The following supplement accompanies the article

Estimating propagule pressure and viability of diatoms detected in ballast tank sediments of ships arriving at Canadian ports

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Results & Discussion: Methodological insights

Sediment screening:

Assessment by smears was a helpful first step in screening samples for more in-depth analysis. Although it is not possible to estimate the probability of false negatives (samples with live diatoms that were not selected), the presence and appearance of cells in smears were good indicators of their abundance in the actual cell counts (Fig. 1). Smears with frustules with intact protoplasm tended to have higher number of cells in the actual counts than smears selected because of the presence of frustules with dark-brown, amorphous content (structures used solely as an indicator of the possible presence of live cells). There were three exceptions: samples WC-24, EC-45, and WC-33 turned out to have high cell numbers, even though only frustules with dark-brown, amorphous content were found in the smears. Only five samples, of the 54 preserved samples targeted for detailed microscopic analysis, did not have any live diatoms detected by our methods.

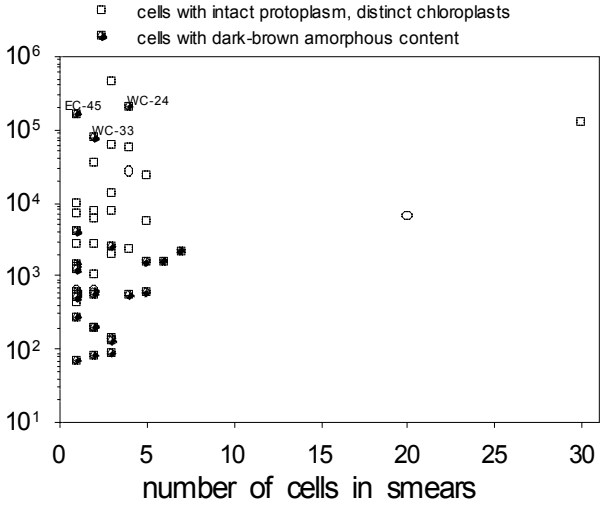

Fig. 1. Relationship between numbers of presumed live cells detected in smears of unprocessed sediment samples (x-axis) *vs*. abundance of cells with intact protoplasm per g (w.w.) after samples were subjected to density gradient centrifugation (y-axis). Only the 49 preserved samples with live diatoms are included in this analysis. See text for comments about the three samples noted (WC-24, EC-45, WC-33).

Sediment processing:

Here we present the protocol used for sorting diatoms from the sediment in more detail and data on the efficacy of the method.

The step-by-step procedure used in the density gradient centrifugation method conformed fairly closely to that described by Bolch (1997), using Milli-Q water for preserved samples and sterile filtered seawater for live samples, as follows: 1ml of wet sediment was increased to 20 ml by adding Milli-Q/seawater in a test tube, this slurry was shaken for 2 minutes (Vortex Genie 2, set on 3) and sonicated for 1 minute (Branson1200) to dislodge cells from aggregates, filtered through a 100 µm sieve, and collected onto a 10µm sieve; this material was then resuspended in 10–20 ml of Milli-Q/seawater in a fresh test tube, carefully loaded with a pipette on top of 10 ml of Ludox or sodium polytungstate (SPT) in a 50 ml screw-capped polyethylene centrifuge tube, and centrifuged for 10 minutes at 2000 rpm (using a swing-out rotor and slowly increasing/decreasing the rotation to the desired speed); the interface with diatoms and most of the overlaying water were recovered with a pipette and placed into a fresh tube to which 20–30 ml of Milli-Q water/seawater was added to dilute the Ludox or SPT, and centrifuged again for 10 minutes at 2000 rpm to pellet the cells so that the overlaying water could be discarded; the recovered cells were resuspended with Milli-Q and Lugol's solution to 10 ml; the sediment pelleted below the gradient was also rinsed as the recovered cells and resuspended with Milli-Q and Lugol's solution to 5 ml.

This was an effective method to recover preserved diatoms from the sediment (83% of samples ≥ 90% recovery, 63% of samples ≥ 98% recovery) (Fig. 2) as previously demonstrated for dinoflagellate cysts (Bolch 1997, 82% recovery). The highest scavenging effect of centrifugation (ca. only 70–80% recovery) was found in those few samples (10%) that had a high relative contribution of heavily silicified diatoms, often chain-forming species, such as *Aulacoseira* spp., *Neodenticula seminae*, *Paralia* spp. and resting spores. Inspection of the pellet is, therefore, recommended in these circumstances.

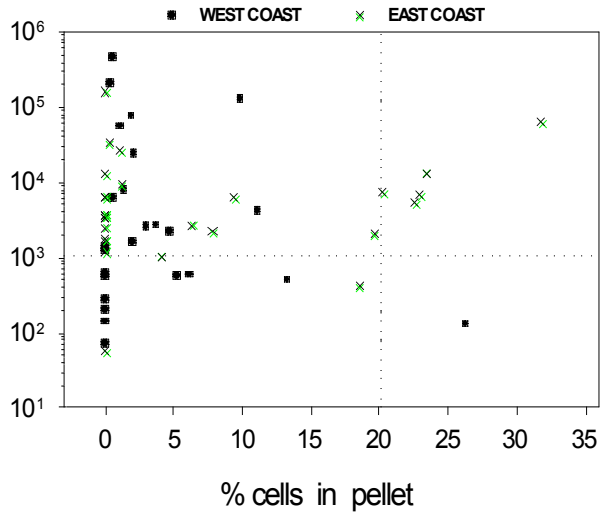

Fig. 2. Relationship between abundance of cells with intact protoplasm (y-axis) and the percentage of cells in the corresponding sediment pellets (x-axis) for each Canadian coast. Only the 49 preserved samples with live diatoms are included in this analysis. For future reference, samples above and to the left of the dotted lines indicate which samples would necessarily require detailed inspection of the pellet, as discussed in the text.

Microscopic evaluation:

In all counts that achieved ≥ 10^4 cells/g w.w., and in some that were ca. 10^3 cells/g w.w., the coefficients of variation between replicates were no greater than 15–20% (Fig. 3). Samples with cell numbers ≤ 10^3 cells/g w.w had a larger error, which is expected in microscopic assessments of infrequent organisms that reproduce exponentially and/or form colonies; nevertheless, numbers between replicates were within the same order of magnitude. Using Shaw's (1964) probability table to calculate the detection limits of infrequent species as a function of total cell counts gives an estimate of the resolution power of this data set for the early detection of NIS, a key component in bioinvasion risk assessments (Reaser et al. 2008). In 60% of the samples (those with ≥ 10^3 cells/g w.w.), we were able to detect taxa that comprised at least 6% of the assemblage and in 22% of these our detection level reached taxa that comprised 1% (Fig. 1 in the main text). There is room for improvement by increasing counting effort. On one hand, this may not seem cost-effective in terms of management since the "problem" samples are those from ships with the lowest propagule numbers. On the other hand, if events of low magnitude introductions are frequent enough, this may represent a high enough propagule pressure (and introduction potential) that may justify the time invested in achieving more accurate counts.

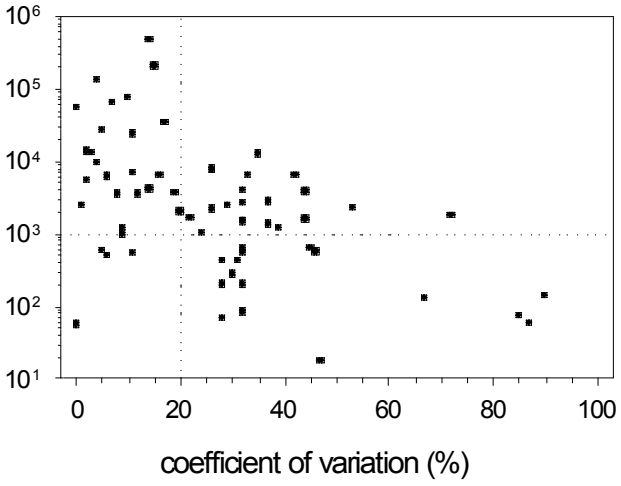

Fig. 3. Relationship between abundance of cells with intact protoplasm (y-axis) and the respective coefficient of variation (CV= [standard deviation/mean]*100) between replicates (x-axis). Only the 61 samples with live diatoms are included in this analysis. Dotted lines are references for lower/higher CV values, as discussed in the text.

Viability assessment:

Although SPT has been shown to be harmless to dinoflagellates (Bolch 1997), all 6 live diatom samples prepared with SPT prior to incubation into fresh media did not show diatom growth. Diatoms grew in all replicates of these same samples that were just sieved and rinsed with sterile filtered seawater before inoculation. Our data do not allow discerning between acute short-term effect (hours) and chronic long-term effect (days) of SPT on diatoms. Here we report the longer-term effect – no growth. One may speculate that, if there is also an immediate short-term effect, then our cell counts of SPT-treated samples analysed with the vital stain might have underestimated cell viability, which would bring fluorescein diacetate (FDA) values closer to those we found for autofluorescence and protoplasm (compare indicators of viability in Fig. 5 in the main text).

The visualization of the FDA stain was straightforward in our case, probably due to the use of a filter set that blocked the emission of the chlorophylls that can mask the FDA signal and has caused problems in other studies (Garvey et al. 2007). The use of FDA is not a flawless method. There are some species with known false negatives (references in Garvey et al. 2007) and there is evidence for signal interference from green autofluorescence (Tang & Dobbs 2007). Occasionally, some cells in *Paralia* spp. chains in our samples gave faint, dubious results and were thus not considered as viable (a possible source of false negative). Nevertheless, it proved to be superior to other options of indicators of cell viability in experiments to determine the efficiency of ballast water treatments (Reavie et al. 2010). One disadvantage of FDA is lack of resolution for species identification in comparison with the refinement possible with transmitted light microscopy. However, ballast water regulations for ships under Canadian jurisdiction target mostly abundances by size classes regardless of species identity (Transport Canada 2006), with the possible exception of harmful biota. As is often the case, the best method (or toolbox of methods) of choice depends on the objective/goal intended.

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