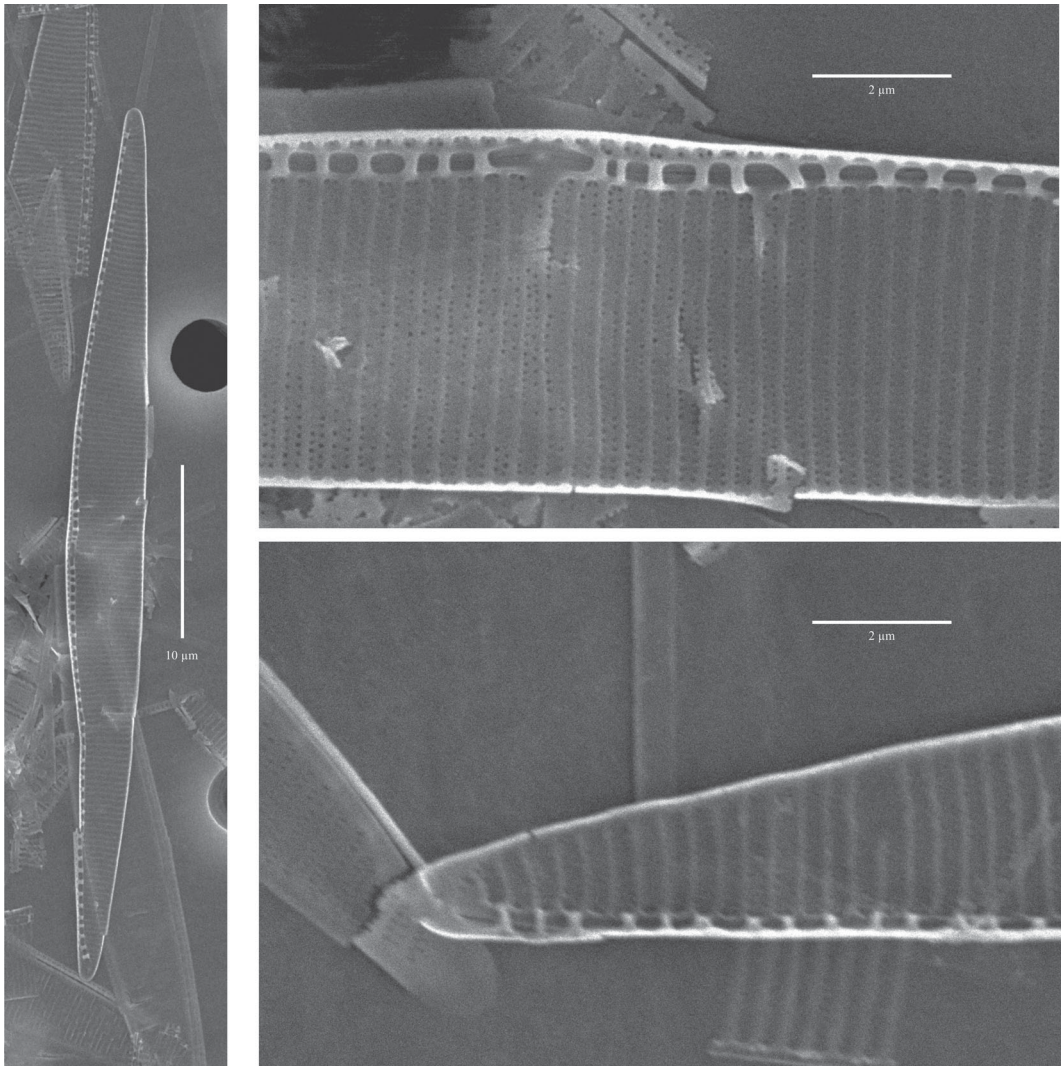
cycle, with a photon flux of 60 µmol photons m^{-2} s^{-1}. Established cultures were transferred to borosilicate Erlenmeyer flasks with 150 mL of f/2 medium and grown at 15°C in a 12:12-h light:dark cycle, with a photon flux of 80 µmol photons m^{-2} s^{-1}. Mass cultures of each strain were grown in borosilicate bottles with 10 L of f/2 medium under the above conditions. For cell counts, 2 mL aliquots from each culture were taken daily for 7 days. Each aliquot was diluted with 10 mL of filtered seawater (0.45 µm) and preserved with one drop of Lugol's solution (0.5%v/v). Culture densities were quantified by the Utermöhl method (Hasle 1978) using 2 mL chambers (Hydrobios, Germany) and a CKX 31 inverted microscope at 40 × magnification (Olympus, Tokyo, Japan).

Taxonomic analyses

Scanning electron microscopy (SEM) was used to perform detailed morphological analyses of the *Pseudo-nitzschia* cultures. Organic matter was removed from the frustules following (Lundholm et al. 2002b). The cleaned material was retained on a 5.0 µm Isopore (Merck KGaA, Darmstadt, Germany) membrane filter, washed with distilled water to remove salts and preservative, and dehydrated through an ethanol series (30, 50, 75, 95 and 100%) followed by 100% hexamethyldisilazane. After being air dried overnight, specimens were gold coated in a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a Phillips XL30 or FEI Quanta 200 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

Sample preparation and chromatographic analysis

The presence of DA (cellular content) in the extracts was determined following a previously validated method (Regueiro et al. 2011). Sample analyses were performed using LC–MS/MS with a Surveyor MS HPLC quaternary pump and a Surveyor autosampler coupled to a Deca



Figs 2–4. *Pseudo-nitzschia subpacifica*, Isla San Lorenzo. SEM. Fig. 2. Whole valve, Fig. 3. Valve end. Fig. 4. Details of the valve structure and the central interspace.

XPplus ion trap mass spectrometer through an electrospray API (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was carried out in a core–shell reversed-phase column Kinetex C18 (50 × 2.1 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA), maintained at 35°C. The limit of detection of the technique was 2 ng mL^{-1}.

Results

Four strains of *Pseudo-nitzschia* species were isolated from Isla San Lorenzo samples and one strain from Bahía de Paracas. No culture strains were obtained from Bahia de Pucusana samples.

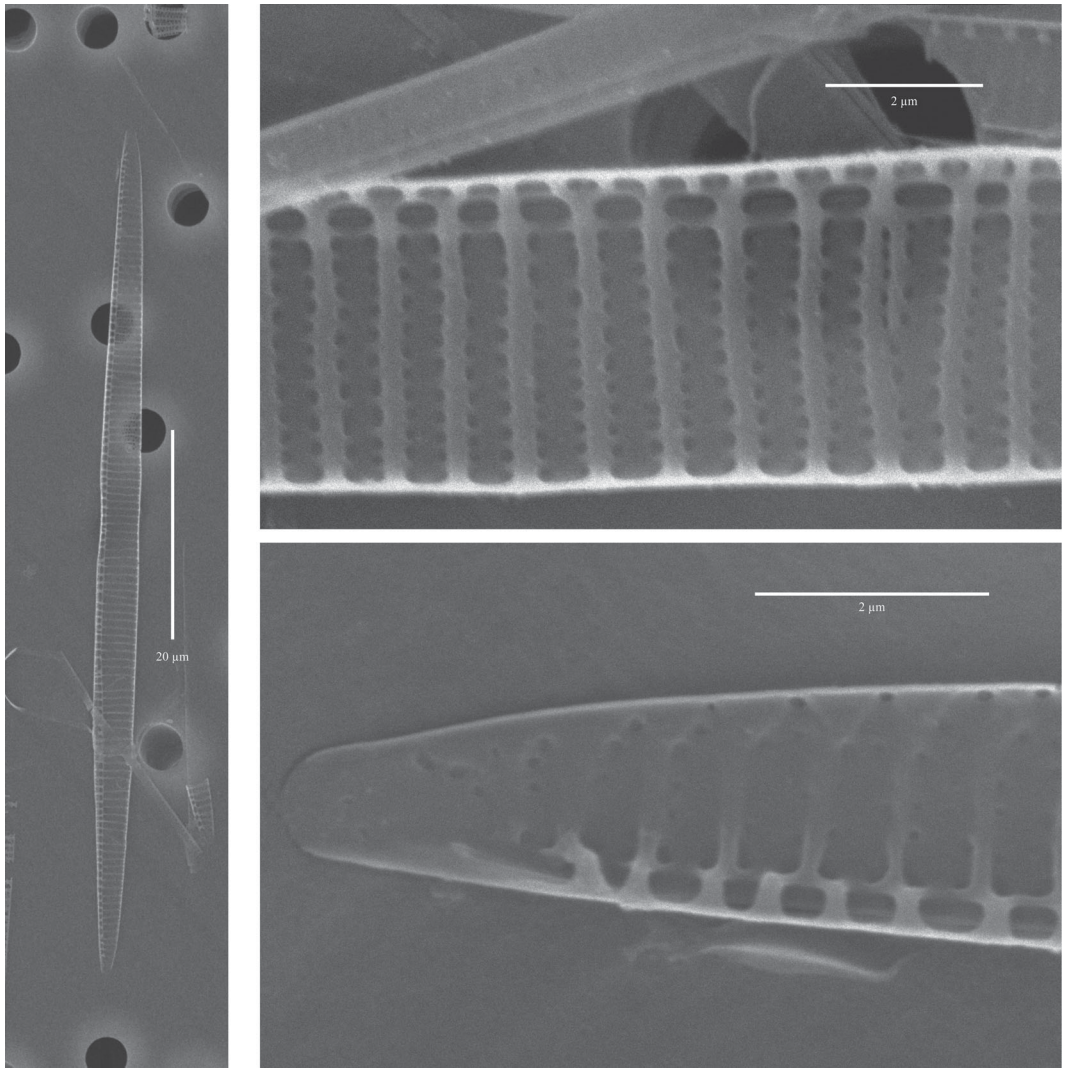
Taxonomy

Pseudo-nitzschia subpacifica (Hasle) Hasle

From the morphological examination using SEM, culture IMP-BG-035 from Isla San Lorenzo was identified as *P. subpacifica*. Individual cells were mainly lanceolate in shape, asymmetric in valve view, with one convex side, and the other varying from somewhat convex to nearly straight (Figs 2–4). The apical axis was 42–52 µm long, and the transapical axis 2.1–4 µm. There were 28–33 striae per 10 µm, and 15–17 fibulae per 10 µm. A central nodule was present, and the striae were formed of two rows of rounded poroids separated by a uniform area (Fig. 4).

Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle

The morphological examination of the IMP-BG-042, IMP-BG-043, and IMP-BG-044 cultures from Isla San Lorenzo, and IMP-BG-048 from Bahía Paracas, showed overall agreement with the descriptions of *P. pungens*. The cells were mainly linear-lanceolate in shape, with pointed apices in the valve view (Figs 5–7). Cell length ranged from 80 to 130 µm, and width from 2.1 to 4 µm (Fig. 5). There were 12–15 striae per 10 µm, comprising two rows of rounded poroids (Fig. 7) separated by a uniform area. The



Figs 5–7. *Pseudo-nitzschia pungens*, Isla San Lorenzo. SEM. Fig. 5. Whole valve. Fig. 6. Valve end. Fig. 7. Details of the valve structure.

number of fibulae in 10 µm varied from 12 to 15. No central interspace or nodule was found (Fig. 7).

Cultures

The cultures of strains IMP-BG-035 (*P. subpacifica*), IMP-BG-042, and IMP-BG-048 (*P. pungens*) were grown in 9 L volumes to accumulate sufficient biomass to perform toxin analyses. The strains showed good growth with aeration and were harvested and lyophilized on the sixth day of culture, with the following results: IMP-BG-035-245 – 455 cells/mL (0.4 g dry weight: DW); IMP-BGA-042 (*P. pungens*) – 264,064 cells/mL (0.36 g DW); IMP-BG-048 (*P. pungens*) – 127,945 cells/mL (0.22 g DW).

Toxin analyses

Neither *P. subpacifica* (IMP-BG-035) nor. *P. pungens* strains were found to contain DA in detectable amounts by HPLC–MS/MS.

Discussion

Distribution and taxonomy

This study provides the first report of *P. subpacifica* in Peruvian coastal waters, collected from San Lorenzo Islands, Callao in February, 2013 with a sea surface temperature of 17°C. Along the pacific coast of America, this species has been previously reported from the Monterrey Bay, USA (Buck et al. 1992, Fernandes et al. 2014). Specimens from our culture agree with the descriptions given by Hasle (1965), Hasle and Syvertsen (1997), Churro et al. (2009), Fernandes et al. (2014) and Rijal Leblad et al. (2013) (Table 1). However, some differences were found with the specimens collected in Canada (Kaczmarska et al. 2005a, b). In our cells, the transapical and apical axes are narrower and shorter. Similar differences were found with descriptions given by Lü et al. (2012) of cells collected from China, which had narrower transapical axes than our specimens.

Pseudo-nitzshia pungens is a cosmopolitan species (Casteleyn et al. 2008) that has been previously reported from coastal Peruvian waters (Hasle 1965). Based on its taxonomic features, our species possibly correspond to *P. pungens* var *averiensis*, Lundholm, Churro, Carreira, and Calado reported by Churro et al. (2009) and also by Rijal Lebland et al. (2013) from M'diq Bay, Morocco. Girdle band and valvocopula details were not recorded in the latter; however, the stria and fibula densities, number of poroid rows, and poroid density are similar to those recorded by Churro et al. (2009) (Table 2).

Toxicity/toxin content

The culture of *P. subpacifica* was not found to contain DA at detectable levels (Table 3). The lack of DA has also been observed in strains from other locations, such as Ria

Table 1. Comparison of morphometric data between Peruvian strains of *P. subpacifica* with some strains obtained from different locations around the world.

P. subpacifica (Hasle) Hasle	Apical length (µm)	Valve width (µm)	Fibulae in 10 µm	Striae in 10 µm	Row of poroids	Poroids in 1 µm	Central nodule
Isla San Lorenzo, Perú IMP-BG-035	42–52	4.7–5.3	17	30	2	8–9	+
Gulf of Maine, USA (Fernandes et al. 2014)	36–68	3.8–5.8	17–21	29–33	2	8–9	+
Bay of Fundy, Canada (Kaczmarska et al. 2005a, b)	72–88	5.7–7.2	15–20	27–30	2	7.5–10	+
Chesapeake bay and the Gulf of Panama (Hasle, 1965; Hasle and Syvertsen, 1997)	33–70	5–7	15–20	28–32	2	9–10	+
Aveiro Lagoon, Portugal (Churro et al. 2009)	37–58	4.1–5.8	16–20	28–32	2	7–10	+
Australia (Hallegraeff, 1994)	50–119	4.5–7	15–17	26–31	2	6–8	+
Guangdong, China (Lü et al. 2012)	43–59	3.6–4.6	17–19	na	2	9–10	+
M'diq Bay, Morocco (Rijal Leblad et al. 2013)	45.2–60.1	4.9–6.1	17–20	28–32	2(3)	9–10	+

Table 2. Comparison of morphometric data between Peruvian strains of *P. pungens* with some strains obtained from different locations around the world.

Species	Reference	Apical length (µm)	Valve width (µm)	Fibulae in 10 µm	Striae in 10 µm	Row of poroids	Poroids in 1 µm	Band striae in 10µm	Valvocopula striae/poroids
P. pungens	Isla San Lorenzo, y Bahia Paracas. Perú: *IMP-BG-042, IMP-BG-043, IMP-BG-044, IMP-BG-048*	80–130	2.1–4	12–15	12–15	2–3	3–4	nd	nd
P. pungens var. *averiensis*	Aveiro Lagoon, Portugal (Churro et al. 2009)	47–100	2.7–3.7	13–16	13–16	2–3	3–5	21–25	1 split poroid
P. pungens var. *averiensis*	M'diq Bay, Morocco (Rijal Leblad et al. 2013)	nd	2.9–3.1	16	16	2	4	nd	nd
P. pungens var. *cingulata*	Villac & Fryxell (1998)	71–140	2.8–4.5	10–14	10–14		3–4.5	20–24	nd
P. pungens var. *cingulata*	Aveiro Lagoon, Portugal (Churro et al. 2009)	87.9–110.8	3.5–4.7	11–15	10–13		3–5	nd	nd
P pungens var. *pungens*	Yeddo Bay, Japan	110	2.7–3.5	8.6–11.6	8.6–11.6		2–3	11.6–18.9	1 poroid
P. pungens var. *pungens*	Clade I (var. *pungens)* Churro et al. 2009)	24.4–121.0	2.4–4.2	10–14	9–13		2–4	nd	1 poroid

de Arousa, Spain (strain RdA8) and Port Shelter, Hong Kong (strain Zhenbo7B) (Lundholm et al. 2002a). Currently, only Fernandes et al. (2014) confirmed using ELISA that four strains of *P. subpacifica* from the Gulf of Maine (USA) were capable of producing low levels of DA in culture (0.06–1.1 ng mL^{-1}). The capacity of *P. subpacifica* to produce this toxin and the detection limits of our chromatographic technique suggest that we should not rule out the possibility that this species could produce DA. Therefore, more research is needed to determine whether other strains of *P. subpacifica* isolated from different locations along the Peruvian coast could produce this toxin.

None of the cultured strains of *P. pungens* produced DA in detectable amounts (Table 3). Nor has the toxin been detected in cells of this species from other areas of the world, including Cardigan Bay, Canada (Bates et al. 1998), Santa Cruz and Pacific Grove, Pacific coast of USA, Galveston, Gulf of Mexico, USA, Massachusetts, Atlantic Coast of USA, Sendai, Japan, Dangpen Bay, China (Villac & Fryxell 1998), Changjiang River, China (Li et al. 2005), Aveiro coastal lagoon, Portugal (Churro et al. 2009), Malaysian waters (Lim et al. 2010), and the Catalan coast, Spain (Quijano-Scheggia et al. 2010). At present, only a few strains of *P. pungens* have been recorded as DA producers.

Rhodes et al. (1996) described the production of DA for the first time in strains of *P. pungens* obtained from Marlborough sound, New Zealand, with a concentration of 470 pg $cell^{-1}$. Calu et al. (2009) recorded low levels of DA in strains obtained from the Bay of Crozon, France, approximately 0.40 pg $cell^{-1}$ and Moschandreou et al. (2012) reported the presence of trace levels of DA in a strain isolated from Greek coastal waters, with between 0.2 and 0.8 ng DA mL^{-1}. The detection of non-toxic strains of *P. pungens* from Isla San Lorenzo does not necessarily mean that other regional populations or strains of this species in Peru cannot produce DA. In fact, Rhodes et al. (1996) reported toxic and non-toxic strains of *P. pungens* from different locations in New Zealand and suggested that such factors as nutrients or genetic variability could be involved in toxin production.

Tammilehto et al. (2015) and Haróardóttir et al. (2015) found that the toxicity of *P. seriata* (Cleve) H. Peragallo increased significantly (3300%) in the presence of grazing adult females of *Calanus finmarchicus* and *C. hyperboreus*. On the other hand, initial tests showed that *Pseudo-nitzschia obtusa* (Hasle) Hasle & Lundholm did not produce DA at detectable levels, but surprisingly it was revealed to be toxigenic when induced by copepodites. Our results suggest that the presence of the non-toxic *Pseudo-nitzschia* species is common along the central coast of Peru. However, more research is needed with other strains from different locations, giving special attention to *P. australis,* which is the most common toxic *Pseudo-nitzschia* species in South America, previously reported from the north of Chile (Álvarez et al. 2009, Trainer et al.

Table 3. Measurements of DA in the Peruvian strains of *Pseudo-nitzschia*.

Strain	Species	Date	Sample location	DA ng mL^{-1}
IMP-BG-035	*P. subpacifica*	05/11/12	Isla San Lorenzo	nd
IMP-BG-042	*P. pungens*	05/11/12	Isla San Lorenzo	nd
IMP-BG-043	*P. pungens*	07/14/12	Isla San Lorenzo	nd
IMP-BG-044	*P. pungens*	09/30/12	Isla San Lorenzo	nd
IMP-BG-048	*P. pungens*	11/14/12	Bahía Paracas	nd

nd, DA not detected (< 100 ng mL^{-1}).

2012). This was described for the first time in Peru by Hasle (1965).

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