The potentially toxic diatom *Pseudo-nitzschia* H. Peragallo in the Paraná and Santa Catarina States, Southern Brazil

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ABSTRACT - A taxonomic survey of the diatom *Pseudo-nitzschia* H. Peragallo was carried out in the Paraná and Santa Catarina coasts, Southern Brazil. Samples were taken in various localities along the coastline using vertical hauls from the bottom to the surface. Electron microscopy revealed five species: the potentially toxic *P. australis* Frenguelli, *P. calliantha* Lundholm, Moestrup & Hasle, *P. multiseries* (Hasle) Hasle and *P. pungens* (Grunow ex Cleve) Hasle (plus *P. pungens* var. *cingulata Villac*), and the non-toxic *P. linea* Lundholm, Hasle & G. A. Fryxell. Southern Brazilian strains of *P. calliantha* and *P. multiseries* have previously been shown to be toxic, raising concerns about a potential contamination of mussels and oysters being commercially grounded in the region. High morphological variability was observed in valve characters of *P. calliantha* and *P. pungens*, in some cases confirmed in the literature. In *P. calliantha* there was a conspicuous differentiation in two morphotypes separated from each other by the width and the shape of the valve, and the density of the poroids. *P. linea* has not previously been found in Brazilian waters, and *P. pungens* var. *cingulata* is a new record in Western Atlantic waters. Future investigations using molecular techniques will elucidate whether the genetic variability corresponds to the morphological variation and unveil the possible existence of semicryptic species of Pseudo-nitzschia inhabiting the South Brazilian coast.

Key Words: *Pseudo-nitzschia*, taxonomy, domoic acid, South Brazil

RESUMO – **A diatomácea potencialmente tóxica *Pseudo-nitzschia* H. Peragallo no Paraná e Santa Catarina, Sul do Brasil.** Um levantamento taxonômico da diatomácea *Pseudo-nitzschia* H. Peragallo foi realizado com base em amostras coletadas no Paraná e Santa Catarina, sul do Brasil. As amostras foram coletadas mensalmente em diversos pontos em arrastos verticais do fundo a superfície. Após observações em microscopia eletrônica, cinco espécies foram encontradas: *P. australis* Frenguelli, *P. calliantha* Lundholm, Moestrup & Hasle, *P. linea* Lundholm, Hasle & G. A. Fryxell, *P. multiseries* (Hasle) Hasle e *P. pungens* (Grunow ex Cleve) Hasle, além da variedade *P. pungens* var. *cingulata* Villac. As espécies *P. calliantha* e *P. multiseries* foram confirmadas como tóxicas, e ocorreram em densidades elevadas nas amostras analisadas, aumentando a preocupação quanto ao risco de contaminação dos moluscos utilizados comercialmente na região. Variabilidade significativa ocorreu quanto à estrutura da valva em *P. calliantha* e *P. pungens*, em alguns casos confirmadas na literatura, e sugerindo a possível presença de espécies semicrípticas. Em *P. calliantha* dois morfótipos foram bastante conspícuos, diferenciados pela largura da valva, número de poróides por estria, e forma da valva. *P. linea* é nova citação para o Brasil, e *P. pungens* var. *cingulata* é inédita para o Atlântico Sul Ocidental. Investigações futuras utilizando técnicas de biologia molecular poderão esclarecer se a variabilidade genética corresponde á variação morfológica observada e desvendar a presença de espécies semicrípticas nas populações de *Pseudo-nitzschia* no Sul do Brasil.

Palavras-chave: *Pseudo-nitzschia*, taxonomia, ácido domóico, sul do Brasil

INTRODUCTION

The diatom genus *Pseudo-nitzschia* H. Peragallo in H. & M. Peragallo comprises a small group of more than 32 marine species distributed worldwide (Thessen, 2007; Amato & Montresor, 2008). Though predominantly cosmopolitan, some species appears to occur only in temperate, tropical or polar waters (Hasle, 2002; Fryxell & Hasle, 2003). The genus was created by H. Peragallo in Peragallo *et* Peragallo (1900) based on *Nitzschia* Hassall species. Afterwards, it was reduced to a section of Nitzschia by Hustedt (1958). Later, Hasle (1994) raised *Pseudo-nitzschia* back to the genus level, taking into account specific morphological features like stepped chains, eccentric raphe system not elevated above the valve face by a keel, and absence of poroids in the walls of the raphe canal. Hasle´s proposal was later supported by molecular data (e. g. Lundholm *et al.*, 2002). Some species like *P. calliantha, P. multiseries, P. pungens* and *P. pseudodelicatissima* reach high concentrations in coastal areas and may eventually form blooms (Kaczmarska *et al.*, 2003).

The interest in this small group of raphid diatoms has increased since the discovering of toxin production by *P. multiseries* during a bloom in Prince Edward islands, Canada in 1987. This first report of a domoic acid poisoning resulted in three people died and about one hundred developed poisoning symptoms. The toxin domoic acid (DA) causes the Amnesic Shellfish Poisoning (ASP) which may affect birds, marine mammals and human beings. The main symptoms are nausea, vomiting, diarrhea and abdominal cramps, but neurological symptoms can in severe cases cause headache, hallucinations, loss of short memory and respiratory malfunctions, and rarely even coma and death. Since 1987, many investigations dealing with taxonomic, toxicological and ecological aspects of *Pseudo-nitzschia* have been published. More recently, molecular tools have been applied to investigate the phylogenetic relationships among the species and the relatedness among populations from different areas in micro- as well as macroscale perspectives (Lundholm et al., 2003; 2006; Casteleyn et al., 2008). Presently, eleven *Pseudo-nitzschia* species have been reported to produce domoic acid (Fryxell & Hasle, 2003; Thessen, 2007).

Pseudo-nitzschia is a common to abundant genus in Brazilian waters, mainly during late spring and summer seasons, when several species can reach concentrations above one million cells per liter (Villac & Tenembaum, 2001; Odebrecht *et al.*, 2001; Fernandes & Brandini, 2004; Mafra *et al.*, 2006; L. F. Fernandes, unpublished data). Though blooms have been recorded in southeast and south Brazilian waters, until 2009 outbreaks of ASP had not been reported in any coastal area of Brazil, including areas used for marine farming. However, in January 2009 the first report of DA concentrations up to 20mg kg^{-1} mussel meat, thus harmful to humans, was recorded in two mussel farms in Santa Catarina state (L. A. Proença, unpublished data). This led to closure of the farms for 25 days. This event raised the concern that ASP could be a potential threat to the marine farms exploiting oysters and mussels for commercial purposes. It is important to consider that all of the South Brazilian coastline and its shelf waters share similar oceanographic conditions, and therefore the risk for harmful *Pseudo-nitzschia* blooms account to the whole region. In addition, in many areas mollusk farming has increased for the last years, a fact that further stress the potential threat posed by *Pseudo-nitzschia* blooms in these areas.

In this study the results of a taxonomic survey of the potentially toxic diatom *Pseudo-nitzschia* in the Paraná and Santa Catarina coasts are presented along with a brief review of the previous studies done in the region.

MATERIAL AND METHODS

The field material was gathered from three different research projects and some occasional samplings carried out in different years along the Paraná and Santa Catarina’s coastline (Fig. 1, Tab. 1).

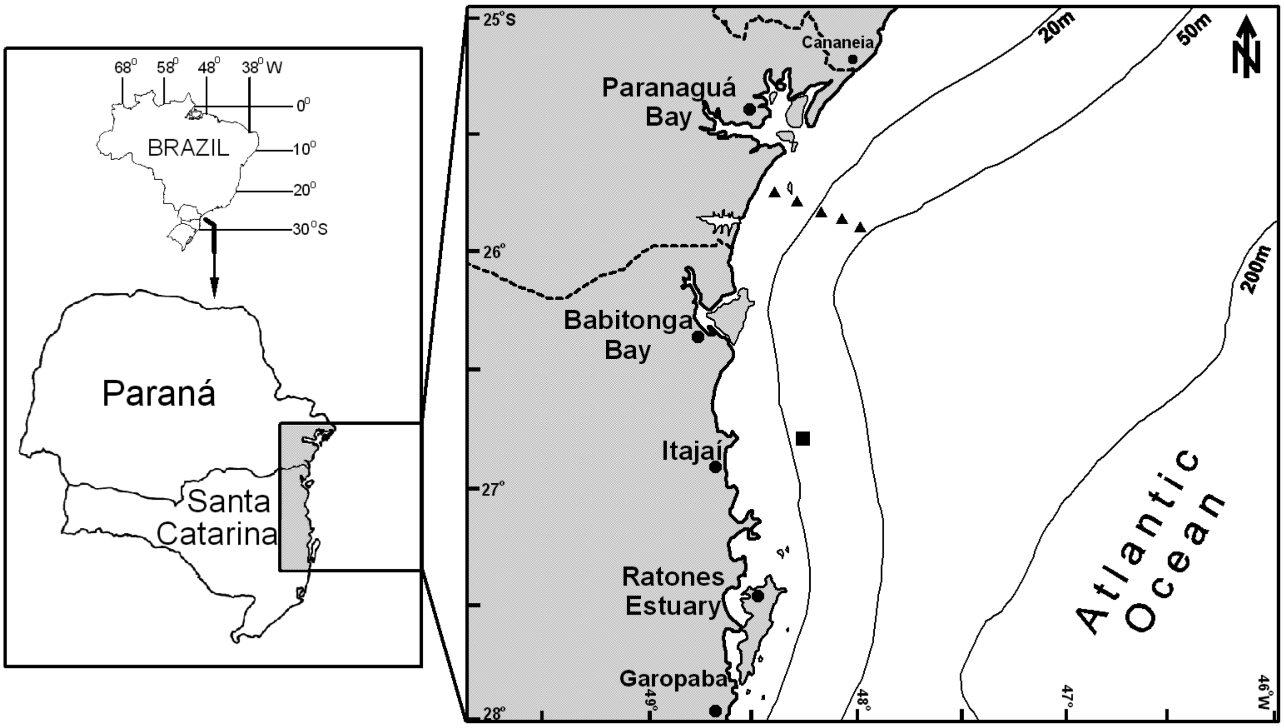


Fig. 1. Map of study region with the approximate locations of the sampling stations. Detailed locations are showed for stations over the Paraná shelf waters (▲) and Itajaí, Santa Catarina (■).

In the shelf waters of Paraná coast, samples were taken monthly in 5 stations with different bottom depths (between 10 and 40 meters) from August 1997 to March 1999. Vertical towings were made from bottom to surface using plankton net with 25 µm mesh size.

In Paranaguá bay, Paraná, samples were taken from 5 stations based on a salinity gradient previously established in other studies (Lana et al., 2001). A 20µm mesh size plankton net was towed from the bottom to the surface in all stations at monthly intervals from August 2002 to September 2003. The samples were screened alive to record delicate or abundant species, and preserved in formaldehyde 2% final concentration.

Occasional samplings were carried out in different locations along the Santa Catarina coast from November 2005 to January 2007: Itajaí city, Ratones River estuary in Florianópolis city, Garopaba and Bay of Babitonga, São Francisco do Sul City (Tab. 1). The original field material and the permanent slides are kept in the Laboratory of Phycology, Department of Botany, Federal University of Paraná, Brazil.

The frustules were cleaned following the technique of Hasle & Fryxell (1970). Permanent slides were prepared using Naphrax (refractive index = 1.74) as mounting media and observed by Light Microscopy (LM) using a Olympus IX-70 equipped with phase and differential contrast systems. A Philips LX30 scanning electron microscope (SEM) was used at 10-20 KV acceleration voltages. For Transmission Electron Microscope (TEM) observations, a small drop of cleaned or distilled water washed material was gently placed onto 150 mesh nickel grids coated with Formvar and Carbon. Grids were air dried and kept in desiccators until the TEM sessions with a Jeol JM120 EXII electron microscope. Terminology followed Hasle et al. (1996), Fryxell & Hasle (2003) and Lundholm *et al.* (2003).

RESULTS

A total of five *Pseudo-nitzschia* species and one variety (Tab. 2) was found in Paraná: *P. australis, P. calliantha, P. linea, P. multiseries, P. pungens* var. *pungens* and *P. pungens* var. *cingulata*. A summary of morphometric features of the 5 species is showed in Table 2. Two of them were exclusively found in the shelf waters off the Paraná coast: *P. linea* and *P. australis* that is, not observed in Paranaguá Bay.

TABLE 1 - Station Locations, Deplit and Intervals of Sampliups use in this study.

Station Locations	Station Labels	Coordinates	Depth at Station	Intervals of Samplings
Shelf Waters of Paraná	E1	25°42’60”S 48°27’60”W	10.0 m	Aug/1997 – Mar/1999
Shelf Waters of Paraná	E2	25°44’10”S 48°21’55”W	15.0 m	Aug/1997 – Mar/1999
Shelf Waters of Paraná	E3	25°46’27”S 48°12’10”W	20.0 m	Aug/1997 – Mar/1999
Shelf Waters of Paraná	E4	25°48’05”S 48°04’85”W	25.0 m	Aug/1997 – Mar/1999
Shelf Waters of Paraná	E5	25°50’20”S 47°55’75”W	40.0 m	Aug/1997 – Mar/1999
Paranaguá Bay, Buoy 12	E1	25°33’63”S 48°20’53”W	13.0 m	Aug/2002 – Sep/2003
Paranaguá Bay, Maciel River	E2	25°33’69”S 48°25’48”W	3.0 m	Aug/2002 – Sep/2003
Paranaguá Bay, Port of Paranaguá	E3	25°29’05”S 48°31’39”W	2.2 m	Aug/2002 – Sep/2003
Paranaguá Bay, Itiberê River	E4	25°30’93”S 48°29’88”W	2.3 m	Aug/2002 – Sep/2003
Paranaguá Bay, Europinha	E5	25°33’96”S 48°38’02”W	1.2 m	Aug/2002 – Sep/2003
Paranaguá Bay, Antonina city	E6	25°24’18”S 48°42’24”W	1.0 m	Aug/2002 – Sep/2003
Itajaí, Santa Catarina	SM-I-1	26°55’00’S 48°34’00”W	20.0 m	10/Nov/2005
Itajaí, Santa Catarina	SM-II-1	26°55’00’S 48°34’00”W	20.0 m	16/Jan/2006
Bay of Babitonga, São Francisco	--	26º14´05”S 48º39´14”W	2.3 m	28/Jan/2007
Ratones R. estuary, Florianópolis	--	27°27´45”S 48°32´11”W	1.4 m	23/Jan/2007
Garopaba, Santa Catarina	--	28°01´00”S 48°37´32”W	2.8 m	16/Jan/2007

P. australis occurred in May and July exclusively. The remaining species were very abundant from November to February, and almost absent during the remaining months.

In Santa Catarina only *P. calliantha, P. multiseries* and *P. pungens* were found, most likely reflecting the paucity of samplings when compared to the number in Paraná state. The three species occurred in all the samples taken in Sao Francisco, Itajaí, Florianópolis and Garopaba. Different morphotypes of *P. pungens* were found in Itajaí regarding poroids structure.

Pseudo-nitzschia australis Frenguelli *in* Diatomeas del Golfo de San Matías. *Rev. Museo de La Plata*, N.S., (Bot.) v. 2 n. 10, p. 201-226. 1939.

(Figs. 2-14)

The valves are lanceolate with subrostrate apices (Figs 2, 3, 5). Valve shape is clearly asymmetrical, with one margin almost straight and other convex (Figs 3, 6). Apical axis counts 70.4-84.0 µm; transapical axis 7.5-8.0µm. The raphe system is marginal and continuous, that is, no central nodule is present (Fig. 7). Fibulae are regularly spaced with a density of 12-

TABLE 2 - Morphometric data of *Pseudo-nitzschia* species found in coastal waters of South Brazil.

	P. australis	*P. calliantha* (morphotype 1)	*P. calliantha* (morphotype 2)	*P. linea*	*P. multiseries*	*P. pungens*	*P. pungens var. cingulata*
Valve shape	Lanceolate	Linear, gradually tapering	Linear, tapering near the apices	Linear	Linear to lanceolate	Linear to lanceolate	Linear to lanceolate
Length (µm)	70.4-84.0	81-110	60-105	17-18	74-123	84-165	89-122
Width (µm)	7.5-8.0	4.0-5.0	1.6-2,2	2.5-3.0	3.0-5.0	3.0-5.0	3.0-4.0
Interstriae/10µm	12-19	22-26	35-37	32-34	9-13	13-16	12-14
Fibulae/10µm	12-17	11-18	18-21	20-24	10-14	13-18	13-14
Poroids/1µm	4-5	5-6	4-5	10-12	5-6	2-3	3-4
Rows of poroids	2	1	1	2	3-5	2	2
Central interspace	absent	present	present	absent	absent	absent	absent

17 in 10µm (Figs 8, 9). Terminal fissures are simple, surrounded by a discrete helictoglossa (Fig. 9). Valve striae count 12-19 in 10µm; stria contains two rows of poroids (Fig. 10). Poroids are rounded, 4-5 in 1 µm, placed close to the interstriae, leaving a hyaline area between the two rows (Fig. 10). In the middle of the valve, poroids of the two rows alternate with each other. At the apices the interstriae vary, some are modified into branches reaching the valve edge, along with accompanying poroids (Figs 11-13); others are not branched (Fig. 14).

Occurrence in the samples: Paraná shelf waters (E1, E2, E3, May and July 1998).

Comments: No morphometric differences were observed between our material and records from elsewhere (Hasle et al., 1996; Fryxell & Hasle, 2003 and references therein) and nearby coastal waters of Argentina, where the valves were 70-116µm long, 6.5-10µm wide with 12-19 striae in 10µm (Ferrario et al., 1999; 2002; Sunesen et al., 2009).

Pseudo-nitzschia calliantha Lundholm, Moestrup & Hasle (morphotype 1) *in* A study of the *Pseudonitzschia pseudodelicatissima/cuspidata* complex (*Bacillariophyceae*): what is *P. pseudodelicatissima*? **J. Phycol**. v. 39, p. 797-813. 2003.

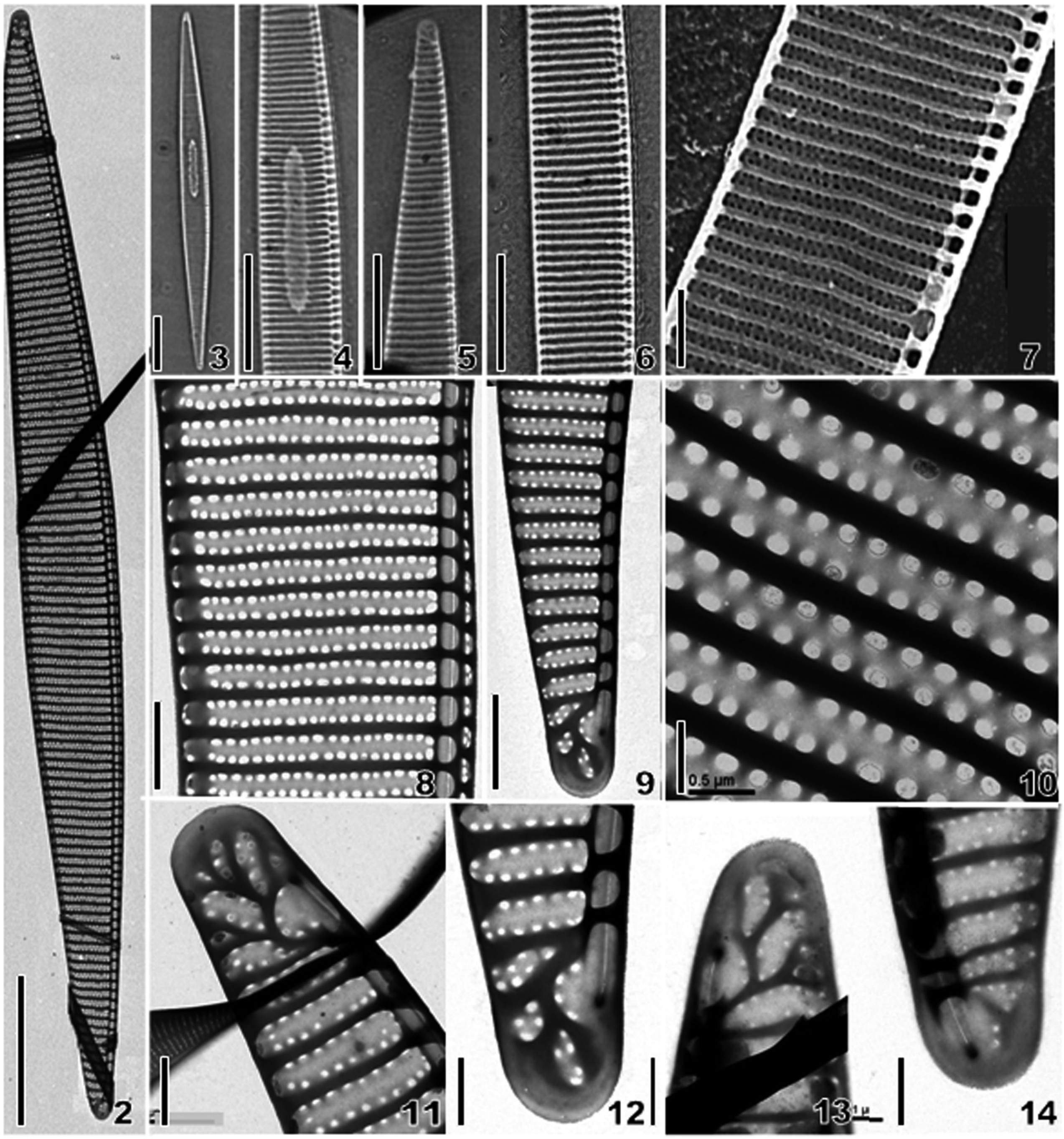
(Figs. 15-26)

Valves are linear to lanceolate with parallel sides and subacute apices (Figs 15-18); 81-110µm apical axis; 4.0-5.0µm transapical axis. Striae and poroids are only faintly resolved in phase contrast, but conspicuous under polarized contrast (Fig. 16). Valve surface is striated; 22-26 in 10µm. Each row of poroids has 5-6 poroids in 1µm (Figs 19-22). Poroid is perforated by 6-9 sectors, usually surrounding a central one (Fig. 23). Raphe system marginal composed of two branches. Central nodule is placed in the larger central interspace. Terminal endings are simple, surrounded by helictoglossa (Figs 21-23). Fibulae count 11 to 18 in 10µm. Mantle very shallow, bearing one simple row of longitudinal elongated poroids (Fig. 23). Cingulum has 3-4 narrow bands (Figs 23, 24). Valvocopula and adjacent band have similar construction. Interstriae separate groups of three rows of poroids, each row with 4-6 poroids, except for the middle row with 2-4 poroids (Figs 25, 26a). The third and fourth bands have a fewer number of poroids, usually two in each row (Fig. 24).

Occurrence in the samples: Paraná shelf waters (E1, E2, E3; November to December, February and March 1998 and 1999), Paranaguá Bay (E1, E3, E5; January and February 2003), Itajaí (SM-I and SM-II), Ratones River, Bay of Babitonga, Garopaba.

Comments: The morphotype 1 differs from the original material of *P. calliantha* (see Lundholm *et al.*, 2003) for the latter is narrower (width 1.4-1.8µm) and has a higher density of interstriae (34-39 in 10µm) than in our material.

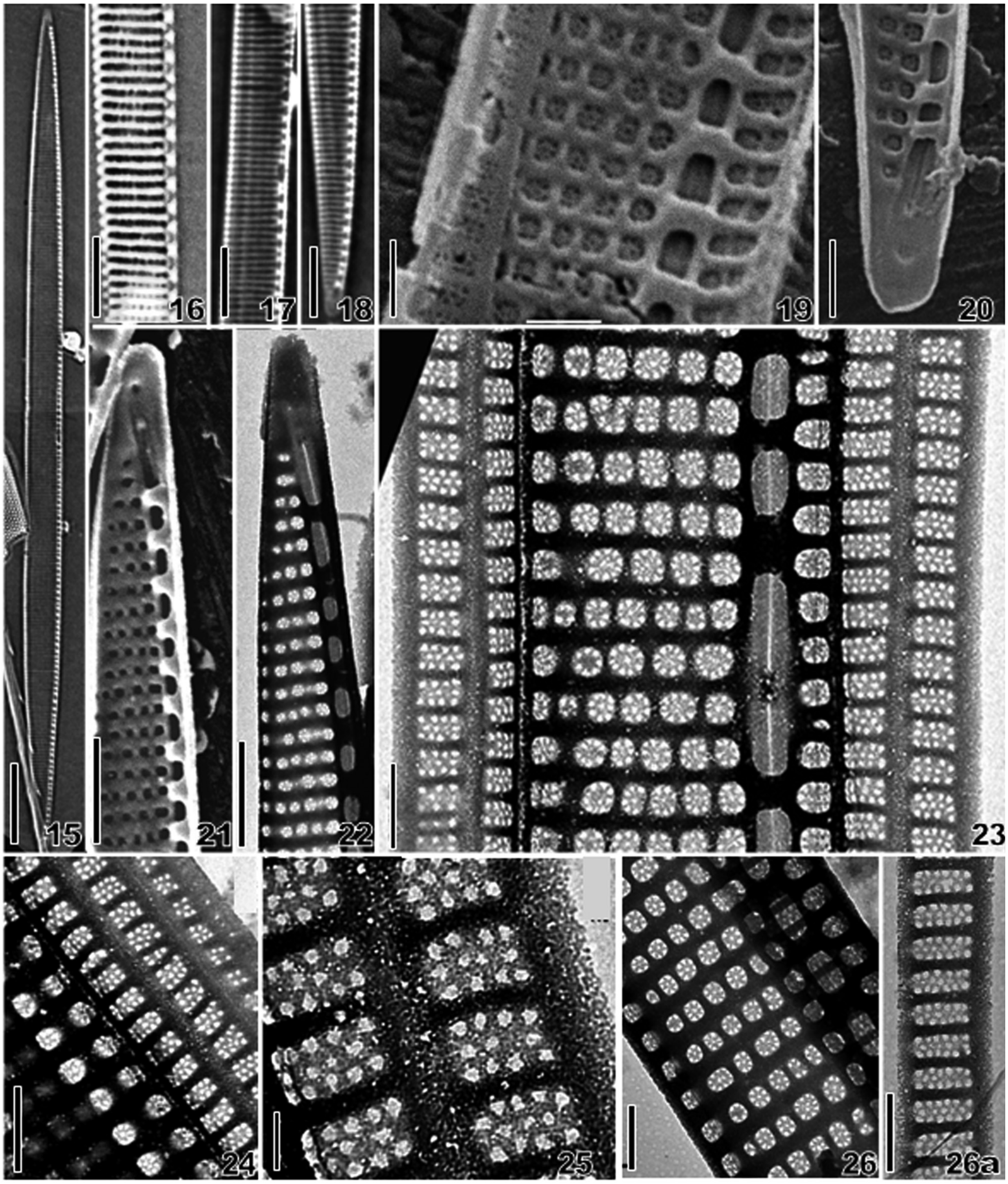
Material from Itajaí (Figs 26, 26a) has wider interstriae on the valve; in other words, the striae are more spaced one to each other, and poroids are almost quadrangular. The height of the valvocopula is higher than in the material from the Paraná coastal water (Figs. 15-25). Additionally, the interstria separates groups of three rows of poroids in the valvocopula, the external ones with six poroids, and the central one with three poroids.



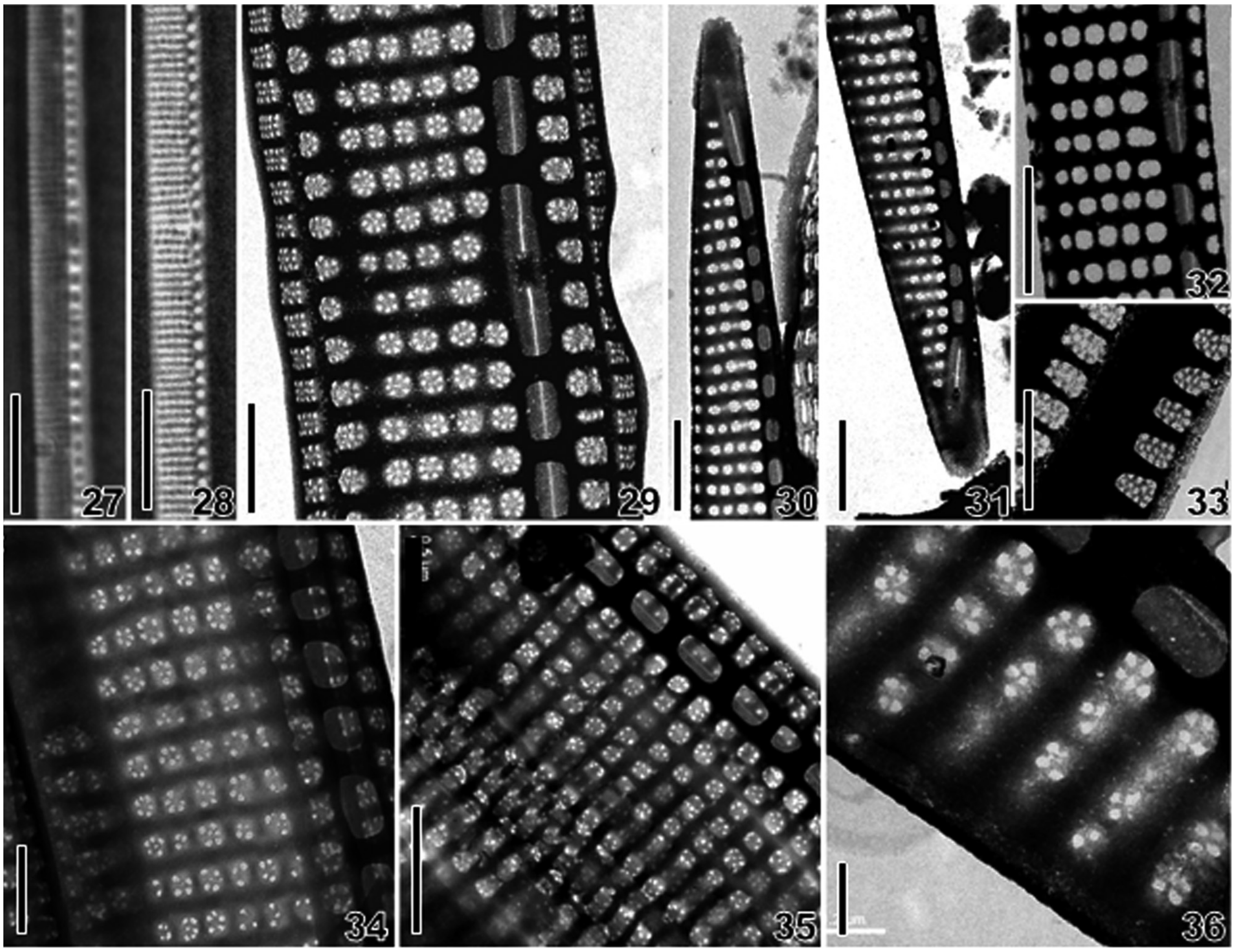
Figs. 2-14. *Pseudo-nitzschia australis*; valve views; **2.** Apically asymmetric valve (TEM); **3-6.** Valve views in different foci (LM); **7.** Central region; SEM; **8.** Central region. Note matching between interstriae and fibulae; **9.** Apical region; **10.** Detail of poroids arranged in two striae; **11-14.** Apices of two different valves illustrating branched interstriae. Scale bars: **Figs. 2-6** = 10 µm; **Figs. 7-9** = 2 µm; **Fig. 10** = 0.5 µm; **Figs. 11-14** = 1 µm.

Pseudo-nitzschia calliantha Lundholm, Moestrup & Hasle (morphotype 2)
(Figs. 27-36)

Valves are linear lanceolate with parallel sides (Figs 27, 28), turning convergent in the last one third of the valve (Figs 30, 31); apical axis 60-105µm, transapical axis 1.6-2.2µm. Valve surface is striated; 35-37 in 10µm. Each stria is composed of rounded to subrectangular poroids; 3-5 per stria (Fig. 29). Poroids are not visible under standard LM or phase contrast, except when using polarized contrast (Fig. 28). They are only discernible in SEM, but best resolved in TEM. Each poroid is perforated by 5-6 (rarely 8-9) small openings (Fig. 29). Mantle is very



Figs. 15-26. *Pseudo-nitzschia calliantha* morphotype 1. **15**. Valve with parallel sides (LM); **16**. Valve center with conspicuous poroids; differential contrast; **17, 18**. Valve views (LM). Note larger interspace in Fig. 17; **19-21**. Internal views (SEM); **22**. Detail of apex (TEM); **23**. Central region showing central nodule and larger interspace. Note girdle bands; **24, 25**. Girdle bands in detail. **26, 26a.** Material from Itajaí. **26**. Valve with wide interstriae; **26a**. Valvocopula with three and six rows of poroids. Scale bars: **Fig. 15** = 10 µm; **Figs. 16-18** = 5 µm; **Figs. 16, 20, 23, 24, 26, 26a** = 0.5 µm; **Figs. 21, 22** = 1 µm; **Fig. 25** = 0.2 µm.



Figs. 27-36. *Pseudo-nitzschia calliantha* morphotype 2; 27, 28 (LM); 29-36 (TEM). **27, 28**. Central regions of valve. Larger central interspace is conspicuous; **29**. Central region with nodule and striae composed of 3-4 poroids; **30, 31**. Two apices of a same valve; **32**. Thinner valve from Itajaí material; **33**. Detail of two bands; **34-36.** Valve view of specimens with variable number of sectors in the poroids. Scale bars: **Figs. 27-28** = 4 µm; **Figs. 29, 34** = 0.5 µm; **Figs. 30-32, 35** = 1 µm; **Fig. 33** = 0.8 µm; **Fig. 36** = 0.2 µm.

shallow having one longitudinal row of slightly elongated poroids (Fig. 29). The raphe system is marginal with two branches; central nodule well delimited by a larger interspace (Fig. 33). The central endings are simple; terminal endings surrounded by helictoglossa (Figs 30, 31). Fibulae count 18 to 21 in 10 µm; interspaces irregularly placed. The valvocopula has groups of three rows of poroids separated by silica ribs (Fig. 33). Other bands were not observed.

Occurrence in the samples: Paraná shelf waters (E1, E2, E3; November to December 1998 and 1999).

Comments: The morphotype 2 agrees fairly well with the morphological features of P. calliantha´s original material (Lundholm *et al.*, 2003). Only the width is slightly higher in morphotype 2 than in the latter (1.4-1.8µm).

Besides the two morphotypes described above, certain variability regarding the areola structure was recorded as well. Some valves (Figs. 34-36) showed either poroids with a smaller number of perforations (2-5) and poroids bearing 6-9 perforations. Finally, several bands presented larger hyaline strips, occupying about the half of the width of the band. The remaining half was composed of a row of longitudinal poroids.

Pseudo-nitzschia linea Lundholm, Hasle & G. A. Fryxell *in* Morphology, phylogeny and taxonomy of species within the *Pseudo-nitzschia americana* complex (*Bacillariophyceae*) with descriptions of two new species, *Pseudo-nitzschia brasiliana* and *Pseudo-nitzschia linea.* **Phycologia**, v. 41, n. 5, p. 480-497. 2002.

(Figs. 37-45)

Cells are solitary or in short chains of 2-3 cells (Fig. 37); some chains were epiphytic on setae of *Chaetoceros* sp.. Frustules are weakly silicified. Valves are linear (Fig. 38) with blunted apices, almost quadrangular (Figs 39, 40); 17-18 µm apical axis, 2.5-3.0 µm transapical axis. The valve surface is striated (Figs 41, 42); 40-44 striae in 10µm forming double rows of poroids enclosed by interstriae. Poroids are almost indistinct due to the poor valve silicification; 10-12 in 1µm. Near the apices, the interstriae are branched and arranged obliquely, occupying all the apex area (Fig. 43). Raphe system is marginal, continuous, with fibulae counting 20-24 in 10 µm, leaving irregularly interspaces between each other (Fig. 44). Two interstriae are merged together in each fibula (Fig. 45). Cingulum is composed of 3-4 bands (Fig. 43). No band structure could be observed for the valves were weakly silicified.

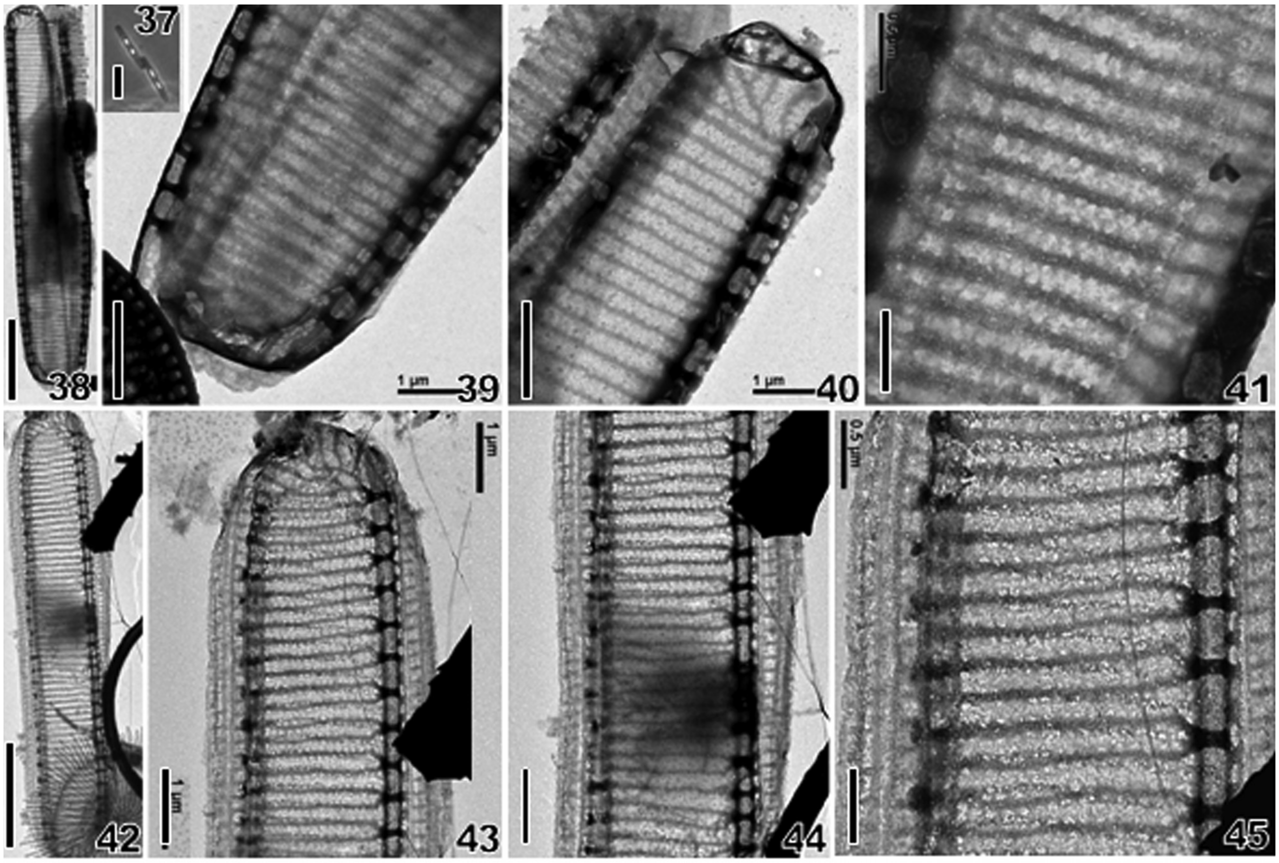
Occurrence in the samples: Paraná shelf waters (E1, E2, E3; November to December 1998 and 1999), Paranaguá Bay (E3, E5; January and February 2003).

Comments: Our specimens matched with those found by Lundholm *et al.* (2002) by having the same linear valve shape and similar dimensions of apical and transapical axes. The number of fibulae was higher than the reported in Lundholm's (18-22 in 10µm). *P. americana* (Hasle) G. A. Fryxell *in* Hasle is closely related to *P. linea*, but its valves are linear lanceolate, larger (16-42µm) and wider (2.5-4.0µm) than in *P. linea* (Lundholm *et al.*, 2002).

Pseudo-nitzschia multiseries (Hasle) Hasle *in Pseudo-nizschia pungens* and *P. multiseries* (*Bacillariophyceae*): nomenclatural history, morphology and distribution. **J. Phycol.** v. 31, p. 428-435. 1995.

(Figs. 46-58)

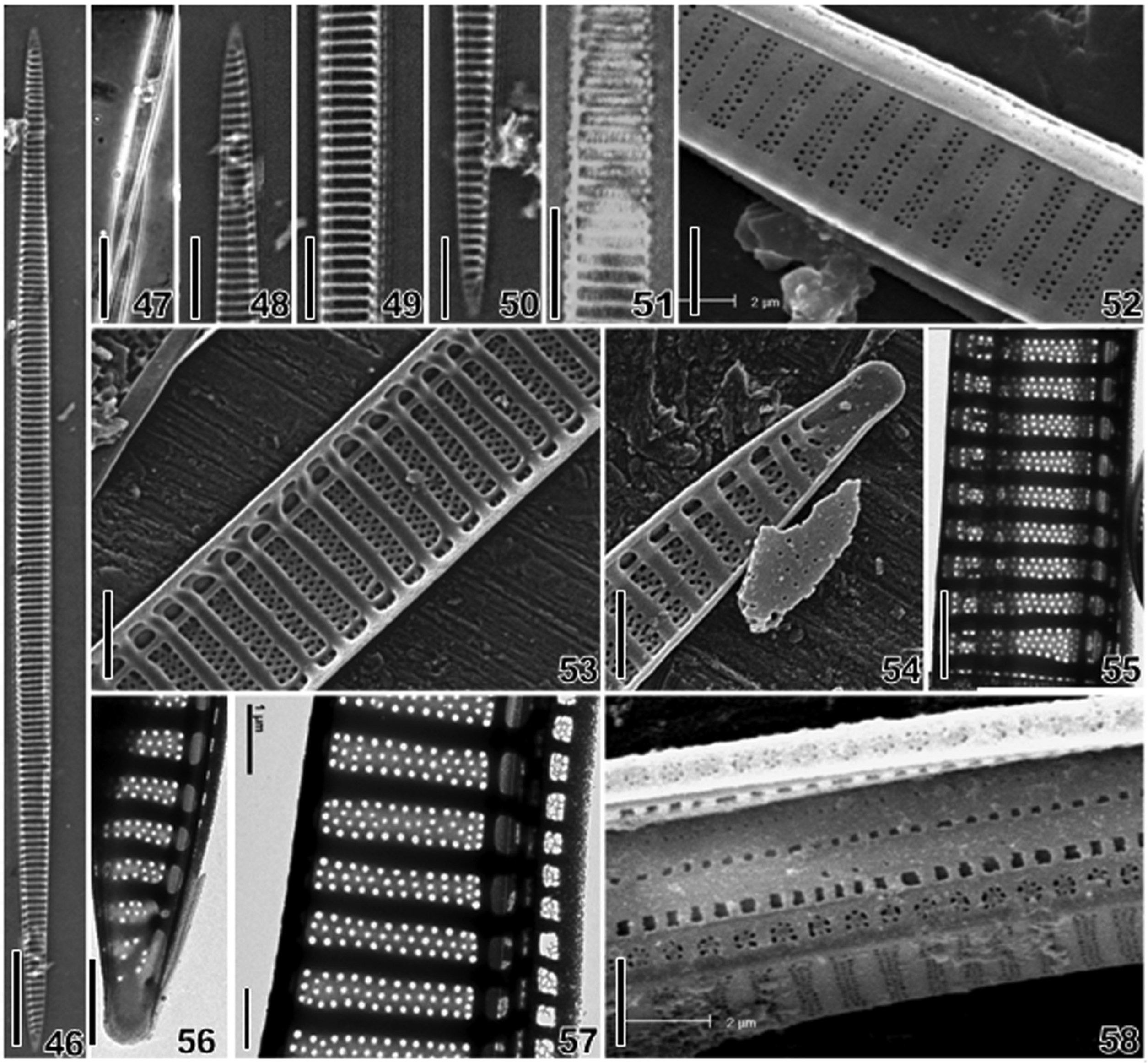
Valves are linear-lanceolate (Fig. 46) with acute apices (Figs 47, 48, 50); 74-123µm apical axis, 3.0-5.0µm transapical axis. Interstriae are visible in



Figs. 37-45. *Pseudo-nitzschia linea*; 37. Two-celled colony (LM); **38-45** (TEM). **38**. General view of frustule; **39, 40**. Different views of apex; **41**. Central region; 42. Valve view; 43, 44. Valve view, showing interstriae and fibulae; **45**. Detail of central region. Each fibula matches two merging interstriae. Scale bars: **Fig. 37** = 15 µm; **Figs. 38, 42** = 5 µm; **Figs. 39, 40, 43, 44** = 1 µm; **Figs. 41, 45** = 0.5 µm.

L.M. but not the striae (Fig. 49). Under the polarized contrast, the poroids are seen like transverse striae regarding the interstriae (Fig. 51). Striae are composed of small poroids, and organized in groups of 3-5 (rarely 1-2) rows of poroids enclosed by interstriae (Figs 52, 55). Poroids count 5-6 in 1µm. Interstriae are visible under LM; 9-13 in 10µm. Each interstria is coincident with a fibula (Fig. 53). Raphe system is marginal, continuous, with simple terminal fissures (Figs 54, 56). Fibulae are regularly spaced to each other, leaving equal sized interspaces (Figs 53, 54); 10-14 in 10µm. The shallow mantle has 5-6 poroids arranged in circular pattern. Cingulum has a variable number of bands, each one with single rows of longitudinal poroids (Fig. 58). At least the valvocopula has each poroid divided in sectors by a delicate silica membrane (Fig. 57).

Occurrence in the samples: Paraná shelf waters (E1, E2, E3; November to December, February and March 1998 and 1999), Paranaguá Bay (E1, E3, E5; January and February 2003), Itajaí (SM-I and SM-II), Ratones River, Bay of Babitonga, Garopaba.



Figs. 46-58. *Pseudo-nitzschia multiseries*; **46-51** (LM). **46**. General valve view; **47**. Overlapping of two cells; **48-51**. Details of a same valve. In Fig. 51, striae appear like in longitudinal pattern; **52, 54, 58** (SEM); **52**. External view; **53, 54**. Internal views. Note prominent interstriae; **55-57** (TEM); **55**. Central region. **56**. Apex of valve; **57**. Detail of center. Valvocopula is at right; **58**. Frustule in girdle view, exposing mantle poroids and bands of cingulum. Scale bars: **Fig. 46** = 10 µm; **Fig. 47** = 20 µm; Figs. **48-51** = 5 µm; **Figs. 52-56, 58** = 2 µm; **Fig. 57** = 1 µm.

Pseudo-nitzschia pungens (Grunow *ex* Cleve) Hasle *in* Nomenclatural notes on marine planktonic diatoms. The family *Bacillariaceae*. **Nova Hedwigia**, Beiheft n. 106, p. 315-321. 1993.

(Figs. 56-67, 69-71, 73)

Valves are linear-lanceolate with acute apices (Figs 59-62), 84-165µm apical axis; 3.0-5.0µm transapical axis. Sides gradually converge in the last one third of the valve. Poroids are visible under phase contrast (Fig. 61) and polarized light (Fig. 63) in most of the valves. Valvar surface has striae composed of two rows of poroids bordered by heavily silicified interstriae (Figs 64, 65). The interstriae are approximately coincident with the fibulae, 13-16 in 10µm. Poroids are in contact with the interstriae, and arranged in opposite sides to each other (Fig. 60). In some valves, an alternate pattern was recorded (Figs 68, 69). The number of poroids (2-3 in 1 µm) in each row was quite variable, due to the different sizes of poroid itself (Figs 66-69, Fig. 68 for *P. pungens cingulata*, see below). Poroids also were missing in many striae in a few valves (Figs 70, 71). Raphe system is continuous, marginal, with fibulae regularly spaced and about the same size, 13-18 in 10µm (Figs 64, 65). Mantle is shallow, composed of one single row of longitudinal elongated poroids, each one with one transverse silica bar. Cingulum has 3-6 bands, each one with one longitudinal row of poroids. Valvocopula has one longitudinal row of simple poroids, 17-18 in 10µm (Fig. 73), or divided into more sectors (Fig. 72 for *P. pungens cingulata*, see below).

Occurrence in the samples: Paraná shelf waters (E1, E2, E3; December, February and March 1998; November to March 1999), Paranaguá Bay (E1, E3, E5; November to February 2003, April 2003), Itajaí (SM-I and SM-II), Ratones River, Bay of Babitonga, Garopaba.

Comments: The morphometric characters of *P. pungens* were very close to those found in the remaining Brazilian waters and the Argentinean coasts (Ferrario *et al.*, 2002).

Pseudo-nitzschia pungens (Grunow e Cleve) Hasle var. *cingulata* Villac *in Pseudo-nitzschia pungens* var. *cingulata* var. nov. (*Bacillariophyceae*) based on field and culture observations. **Phycologia**, n. 4, p. 269-274. 1998.

(Figs. 68, 72)

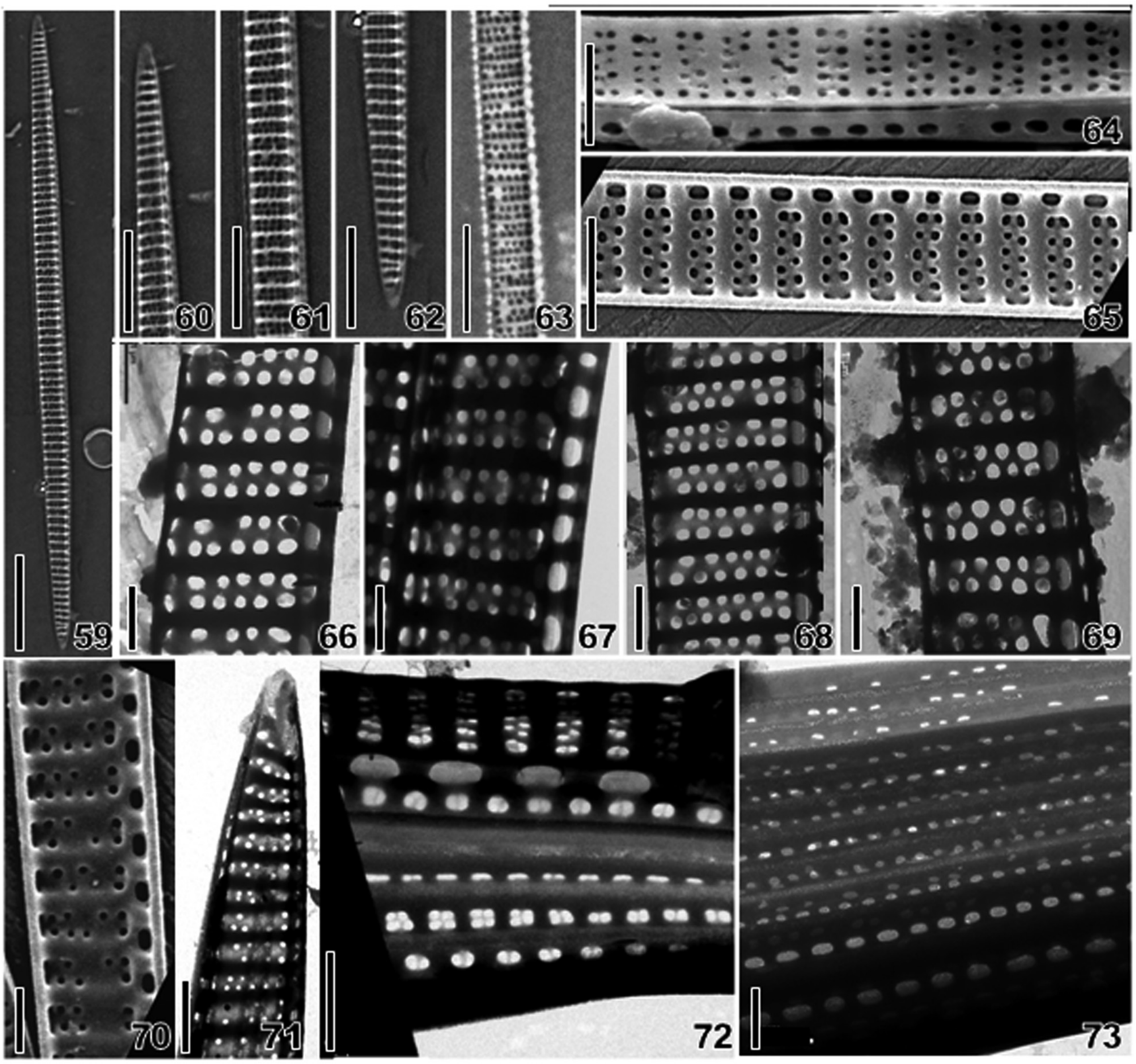
Valves are linear to lanceolate with acute apices, 89-122µm apical axis; 3.0-4.0µm transapical axis; 12-14 interstriae in 10 µm; two rows of 3-4 poroids in 1µm (Fig. 68); 13-14 fibulae in 10µm, equally spaced. Cingulum has 2-3 bands (Fig. 72). Valvocopula (Fig. 72) has 21-25 quadrangular poroids in 10µm, each one divided into 2 or 4 sectors by silica bars (or rotae after Casteleyn *et al.*, 2008).

Occurrence in the samples: Paraná shelf waters (E1, E2; February and March 1998), Paranaguá Bay (E5; February 2003).

Comments: The main differences between these varieties are the number of poroids in 10 µm on the valvocopula, larger in *P. pungens* var. *cingulata* than in *P. pungens*, and the number of rows of poroids in the bands, one in *P. pungens* (see review of Sar et al., 2006) and 2-4 in *P. pungens* var. *cingulata* (Villac *et al.*, 1998). Our material identified as *P. pungens* var. *cingulata* agrees quite well with the measurements of original material described by Villac et al. (1998). To date, We have interpreted the poroids of the valvocopula as one poroid occluded by rotae, usually comprising 2 or 4 radial bars, a view also followed by Casteleyn *et al.* (2008). The morphological variability recorded in our *P. pungens* material matches the Clade I (for *P. pungens* nominate variety) and Clade II (for *P. pungens* var. *cingulata*) of Casteleyn *et al.* (2008). Only the valve illustrated in our Fig. 69 differs from their material for the poroids have a conspicuous alternate pattern. Hallegraeff (1994, his figs 3d-3h) also observed a high variation regarding the number of poroids and their pattern in the striae.

DISCUSSION

The genus *Pseudo-nitzschia* has been one of the most intensively studied diatom genera regarding its molecular biology and phylogenetics due the production of toxin by some species. Four species recorded in this work were found to be toxic in the literature: *P. australis, P. calliantha, P. multiseries* and *P. pungens*. *P. calliantha* and *P. multiseries* can produce high levels of domoic acid, and have been responsible for contaminating mussel grounds and killing birds and cetaceans around the world. In fact, some strains isolated from the same material we analyzed in this work proved to be toxic (Mafra *et al.*, 2006), raising concerns about the potential threat to the ecosystem and commercial fisheries in Paranagua bay. Many small mussel and oyster grounds are used by about 1,000 local fishermen families, granting their subsistence. *P. calliantha* was very abundant or dominant among the phytoplankton in the Paraná shelf waters from 1997 to 1999 in early



Figs. 59-73. 56-67, 69-71, 73 *Pseudo-nitzschia pungens;* **68, 72** *P. pungens* var. *cingulata*. **59-63.** Pictures of a same valve (LM). Two rows of poroids are readily recognizable; **64**. External view, also showing a shallow mantle (SEM); **65**. Internal view; **66-69**. Examples of different poroid densities and arrangements: opposite in Figs 66-68, and alternate in Fig. 69; **70, 71**. Internal views illustrating rows with missing poroids; **72**. Frustule in girdle view showing elongate poroids of mantle and cingulum. Band has one row of poroids divided into 2 or 4 sectors; **73**. Frustule in girdle view showing bands with single rows of elongated poroids, particularly the valvocopula. Scale bars: **Fig. 59** = 10 µm; **Figs. 60-63** = 5 µm; **Figs. 64, 65** = 2 µm; **Figs. 66-73** = 1 µm.

summer (Fernandes *et al.*, 2008) reaching densities up to one million cells/liter. In Paranaguá Bay, Mafra *et al.* (2006) isolated cells that came out toxic. A yet not identified species of the *P. pseudodelicatissima* complex also occurred in blooms in Santa Catarina state in January 2009, contaminating commercial mollusks in some mussel grounds (L. A. Proença, personal communication). *P. multiseries* was a very abundant species during late summer 1998 in Paraná shelf waters, reaching concentrations about 0.5x10^6 cells/liter (Fernandes *et al.*, 2008).

P. australis is a potentially toxic species that has been found southwards along the Rio Grande do Sul state, Brazil (Odebrecht *et al.*, 2001) and Argentina coast (Ferrario *et al.*, 2002), usually reaching higher concentrations in late Autumn and Winter. A bloom of about 2 million cells per liter was recorded in 2007 in the same area where a massive mortality of 61 right whales (*Eubalaena australis)* took place in Peninsula Valdez, a nursery ground for whale calves (Uhart et al., 2008). Negri et al. (2004) also recorded a bloom (up to 2.8x104 cells/liter in July 2000) at a fixed station near Mar del Plata. At that time, domoic acid was detected in mussels (*Mytilus edulis*, 7.7μg/gram) and in commercial pelagic fish *Eugraulis anchoita* (4.9μg/gram).

Procopiak *et al.* (2007) reviewed the literature on *Pseudo-nitzschia* in Parana waters. In total, six species were reported: *Pseudo-nitzschia australis* Frenguelli, *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle, *Pseudo-nitzschia delicatissima* (Cleve) Heiden, *Pseudo-nitzschia multiseries* (Hasle) Hasle, *Pseudo-nitzschia pungens* (Grunow & Cleve) Hasle and *Pseudo-nitzschia seriata* (Cleve) H. & M. Peragallo. The presence of *P. delicatissima* and *P. seriata* were, however, not previously confirmed by electron microscope, a crucial tool to precisely identify the two species. This raises the possibility for misidentifications. Besides, taking into account that *P. seriata* is known to be restricted to the Northern Hemisphere (Hasle, 2002), the record of Moreira-Filho *et al.* (1990) for Paraná waters should most likely be incorrect. The remaining species were confirmed by electron microscope (like in Procopiak *et al.*, 2007). *P. pungens* and *P. delicatissima* have previously been reported in Santa Catarina waters (Moreira-Filho *et al.*, 1990), but not confirmed with electron microscopy.

Five species were found in our material from a total of ten species recorded in Brazilian Waters so far. They are *P. australis, P. brasiliana, P. delicatissima, P. fraudulenta, P. heimii, P. cf. multiseries, P. pseudodelicatissima* (most likely *P. calliantha*, see below), *P. pungens*, and *P. cf. subfraudulenta* (Moreira-Filho *et al.*, 1995 and 1999; Villac & Tenenbaum, 2001; Villac *et al.*, 2005; Odebrecht *et al.*, 2001). The species not found in the present study but occurring in neighbor waters northwards are *P. brasiliana, P. delicatissima, P. heimii* and *P. cf. subfraudulenta* (Villac & Tenenbaum, 2001; Villac *et al.*, 2005). During an extensive oceanographic cruise along the southward waters of Rio Grande do Sul state, Odebrecht *et al.* (2001) reported five species: *P. australis, Pseudo-nitzschia fraudulenta, P. multiseries, P. pungens* and *P. pseudodelicatissima* (most likely *P. calliantha*). *P. fraudulenta* was not detected in our survey. The size of the nets (25μm) used in the Paraná region and the limited number of geographical locations sampled might explain these discrepancies regarding the number of species. Therefore, it is expected that future studies might report new additions to the *Pseudo-nitzschia* flora in Parana and Santa Catarina. The only confirmed record of *P. calliantha* in Brazil can be found in Moreira (2004) during an ecological study in Cassino Beach, Rio Grande do Sul state. Other authors reported *P. pseudodelicatissima* in South Brazil (Odebrecht *et al.*, 2001) and in Argentina (Ferrario *et al.*, 1999, 2002). However, after the recent review and consequent splitting of *P. pseudodelicatissima* in three species (Lundholm *et al.*, 2003), it might be that the records of *P. pseudodelicatissima* in South America Atlantic waters actually correspond to *P. calliantha*. Unfortunately, It was impossible to ascertain its identity based on the pictures and description furnished in those papers, for the poroid structure of valve and cingulum are neither showed nor described. Conversely, the review of Ferrario *et al.* (2002) describes a "*P. pseudodelicatissima*", giving pictures that do not allow a positive identification. However, the species description quotes valvocopula striae with 4-6 poroids high and 2-3 poroids wide. This description matches the poroids of *P. calliantha* or *P. caciantha actually*. *P. pseudodelicatissima* has valvocopula with rectangular poroids not or quite rarely divided in four sectors (Lundholm *et al.*, 2003). Corroborating our arguments, Almandoz *et al.* (2007) stated that the species identification in Argentinean coast under the name "*P. pseudodelicatissima*" is in fact *P. calliantha*. Therefore, the South America material deserves a further reexamination to check out the true species identity under the *P. pseudodelicatissima* complex. To date, we contacted a number of authors of those papers and they confirmed that their material contained *P. calliantha*, not *P. pseudodelicatissima*. In conclusion, the presence of *P. pseudodelicatissima* can be ruled out in Brazilian and Argentinean waters so far.

During our investigations, great morphological variability was found in valve structure of *P. calliantha* and *P. pungens*, suggesting the possibility of semicryptic species, hidden in the Parana and Santa Catarina populations. Recent works have demonstrated a high genotypic variability in individuals sharing either the same environment or distinct localities. Lundholm *et al.* (2006) investigated populations of *P. delicatissima* from different regions, being able to recognize two new semicryptic species (*P. dolorosa* and *P. decipiens*) inside what had been formerly called *P. delicatissima*. In their study, both the morphological features of the valve and the ribosomal DNA sequencing aided to describe the species. *P. dolorosa* came out very well characterized morphologically, that is, with no or few metric overlapping regarding number of fibulae, poroids in 1µm, width and poroids on the bands. On the other hand, *P. delicatissima* and *P. decipiens* were similar in many of the valve features used to compare the three species (see Table 5 of Lundholm *et al.*, 2006).

Casteleyn *et al.* (2008) recognized three clades of *P. pungens* with specific DNA signatures and frustule morphology. Clade I is widespread geographically, while Clade II is exclusive of Pacific US waters, and Clade III contained rDNA sequences from Vietnam, Mexico and China. This latter were not examined regarding frustule structure by Casteleyn. However, Churro *et al.* (2009) later described the new variety *P. pungens* var. *aveirense* based on morphological and genetic features, which matched with the rDNA signature of Casteleyn´s Clade III, thus placing the variety aveirense in Clade III. Morphologically, Clades I and II differed markedly in structure of girdle poroids and in poroid density in the valve face. Clade I, corresponding to the nominate variety, had valvocopula with simple rectangular poroids and a smaller number of poroids per stria (2-3 in 1µm). In Clade II, the valve had a larger number of poroids (4-5 in 1µm) (see Fig. 4d of Casteleyn *et al.*, 2008). They also were small in size and quite close to the interstriae. The valvocopula had square to rectangular poroids occluded by two or four rotae. Clade II was identical to *P. pungens* var. *cingulata* described in Villac *et al.* (1998). In our work, we found the two varieties with the same morphology of Casteleyn´s and Villac´s materials. In Itajai, Santa Catarina state, both *P. pungens* and *P. pungens* var. *cingulata* (see Figs. 10, 14) were present, whereas in Paraná and other sampling sites only *P. pungens* was detected. To date, Villac *et al.* (1998) also cited a third incipient row of poroid between the two typical striae in *P. pungens* var. *cingulata.* However, Sar *et al.* (2006) also reported the same feature in many valves of *P. pungens*. It might be, therefore, that this character is not stable enough to help differentiating the two taxa. Specimens of Clade III can be characterized by possessing a denser number (21-25 in 10µm) of band striae than in *P. pungens* var. *pungens* (11-18 in 10µm), and almost the same number of *P. pungens* var. *cingulata* (20-24 in 10µm). Moreover, there are two types of poroids composing the bands in *P. pungens* var. *aveirense*: oval poroids and squares ones divided into two or three sectors (Churro *et al.*, 2009 Figs 51 and 51 insert).

Our *P. calliantha* material also presented some variability regarding poroid features, morphometrics in LM and overall shape. Two distinct morphotypes occurred in Paranagua Bay, and both of them bloomed simultaneously in November and December of 1997 and 1998, reaching concentrations above 1.2x10^6 cells/liter (Fernandes *et al.*, 2008). They were easily separated as two conspicuous entities under light microscopy routine screening at 400x magnification (larger transapical axis and number of fibulae and interstriae, and conspicuous interstriae in morphotype 1) as well as regarding their valve structure in electron microscopy (Table 2). Moreover, some valves presented poroids with both 3 to 4 sectors of hymenes (more typical of *P. caciantha*) and 6 to 8 sectors (typical of *P. calliantha*). Kaczmarska *et al.* (2005) recorded an even higher degree of morphological variability in *P. calliantha/P. pseudodelicatissima* populations in the Bay of Fundy, Canada. In their material, the size and number of sectors within a poroid varied from 1-7 sectors in a single valve as well as in different valves. From Kaczmarska *et al.* (2005)'s paper, there might be at least four species in their study region: *P. pseudodelicatissima, P. caciantha, P. calliantha* and the recently described *P. mannii* Amato & Montresor (Amato and Montresor, 2008).

In conclusion, the material examined containing *P. calliantha* and *P. pungens* presented great morphological diversity, detected under the electron microscopes. More research is deserved to effectively elucidate whether there are cryptic species in the populations studied in this work or not. Molecular biology studies coupled with morphological description of the valves will help to recognize the populations as well as to unveil possible new cryptic species. Moreover, since two species have been found to be toxin producers, *P. calliantha* and *P. multiseries*, future molecular biology investigations will be an important achievement, contributing for the improvement of HAB monitoring programs being carried out in South Brazil marine farms, particularly if molecular probes are developed to efficiently detect harmful strains. Finally, given the presence of potentially toxic species and their high abundance in South Brazil waters, the inclusion of the genus *Pseudo-nitzschia* in the currently and future monitoring programs of harmful phytoplankton is highly recommended.

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