

Research Article

High colonization and propagule pressure by ship ballast as a vector for the diatom genus *Pseudo-nitzschia*

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Abstract

Most harmful diatoms belong to the marine, planktonic genus *Pseudo-nitzschia* and are responsible for amnesic shellfish poisoning through the production of domoic acid. Fifteen *Pseudo-nitzschia* species, nine of them toxigenic (approximately 60% of the species found in our samples) were recovered from 185 ship ballast tanks (water and sediment) destined for Canadian ports. Our results demonstrate that the three Canadian coastal regions receive considerable total propagule pressure (1.2×10^{13} , 2.6×10^{13} , 1.5×10^{10} cells from water ballast; 4.5×10^9 , 3.7×10^{11} , 5.3×10^6 cells from sediments), and colonization pressure (15, 11 and 3 species) from these diatoms for Atlantic, Pacific and Great Lakes ports, respectively. Neither ballast water exchange method (intercoastal unexchanged, intercoastal exchanged, transoceanic exchanged), or ship port-of-destination seemed to affect sample groupings recovered by hierarchical clustering and multidimensional scaling. Only weak separations of samples by coastal region, propagule pressure/colonization pressure, and the number of days in the ballast since mid ocean water exchange were recovered. The Canadian Atlantic coast is under particularly high individual propagule pressure from *P. turgidula*, and the Canadian Pacific from *P. seriata*. Both species are toxigenic and not yet reported from either region. Alien to the Atlantic Ocean, highly toxigenic *P. australis* and *P. turgidula* have been recently found in Scottish waters, but not yet in Atlantic Canada, with the former relatively common in our ballast water samples. A greater number of species may be dispersed by ballast waters than by sediments because lightly silicified and narrow-valve species were absent in our sediment samples. However, these hardy survivors in the ballast sediments may be better adapted to tolerate suboptimal growth conditions when introduced to non-native regions and/or environments. *Pseudo-nitzschia delicatissima*, found in Great Lakes ship ballast waters, is known for its tolerance of brackish waters and may be one of the potentially toxigenic coastal species well suited for establishment in the Great Lakes, as has been the case for some other coastal marine organisms.

Key words: non-indigenous species, harmful diatoms, ship ballast, *Pseudo-nitzschia*, propagule pressure, colonization pressure

Introduction

The list of organisms recognised as non-native in the coastal waters throughout the world is growing, as is the number of harmful algal blooms. Many of the factors contributing to these two phenomena are relatively well recognised. The first is attributed to the increase in volume and number of introductions of exotic organisms carried to distant, non-native geographies as a result of the growing volume of global commerce (Gollasch et al. 2007; Lockwood et al. 2009). The second is related to human-mediated

modification of the coastal environment; e.g., the greater influx of terrestrial crop-plant mineral nutrients (Anderson et al. 2008; Anderson et al. 2012), among other recent changes to the global environment (Hallegraeff 2010; Bothwell et al. 2014). Both phenomena are most notable in the regions of main ports and large cities and co-occur in some regions, but considered together have received relatively little scientific attention. Here we evaluate the potential of ship ballast (water and sediment) to contribute to the dispersal of members of the genus *Pseudo-nitzschia*. Some members of this genus are known to be associated with amnesic shellfish poisoning caused by the

production of the heterocyclic amino acid, domoic acid. Recently, six coastal marine diatom species from several other genera have been found to be a source of another neurotoxic amino acid, beta-Methylamino-L-alanine (BMAA), previously known only to be produced by cyanobacteria (Jiang et al. 2014).

New policies and procedures aiming to reduce the human footprint on the environment (IMO 2004), e.g., more prudent agricultural practices, management of ship ballast or monitoring occurrence of harmful algae, are measures to slow down the rate of introduction of non-indigenous organisms and to protect the health of both the ecosystems and humans. Indeed, since the first toxigenic outbreak of *Pseudo-nitzschia* in Eastern Canada in 1987, no more humans have been reported seriously harmed by domoic acid (Todd 1993), despite repeated occurrences of harmful algal blooms (HABs) worldwide. However, humans are not the sole, high level consumers of coastal primary production and consequently, the only harmed target of associated toxins.

Domoic acid (DA) is a neurotoxin that bio-accumulates in animal tissues and with each consumer level, often passed on all the way up to vertebrate consumers (Montie et al. 2012) for whom it may be harmful or even lethal. Bio-accumulated DA doses and/or chronic exposure may result in impairments to vertebrate consumer fitness (Montie et al. 2012) and thus potentially cascade these effects (Powell et al. 2013; Saterberg et al. 2013; Yelenik and D'Antonio 2013) onto their population size and possibly genetics (Fitzpatrick et al. 2010) with subsequent effects on primary producers regulated by these specific top consumers.

Diatoms are one of the most species rich and abundant microeukaryotes in ship ballast (Carlton and Geller 1993; McCarthy and Crowder 2000; Klein et al. 2010; Briski et al. 2012). Even so, the species richness, abundance and survival of *Pseudo-nitzschia* in ship ballast waters and sediments have not been examined systematically in commercial vessel ballast. Although this readily recognisable genus is noted when present, only a few published reports have informed on the identity of species found or analyse their relationships to ballast tank history or environment (Zhang and Dickman 1999; Burkholder et al. 2007; Lang and Kaczmarek 2012). More frequently, these diatoms are only reported in the regional literature (Subba Rao et al. 1994; Macdonald and Davidson 1997; Wetsteyn and Vink 2001; Carver and Mallet 2002).

Therefore, the aim of this study was to estimate colonization pressure (CP, number of species transported) and their respective abundance, or propagule pressure (PP) of individual species and the total for the genus *Pseudo-nitzschia* exerted on Canadian ports. We related species richness and their respective abundances to the ship ballast water management (BWM) strategies executed prior to their arrival at the three destined Canadian coastal regions: Vancouver (WC), selected ports in Atlantic Canada (EC) and Great Lakes (GL). Particular attention was given to toxigenic and non-indigenous species.

Methods

Field protocols

All samples were collected by the same Canadian Aquatic Invasive Species Network (CAISN) sampling team. Ballast water samples were taken at arrival, from vessels at Atlantic (EC), Pacific (WC) and Great lakes (GL) ports in Canada in 2007 (as listed in Briski et al. 2012) for ballast waters and in 2007 and 2008 for ship ballast-tank sediments (listed in Villac and Kaczmarek 2011); the majority were collected during spring and summer. Ballast water and sediment samples did not originate from the same voyages or ballast tanks. All vessels examined observed IMO (2004) convention for the control and management of ships' ballast water and sediments. Therefore, once in Canadian ports, all were in the position to de-ballast if needed.

Ballast water samples were categorised according to the BWM strategies applied to the sampled tanks during the voyage immediately prior to the vessel's arrival to Canadian ports. The categories were as follows: ICU (intercoastal unexchanged), ICE (intercoastal exchanged) and TOE (transoceanic exchanged). Each ballast tank water sample was integrated from casts of a 5 L Niskin bottle collected from three depths (0, 2 and 4 m), pooled in a 20 L carboy container and thoroughly mixed. From this, 3 L of water was sub-sampled for diatom examination and enumeration, preserved with acidified Lugol's solution and shipped to our laboratory. The majority of ships sampled were bulk carriers. The age of ballast water in the tanks was calculated in days following exchange for the tank (if performed) and ranged between 7 and 33 days. Ship ballast sediments were processed as described in Villac and Kaczmarek (2011), optimised specifically for recovery of diatoms from such sediments.

Table 1. 2007 ballast water and 2007-08 ballast sediment samples. First value indicates number of samples where at least one *Pseudo-nitzschia* species was encountered; second value indicates total number of samples examined; percent of samples with *Pseudo-nitzschia* in parentheses. EC = East Coast; GL = Great Lakes; WC = West Coast; ICE = intercoastal exchanged; ICU = intercoastal unexchanged; TOE = transoceanic exchanged.

	2007 ballast water	2007-08 sediments
All samples	42 / 67 (63)	9 / 57 (16)
By region:		
EC	21 / 24 (88)	2 / 24 (8)
GL	2 / 13 (15)	1 / 8 (13)
WC	19 / 30 (63)	6 / 25 (24)
By exchange method:		
ICE	9 / 12 (75)	1 / 17 (6)
ICU	4 / 9 (44)	2 / 14 (14)
TOE	29 / 46 (63)	6 / 26 (23)

Sample processing

A total of 185 ship ballast tanks were examined (Table 1): 67 ballast water and 118 ballast sediments samples were taken during 2007 and 2008 from ships that came to Canadian ports on the West (WC), East coast (EC) and Great Lakes (GL); possibly the largest such sample set thus far examined. Of these, all 67 ballast water samples contained live diatoms (24 EC, 13 GL, 30 WC), but only 57 of sediments samples contained live cells (24 EC, 8 GL, 25 WC; Villac and Kaczmarzka 2011), making a total of 124 samples with live diatoms examined for the presence of *Pseudo-nitzschia* species.

Water samples were carefully decanted and contents concentrated to a final volume of 20 mL and stored at 4 °C until processed following Klein et al. (2010). Identification and enumeration were performed on a 10 mL sub-sample of the concentrate in a settling chamber (Hydrobios, Kiel, Germany) and expressed as cells L⁻¹ or cells per ballast volume discharged as required. Autofluorescence of the chloroplast was taken as a proxy of cell viability at the time of fixation, and only fluorescing cells were counted. To reverse the quenching of chloroplast autofluorescence by Lugol's fixation (Klein et al. 2010), 4 drops of a saturated sodium thiosulfate solution in distilled water were added to the sub-sample prior to settling in the chamber.

Ballast sediment samples were processed as described in Villac and Kaczmarzka (2011). For all samples, cell counts were carried out using a Zeiss Axiovert 200 inverted microscope with epifluorescence illumination (HBO 50/AC, Mercury Shortarc) using 20x or 40x objectives as

required. Using light microscopy (LM), fluorescing *Pseudo-nitzschia* cells in stepped chain colonies were identified to the lowest practical taxonomic level. These counts are conservative because only cells in stepped colonies were counted; taking the colony formation mode as a diagnostic character for this genus; although see Kaczmarzka et al. (2008) to note the propensity for a single cell life-style among some *P. delicatissima* clones and other single-cell species with *Pseudo-nitzschia* valve morphology (Kaczmarzka and Fryxell 1994).

Cell morphometrics and scanning electron microscopy (SEM) were used to refine species identification of LM counts. After LM counts were completed for water samples, the contents of the settling chambers were prepared for SEM examination by acid cleaning using a protocol by Drebes (1974). SEM preparation protocol for diatoms from sediments is described in Villac and Kaczmarzka (2011). Cleaned diatoms were dispersed onto 3 µm pore-size polycarbonate filters, dried, affixed to 10 mm diameter aluminum stubs and sputter coated with ca. 15 nm gold. SEM was performed using a JEOL JSM-5600 SEM operating at 10 kV and 8 mm working distance at the Digital Microscopy Facility, Mount Allison University. SEM results were used to refine species identification and proportion of the species making up lower taxonomic resolution LM counts as described in Kaczmarzka et al. (2005). Members of the genus *Pseudo-nitzschia* were identified to species level in all but two cases: *P. delicatissima*- and *P. pseudodelicatissima*-species complexes, with the exception of *P. cuspidata* in the *P. pseudodelicatissima*-complex, which we were able to distinguish from other members of the complex in our SEM images by poroid morphology (Figure 1). Nonetheless, in the text all will be referred to as “species” for simplicity.

Species identities were based on characters of valve morphology as defined in Tomas (1997), Skov et al. (1999), Lundholm et al. (2003), Kaczmarzka et al. (2005), Almandoz et al. (2008) and Kaczmarzka et al. (2008). Propagule pressures for all *Pseudo-nitzschia* species were calculated based on the tonnage estimates given in Lo et al. (2012) for ballast waters and total ship numbers arriving to Canada, while the sediment volume of the tanks examined is given in Villac and Kaczmarzka (2011). Similar calculations using tonnage estimates detailed in Casas-Monroy et al. (2014) produced somewhat higher propagule pressures, but both produced the same order-of-magnitude propagule pressure estimates.

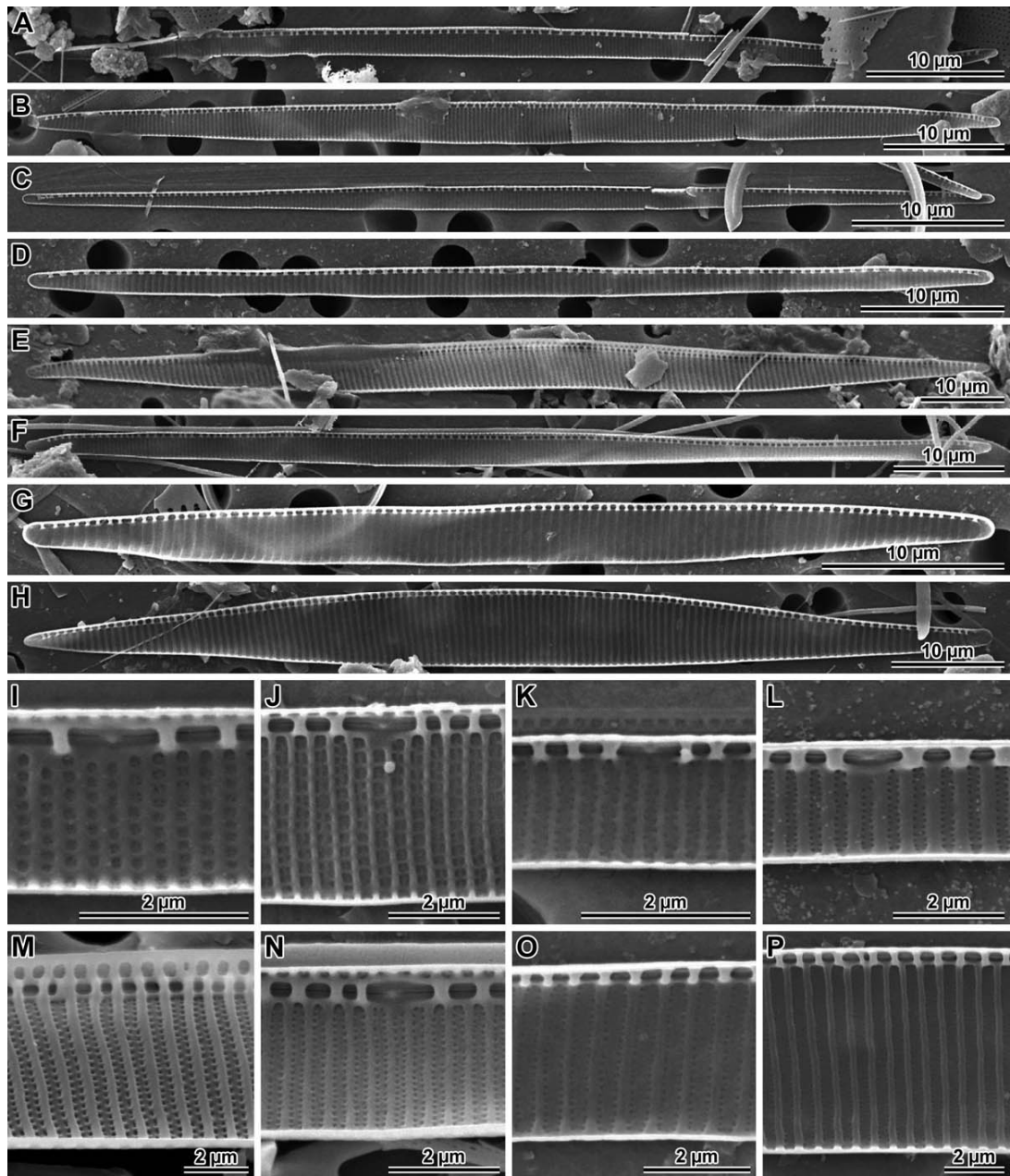


Figure 1. SEM images of selected members of the genus *Pseudo-nitzschia*. (A,I) A member of the *Pseudo-nitzschia pseudodelicatissima*-complex, the whole valve illustrating needle-shaped valve outline (A), and its mid-section detailing central interspace, striae and microarchitecture of poroids (I). (B,J) *Pseudo-nitzschia cuspidata*, complete valve outline (B), and its mid-section detailing central interspace, striae and microarchitecture of poroids (J). Compare several small sectors in each poroid in (I) with larger, usually two, elongated sectors in the poroids in (J). (C,K) *Pseudo-nitzschia delicatissima*, valve outline showing consistently tapering valve margins (C), and its mid-section detailing central interspace and two rows of poroids per striae (K). (D,L) *Pseudo-nitzschia turgidula*, with valve outline showing three areas of swelling (central, and near each valve end, D) and valve mid-section detailing central interspace and the structure of striae (L). (E,M) *Pseudo-nitzschia australis*, lanceolate valve (E), and its mid-section detailing striae consisting of large poroids, note the absence of central interspace (M). (F,N) *Pseudo-nitzschia heimii*, lanceolate valve outline (F), and valve mid-section showing central interspace and the structure of striae (N). (G,O) *Pseudo-nitzschia obtusa*, valve with bluntly obtuse ends (G), and its mid-section showing absence of central interspace and the structure of striae (O). (H,P) *Pseudo-nitzschia seriata*, an almost fusiform valve (H), and valve mid-section showing the absence of central interspace and the structure of striae (P). The third and at times fourth row of poroids in the center of the striae are only barely visible due to incomplete cleaning of the valve.

Statistical treatment

Both ballast water and sediment data for *Pseudo-nitzschia* species was sparse and highly non-normal even after log and other normalizing transformations. In addition, the mixed-species and composite nature of diatom assemblages in both types of samples (representing a number of voyages and exchanges) questions the applicability of statistical methods developed for natural biological community ecology such as principal components or canonical correspondence analysis. Nonetheless we used these methods in reconnaissance analyses, and found that they did not contribute any significant new or contradictory insights compared to the methods that produced the results presented below. Following reconnaissance analyses, we chose hierarchical clustering, analysis of similarity (ANOSIM) and multidimensional scaling (MDS) following the logic of Clarke and Warwick (2001). ANOSIM and MDS are non-metric techniques that make few, if any, model assumptions about the form of the data or interrelationships between samples (as is likely to happen in ship ballast tanks). ANOSIM is a non-metric analog to ANOVA and provides statistical tests to determine if there are differences between various factors within a data set. MDS provides an n-dimensional ordination of samples (similar to PCA or CCA) but without concerns over the suitability of the data for analysis as would be the case for ballast samples that are more chance-driven, mixed origin species assemblages of surviving biota. The purpose of MDS is to construct a “map” or configuration of the samples, which attempts to satisfy all of the conditions imposed by the rank (dis)similarity matrix; e.g., if sample 1 has a higher similarity to sample 2 than to sample 3, then sample 1 will be placed closer on the map to sample 2 than to sample 3. All analyses were performed on a Bray-Curtis similarity matrix. Results presented here originated from $\log(x+1)$ transformed data, but results obtained from untransformed abundance data produced similar results with no new insights into relationships between samples. The small and sparse data set for sediments harbouring *Pseudo-nitzschia* species produced insignificant results in clustering, ANOSIM and MDS analyses and so we relate sediment data only qualitatively to the water ballast results. Similarly, comparisons with GL samples in ballast water are presented here for the sake of completeness of analyses, even

though there were only two GL samples with *Pseudo-nitzschia* cells in the data set.

Results

Colonization and propagule pressures

Among 67 ballast waters samples examined, 42 (21 EC, 2 GL, 19 WC; 9 ICE, 4 ICU, 29 TOE; Table 1) collectively harboured fifteen different species of *Pseudo-nitzschia* (Supplementary material Table S1). Non-indigenous and/or taxonomically challenging species are illustrated in Figure 1. The fifteen *Pseudo-nitzschia* species found constitute approximately 35 % of the species known in the genus, some with high cell quantities. Cumulatively, up to 1.2×10^{13} , 2.6×10^{13} , 1.5×10^{10} live *Pseudo-nitzschia* cells, or total PP, were calculated to be potentially delivered annually via the waters in ballast tanks for Atlantic, Pacific and Great Lakes ports, respectively. The most species rich (highest CP) were the tanks of WC vessels (15 species), closely followed by EC (11 species). Two tanks arriving at the Great Lakes ports harboured one species each. BWB methods had little effect on the number of species surviving in the tanks of intercoastal vessels; in fact more species were found in exchanged coastal tanks (11 species in ICE vs. 8 in ICU) while still more in TOE vessels (14 species). These results demonstrate that the three Canadian coastal regions may be receiving considerable total propagule and colonization pressure from *Pseudo-nitzschia* diatoms.

Fewer sediment samples contained live *Pseudo-nitzschia*; of 57 samples containing live diatom cells only 9 carried live *Pseudo-nitzschia* detectable using our methods (2 EC, 1 GL, 6 WC; 1 ICE, 2 ICU, 6 TOE; Table 1). In these 9 samples, 5 species were found (Table S1). Most species rich were again WC samples (4 species), followed by EC (2 species) and 1 species was found in sediment from a vessel destined for a GL port. If discharged untreated, 3.7×10^{11} , 4.5×10^9 , 5.3×10^6 cells annually as total PP for *Pseudo-nitzschia* would have entered Pacific, Atlantic and Great Lakes ports through sediment ballast. All the taxa present in the ship ballast sediments were those with large and relatively heavily silicified frustules. Species with delicate and lightly silicified frustules were never found in sediment samples, even as dead (empty) valves. Individual species PP (cell densities in arriving ship tanks) was generally low, with approximately half of the species present at average densities

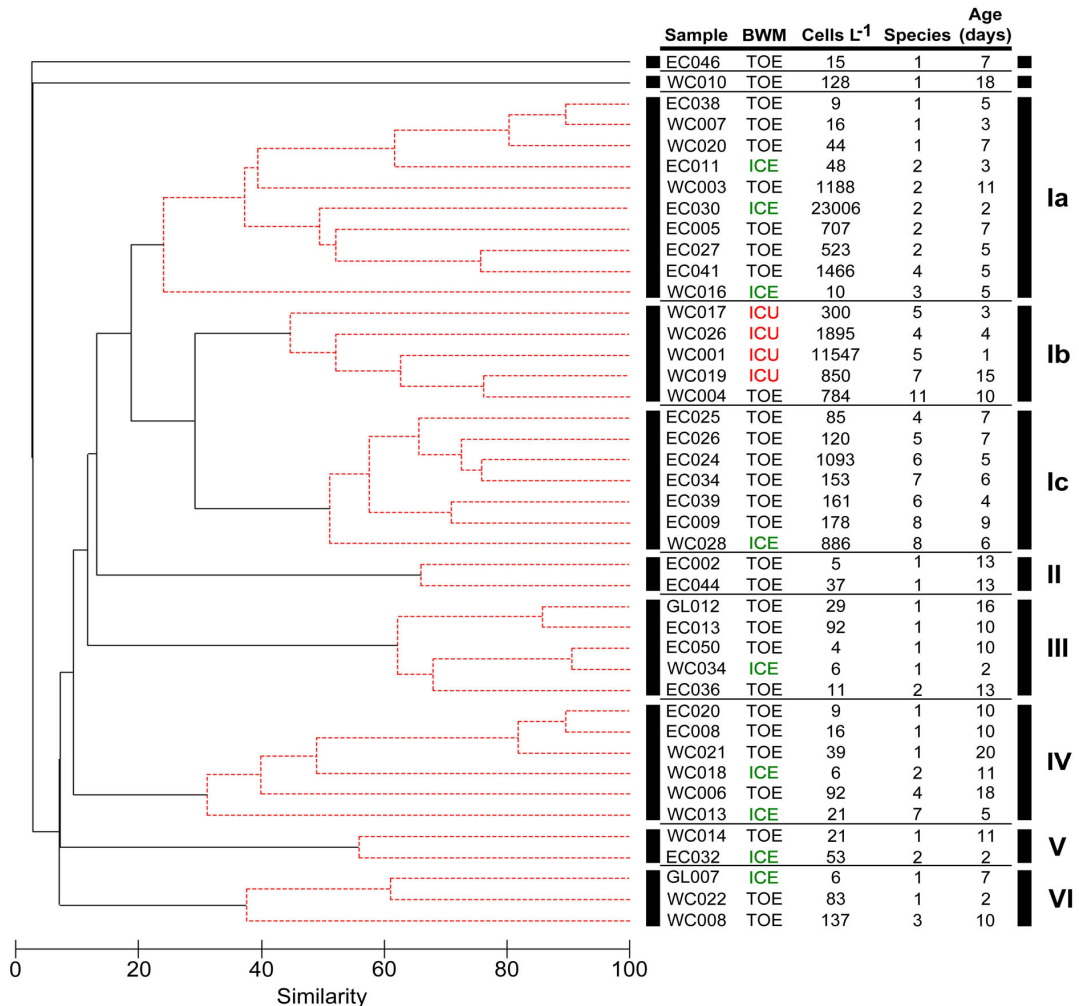


Figure 2. Hierarchical clustering of log(x+1) transformed *Pseudo-nitzschia* ballast water abundance data. Solid black lines in cluster indicate statistically significant branchings, red dotted lines indicate arbitrary clustering. Significant branches divided into 6 major clusters, with cluster I containing subgroups a-c. Each sample identified by sample name (indicating sampling coast: EC = East Coast, WC = West Coast, GL = Great Lakes), ballast water management strategy (BWM) for the sample (TOE = transoceanic exchanged, ICE = intercoastal exchanged, ICU = intercoastal unexchanged), total *Pseudo-nitzschia* cell concentration (cells L⁻¹), number of *Pseudo-nitzschia* species found in sample and age of the water in the ballast tank at time of sampling.

less than 100 cells L⁻¹ of ballast water or per gram (wet weight or w/w) of sediments (Table S2). A few more common species exerted higher individual PP (present in quantities of ~100–300 cells L⁻¹ ballast water or cells g⁻¹ of sediments); with the notable exception of two diatoms, both well known for their toxicity, namely *P. multiseries* and *P. turgidula*. *P. multiseries* was encountered at an average of ~2500 cells g⁻¹ in WC ballast sediments while *P. turgidula* occurred in EC ballast sediments with a average of almost 10,000 cells g⁻¹ (Table S2).

Statistical analyses

Hierarchical clustering of the ballast water samples containing live *Pseudo-nitzschia* cells produced 10 statistically significant groups of samples ranging in size from 1 to 10 samples (Figure 2) and including six main clusters and 2 singletons. The largest of the clusters (Cluster I) contained nearly half of the samples and was further subdivided into three subclusters (Ia, Ib and Ic). In most part, the clustering partially separated samples with multiple species and/or

higher cell densities (medium to high, or 11–100 and 101–1000+ cells L⁻¹, respectively) from others containing 1–2 species and low to medium cell densities (1–10 or 11–100 cells L⁻¹, respectively). Exceptions include EC038-TOE with a few cells of *P. turgidula* clustered with other samples containing this species in the largest cluster, subcluster Ia. Two other samples (WC006-TOE and WC013-ICE, cluster IV) clustered with several other samples, all containing *P. pungens*. Ballast water samples with 1–2 species and low to medium cells densities (1–10 or 11–100 cells L⁻¹, respectively, Table S2) tended to be older in terms of number of days following mid-ocean ballast water exchange (mostly 10–20 days, Clusters II–VI, Figure 2) than those with greater number of species and cells.

There was no obvious separation of samples subjected to different BWM strategies or for different coastal regions (Figure 2); but robust interpretation is difficult owing to the greater number of TOE samples. The strongest clustering was shown by all four ICU samples clustering with a single TOE sample arriving at WC. All ICU samples originated in Seattle (WA, USA) between April and July, while the TOE sample originated in May in Incheon (S. Korea); this clustering thus likely reflects the seasonal status of the ballast source waters. The clustering also illustrates the absence of groupings reflecting the geographic ballast water source. For example, Sub-cluster Ia grouped ship tanks with water exchanges between the port of Vancouver and the last ports of call in Japan, China, Seattle, and British Columbia all together with those exchanged between Atlantic Canada and ports in the UK, Eastern Seaboard of the USA and New Orleans in the Gulf of Mexico. Whether there is a tendency for WC and EC samples to cluster together at all will be further explored through the MDS plot below.

ANOSIM for ballast water addressed whether the coast (EC, GL, WC; operating in either Atlantic or Pacific Ocean) or the type of BWM strategy (TOE, ICE, ICU) were factors affecting the *Pseudo-nitzschia* species in the ballast tanks examined. The ANOSIM test statistic ($R = 0.112$) for coasts was significantly larger ($p < 0.01$; only 10 permutations $\geq R$) compared to 999 R values calculated with random permutation of sample labels, so the first null hypothesis of no differences between the coast of voyages destination was rejected. Pairwise tests indicated that only EC and WC samples containing *Pseudo-nitzschia* were statistically different ($p < 0.03$). However

the low value of R (0.088) for this pair indicated that the two were strongly overlapping. The second null hypothesis that BWM does not affect the *Pseudo-nitzschia* species richness and abundance in ship tanks could not be rejected ($R = -0.023$; $p = 0.63$; 629 permutations $\geq R$). Thus, whether ballast water was exchanged and if so, the exchange location (mid-ocean or the coastal region), was irrelevant in determining the similarity (or dissimilarity) in ballast water samples in terms of *Pseudo-nitzschia* abundance and species composition. This conclusion must be somewhat qualified owing to the larger number of TOE samples relative to other tank types, but at a minimum it can be said that the TOE group (46 of 67 samples) was statistically indistinguishable from the 21 intercoastal samples, regardless of their exchange type.

MDS analysis for ballast water

The two-dimensional ordination map produced by multidimensional scaling provided a complementary and somewhat more easily interpreted graphical display of similarities between samples. Three-dimensional plots were also examined but did not provide any further useful information. Two-dimensional “stress” for the presented ordination was 0.13, a level indicating that overall, interpretation of the distribution provides a potentially useful picture, although too much reliance should not be placed on details of the plot (Clarke and Warwick 2001). Plotting MDS scores keyed to factors for each sample (coast, BWM strategy, cell count, number of species and number of days in the tank) again revealed partial and overlapping relationships. Only scores keyed to exchange type and number of species are presented here.

MDS scores plotted by BWM strategy (Figure 3), show that ICE and ICU samples were broadly distributed within the main distribution of TOE samples, with the only notable specific cluster of all four WC ICU samples together with WC004-TOE also recovered in hierarchical clustering (Figure 2). Overall this encoding of the MDS data indicates that the three exchange types are fairly indistinguishable in terms of presence and abundance of *Pseudo-nitzschia* species - i.e. in most cases a particular ICE or ICU sample is as close (similar) to a TOE sample as it is to another sample of its own exchange type.

A “bubble” plot with symbol size reflecting total number of *Pseudo-nitzschia* species (Figure 4) reveal that samples with more species tend to

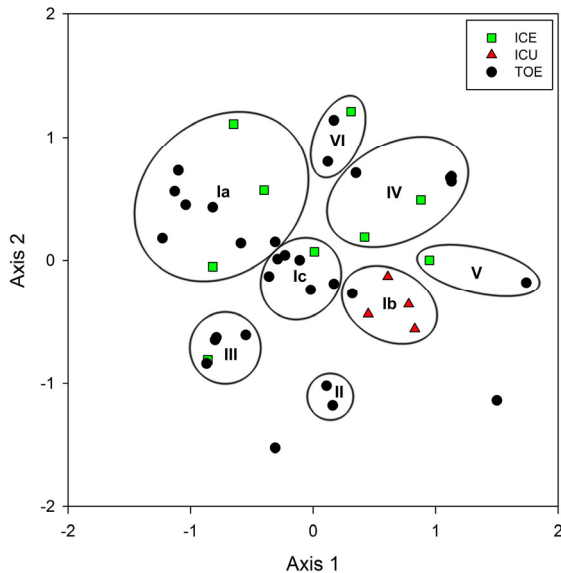


Figure 3. Multidimensional scaling (MDS) biplot of $\log(x+1)$ transformed *Pseudo-nitzschia* ballast water abundance data, by ballast water management (BWM) strategy. ICE = intercoastal exchanged, ICU = intercoastal unexchanged, TOE = transoceanic exchanged. Black ovals (I through VI) surround statistically significant groupings produced by hierarchical clustering (Figure 2).

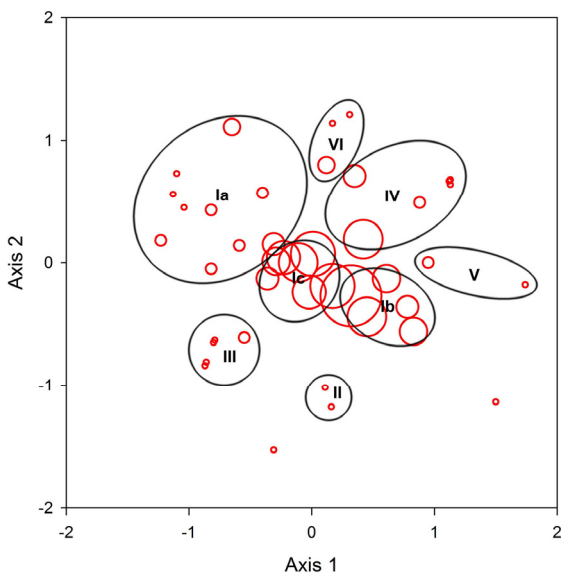


Figure 4. Multidimensional scaling (MDS) biplot of $\log(x+1)$ transformed *Pseudo-nitzschia* ballast water abundance data. Symbol size (open red circles) relative to total number of *Pseudo-nitzschia* species in sample. Black ovals (I through VI) surround statistically significant groupings produced by hierarchical clustering (Figure 2).

cluster in the center of the plot (or Cluster I), while samples with few species are scattered around the periphery (or Clusters II–VI, Figure 2). This pattern was similar in plots where symbol size was determined by *Pseudo-nitzschia* abundance in each sample, and the number of days between last exchange and sampling (not shown). Such a distribution further emphasizes the patterns revealed in hierarchical clustering (Figure 2) and suggests that statistical analyses tend to group the samples that are more similar based on their inherent *Pseudo-nitzschia* abundance, diversity and the age of ballast water rather than geography of the voyages (port of origin and ballast water exchange location) or BWM strategy employed. Thus, the groupings illustrate limited potential of ballast water exchange-based management strategies for mitigation of CP and the total as well as individual PP pressure of *Pseudo-nitzschia* species on Canadian coasts.

Discussion

Colonization pressure, emphasis on non-indigenous taxa

The genus *Pseudo-nitzschia* arriving via ballast waters and sediments exerted an unanticipated high colonization pressure on Canadian ports of destination. Fifteen species detected (~35% of currently known members of the genus) is more than in any previously reported ship ballast examined to species level (Subba Rao et al. 1994; Macdonald and Davidson 1997; Zhang and Dickman 1999; Wetsteyn and Vink 2001; Carver and Mallet 2002; Burkholder et al. 2007; Zvyagintsev et al. 2009) who found 3, 2, 4, 5, 8, 5 and 2 species from this genus in their samples, respectively. Nine of fifteen species found in our tanks are known as toxigenic (Villac et al. 2010, Table S1). The majority of the species arriving at two of the regions (WC, EC) have been reported by earlier plankton studies from Canada (Mather et al. 2010) or neighbouring USA (Horner and Postel 1993; Buck et al. 1992; Trainer et al. 2009) and may be considered native to these regions. This however, does not preclude genetic differences between our native populations and the arriving conspecifics; some cases may be similar to that found in specimens encountered in ballast ship destined for the port of Vancouver who carried natural intervarietal hybrids that were morphologically cryptic with typical *P. pungens* (Casteleyn et al. 2008; Lang and Kaczmarek 2012). Five of the marine species

found in our study are most likely non-native and, based on estimated individual PP in our study, are candidates for establishment in Canadian coastal waters.

Three species, *P. obtusa*, *P. seriata*, *P. turgidula* have not been reported from the WC (the former two) or Canada (the latter). The highly toxigenic potential newcomers deserve more attention. Among these, *P. turgidula* was found in our ship ballast waters (EC and WC) and in the ballast sediments (EC) in relatively high abundance (up to $\sim 15,000$ cells L^{-1} in some samples, Tables S1, S2). In general, this species is thought to have been absent in boreal waters (Hasle 2002, normally occurs between 20–60°S mostly in New Zealand and Australian waters; Hallegraeff 1994; Almandoz et al. 2008), except for a relatively recent appearance in Irish waters (Gallacher et al. 2001). The presence of live *P. turgidula* cells in 9 out of 24 ballast water tanks and 1 of 24 sediment samples from the EC demonstrates a considerable annual individual propagule pressure (7.1×10^{12} cells) this species exerts on Canadian ports, particularly in Atlantic Canada. Another, also toxigenic species, *P. seriata* thus far known only from the Arctic and Atlantic (50–80°N) and not yet reported from the Pacific (Hasle 2002; Lundholm and Moestrup 2006; despite a few claims to the contrary), was found in 6 out of 19 ballast tanks containing *Pseudo-nitzschia* from ships destined for the port of Vancouver (with low or moderate cell densities, up to 160 cells L^{-1}). Its individual propagule pressure may reach at least 4.8×10^{11} cells annually.

Two alien marine species (*P. heimi* and toxigenic *P. delicatissima*) were found in tanks heading for Thunder Bay on Lake Superior (estimated propagule pressure 7.9×10^8 and 3.8×10^9 cells annually, respectively). Among these, toxigenic *P. delicatissima* ought to be of particular concern because it is known for its tolerance of brackish water environments (Parsons et al. 1999). As such it is well positioned to follow some other coastal marine species that have invaded the Great Lakes.

On the Canadian Pacific coast, two other species, *P. obtusa* and toxigenic *P. australis* not yet seen in Canada, showed limited occurrence in our data set, being present only in WC tanks. However, these diatoms are known from the neighbouring coasts, the Northern North Pacific (as *P. seriata* f. *obtusa*, in Kamchatka and Kuril Island) and the American Northwest (Hasle 2002), each found with individual PP estimated at 1.0×10^{11} and 1.7×10^9 cells annually, respectively.

Both diatoms may not yet be reported from Pacific Canada simply due to limited floristic data from this region. In contrast, the Atlantic coast of Canada has been monitored for harmful algae for the past three decades or so, and *P. australis* has yet to be reported from this region (or from the Atlantic ballast data set), but has already been found in Scottish waters (Fehling et al. 2006) where its toxicity caused closures of shellfish harvesting. If our results are any indication of what can happen with any *Pseudo-nitzschia* species, it is likely to reach the East Coast of Canada in the near future.

All these species join two other *Pseudo-nitzschia* species that are very likely recent additions to Canadian diatom communities, namely *P. subpacifica* and *P. fraudulenta*. All these species have large frustules and/or distinct shape and it is unlikely that they were overlooked in the Bay of Fundy (Martin et al. 2001 and references therein) or other studies from the region. Unlike the *P. delicatissima* and *P. pseudodelicatissima* complexes, *P. turgidula*, *P. seriata*, *P. obtusa* and *P. australis*, like *P. subpacifica* and *P. fraudulenta* are distinct and seem to be well defined morphologically and phylogenetically and thus allow a degree of confidence in our current understanding of their biogeography, particularly when electron microscopy aided species identification.

Propagule pressure

It is more difficult to place the total *Pseudo-nitzschia* propagule pressure found here in a broader context because few similar studies are available; although the estimated number of live cells arriving seems astronomical, had they been released to the port waters. A few published studies including cell densities (cells L^{-1} of the sampled ballast waters or gram of sediments) allow for some broad comparisons. Cell densities in our tanks were comparable in magnitude to the individual species maximal cell density range of 2 – 42,259 cells L^{-1} reported by others (Zhang and Dickman 1999; Macdonald and Davidson 1997; Carver and Mallet 2002; Subba Rao et al. 1994; Wetsteyn and Vink 2001; David et al. 2007), although the total propagule pressure would depend on the volume of water and or sediments de-ballasted, which is unknown for the studies cited above. The least that can be said is that cells of *Pseudo-nitzschia* species have been found in many ship ballast tanks and a few ship sediments (although these are examined much

less frequently; Villac and Kaczmarek 2011), at times as frequently as in our report (38% of water tanks examined; Macdonald and Davidson 1997) and in cell densities comparable to those found here; thus total PP of the genus is high at times. Because the taxonomic resolution varied in these studies (LM, TEM, SEM) and was sometimes tentative, individual species PP (including those that are toxigenic) remains poorly known. Species enumeration with the aid of SEM is unparalleled for the size of our data set. Thus, although the natural geographic ranges of the species may be known relatively well, the specific identity of *Pseudo-nitzschia* spp. ballast tank stowaways in many other studies is not.

Although our data represents one of the largest investigations of the transport of potentially toxic and non-indigenous *Pseudo-nitzschia* through ballast water and sediments, it still only represents a part of the year (although at a time when plankton are most actively growing) and involves shipping destinations in only one country. Nonetheless our results indicate that a large degree of chance is involved in assembling *Pseudo-nitzschia* species composition for any particular voyage, and depends on plankton community conditions at the ballast water source and at any subsequent ballast exchange location. Counter-intuitive and rather unanticipated results suggesting that mid-ocean ballast exchange may increase CP of the ballast tank (similar to postulated by Lang and Kaczmarek 2012) may be especially true for those species of *Pseudo-nitzschia* who are also known from offshore sites (Kaczmarek et al. 1986; Kaczmarek and Fryxell 1994; Iriarte and Fryxell 1995). Depending on where mid-ocean water exchange takes place, new *Pseudo-nitzschia* species and/or individuals may be taken up with the re-ballasting waters and delivered to the distant port of call.

While ballast water assemblages carry more significant potentials for non-indigenous *Pseudo-nitzschia* species establishment (higher CP and PP), the sediment samples favoured larger, more thickly silicified species that may be harder and more tolerant of the ballast tank environment than are thinly silicified diatoms. Of the five species recovered from sediments, four (80%) have been shown to include toxic strains, compared to nine of sixteen species in ballast waters (60%). Although our ballast water and sediment samples did not originate from the same ballast tanks (or even the same voyage in most cases), the absence of thinner, more lightly silicified species (even in the post-mortem form,

as empty valves) in ballast sediment samples is an intriguing observation. The implication is that thin-valve species either did not settle into ship tank sediments, did not survive in sediments and their frustules were dissolved, or the much less likely scenario that ballast water from tanks from which sediments were sampled did not contain these thinner-valve species. The latter possibility involves chance alone to cleanly separate our ballast water and sediment data, which is unlikely given the number of samples of both types examined.

Most *Pseudo-nitzschia* species and other diatoms die out in ballast tanks very quickly. Others, however, persist for long periods of time. Forbes and Hallegraeff (2002) successfully cultured other diatom species after transport in ballast water tanks for up to 37 weeks in darkness, while Villac and Kaczmarek (2011) cultured diatoms from ballast sediments, including species not detectable by the LM enumeration method. An unspecified member of the *Pseudo-nitzschia* genus survived ballast water UV-treatment (Liebich et al. 2012) rendering that species (and possibly others) well suited for the means of dispersal despite the varied stresses ship ballast environments impose. This may be particularly pertinent to *Pseudo-nitzschia* species with large and/or heavily silicified frustules that persisted in ship ballast sediments. Thus, the possibility of survival of these potentially “tougher” candidate colonisers should not be overlooked.

CP, PP and ballast management strategies

Our statistical analyses returned only weak relationships between the *Pseudo-nitzschia* CP and PP (individual and total) and the coastal region and to the age of tank waters (lengths of time between exchange and the sampling), reported also for some other biota (Briski et al. 2012). “Coastal region” defined broadly here, in reality represents the oceanic basin because the port ballast water source and the source of the water taken up during exchange occur in the same ocean, at least in our sample set.

Ballast water management strategy (ICE, ICU or TOE) did not affect sample clustering in terms of *Pseudo-nitzschia* colonization or propagule pressures, found also for other organisms (e.g. Burkholder et al. 2007; Briski et al. 2012). Some TOE and ICE treated samples tended to carry higher CP, suggesting that either ballast was taken in a port in which waters contained a blooming population of *Pseudo-nitzschia*, or the

mid-ocean ballast water exchange may have contributed to an increase in CP, perhaps by adding new species and by rejuvenating species accumulating at the tank sediment surface by the influx of new, oxygenated waters and stirring up presumably nutrient rich sediments containing mineralised remains of decomposing organisms from earlier port waters or exchanges. The stochastic nature of *Pseudo-nitzschia* species composition and abundances in the tank waters and sediments is consistent with the mixed-species origin of these diatoms and suggest that predictive insights into the nature of incoming CP and PP for these diatoms and effective strategies to mitigate their influx will be very difficult (if possible) to develop.

Biogeographical considerations

Traditionally, most *Pseudo-nitzschia* species have been regarded as cosmopolitan in their current distribution (Hasle 2002; Smayda 2007). However, the more recent discovery of morphologically cryptic and semi-cryptic *Pseudo-nitzschia* species complexes among the thinly-silicified taxa (*P. delicatissima* and *P. pseudo-delicatissima* species complexes, Amato et al. 2007) combined with toxigenicity varying between strains of the same species underscores the necessity for use of molecular tools (e.g., next generation sequencing) in biogeographical studies of these species complexes in particular. This would allow recognition of influx and monitoring of harmful effects of non-indigenous populations with different physiologies, tolerances and associations with other flora and fauna present in the environment to which they are displaced. If indeed ship ballast dispersed alien species interact differently with local environments, such as that suggested by Kaczmarek et al. (2005) where exotic bacteria might have “stimulated” *P. multiseriata* to produce more toxin, consideration should be given to both organisms (bacteria and *Pseudo-nitzschia*) as potentially harmful invaders in the local environment.

Theoretically, just one individual diatom cell arriving to a foreign location is capable of multiplying vegetatively to establish an alien species population. Empirical data however, suggests that the larger the CP and/or PP, the greater the likelihood of successful introduction of a species outside of its natural distribution range (Simberloff 2009; Briski et al. 2012). Our data demonstrate that many cells of exotic species and strains of *Pseudo-nitzschia*, some of which

are toxigenic, arrive with great regularity (albeit with a high degree of unpredictability) to Canadian ports. The fact that we discovered more *Pseudo-nitzschia* species in our samples than any other previous study may be a further indication of this inherent unpredictability. Taken collectively, we observe that our study supports the assertion of Burkholder et al. (2007 p. 513): “...the dogma of plankton cosmopolitanism leads to complacency about potential for continued risk from ballast waters...”. It would seem that indeed anything can happen (and probably will) in the case of ballast tanks and *Pseudo-nitzschia* species.

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Supplementary material

The following supplementary material is available for this article:

Table S1. *Pseudo-nitzschia* species/complexes found in 2007 ballast water and 2007–08 ballast sediment samples by region and ballast water management (BWM) type.

Table S2. Average cell densities per liter for *Pseudo-nitzschia* species found in 2007 ballast water and per gram (w/w) of 2007–08 ballast sediment samples by region and ballast water management (BWM) type.

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