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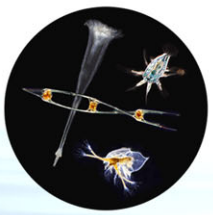

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First observations of living sea-ice diatom agglomeration to tintinnid loricae in East Antarctica

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Tintinnid ciliates are an important link in marine food webs as they feed on phytoplankton and bacteria while providing nutrients to higher trophic levels. Tintinnids are known to agglutinate mineral particles or dead biogenic material such as diatom frustules to their shell-like housing (lorica), however, reasons for this agglutination remain questioned. We report on our observation of agglomeration of the living diatoms *Fragilariopsis curta*, *F. cylindrus*, *F. pseudonana* and *F. rhombica* to loricae of the Antarctic tintinnid ciliates *Laackmanniella naviculaefera* and *Codonellopsis gaussi*. These unusual associations between living diatoms and tintinnids were exclusively observed south of 63.59°S. We discuss the significance of our new finding and generate hypotheses to be tested by future research. It remains unclear where these living diatom–tintinnid associations are initially formed (in or near sea ice or also further north when abundances of *L. naviculaefera*, *C. gaussi*, *F. curta*, *F. cylindrus*, *F. pseudonana* and *F. rhombica* happen to be relatively high); who the beneficiary is in this association; what the exact benefits are; and how they might influence the Southern Ocean carbon cycle. Nevertheless, our observation provides a key step forward towards illuminating the largely unknown ecology of two Southern Ocean-endemic tintinnid species.

KEYWORDS: diatom; tintinnid; *Fragilariopsis curta*; *Fragilariopsis cylindrus*; *Fragilariopsis pseudonana*; *Fragilariopsis rhombica*; *Laackmanniella naviculaefera*; *Codonellopsis gaussi*; sea-ice; Antarctica

INTRODUCTION

Tintinnid ciliates are planktonic protists and an important component of marine food webs. They belong to the microzooplankton (20–200 μm) and are the food source for larger zooplankton such as copepods, krill, mysid shrimp, salps, chaetognaths, juvenile fish, benthic octocorals and

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isopods (Dolan *et al.*, 2012; Dolan *et al.*, 2013 and references therein). Microzooplankton consume up to 70% of the daily annual production (Calbet and Landry, 2004), and although tintinnids specifically play a rather minor role as predators (Dolan *et al.*, 1999), they can be the dominant predator on small phytoplankton and cyanobacteria at times (Karayanni *et al.*, 2005).

The tintinnid lorica is vase-like, i.e. closed or tapered at one end and open at the other (Agatha *et al.*, 2013). Loricae are either hyaline without any particles attached, or hard or soft and partly or completely agglutinated with mineral particles or biogenic material (= agglomerated; e.g. diatom frustules) (Agatha *et al.*, 2013). Agatha and Simon (2012) have shown that tintinnid loricae consist mainly of proteins, however, exact lorica composition (including varying amounts of e.g. proteins, carbohydrates and lipids), the associated influence on benthic food webs and nutrient cycling when dead loricae sediment are still unknown. It is questioned whether agglomerated particles are taken up randomly, reflecting the most abundant phytoplankton species in the environment at the time of agglomeration (Winter *et al.*, 1986; Henjes and Assmy, 2008), or highly selectively, based on particle size and type (1984; Wasik *et al.*, 1996). Takahashi and Ling (1984) even reported that coccoliths (coccolithophore plates) can be arranged on the lorica in a specific way by the tintinnid. Various functions of the lorica have been suggested, most frequently the loricae are reported as providing armour to protect against grazers (Dolan, 2013). However, the lorica may provide further protection by enabling rapid sinking away from grazers (Capriulo, 1982) or by acting as a UV shield (Armstrong and Brasier, 2013). Other studies have suggested the role of the lorica also facilitates flotation in hyaline species, swimming directionality in spike-shaped/elongated forms (Kofoid, 1930; Kofoid and Campbell, 1939; Dolan, 2013), and food-uptake by enhancing fluid motion around the oral cilia as shown for sensory/food-collecting structures of other small zooplankton (Emlet and Strathman, 1985; Dolan, 2013) and by attaching themselves to detrital substrate (Jonsson *et al.*, 2004).

Tintinnids feed by creating micro-scale currents with their propelling cilia at the oral (anterior) end that help to capture prey while simultaneously utilizing these cilia to enable forward (anterior ahead) movement (Montagnes, 2013). The size of prey consumed by tintinnids correlates with the diameter of the mouth, usually being 20% (rarely exceeding 30%) of mouth diameter (Dolan, 2010). The food sources of tintinnids are highly diverse and they are known to consume pico-, nano- and micro-phytoplankton (Montagnes, 2013). Some tintinnids, such as *Laackmanniella*, are believed to suck out and ingest the protoplast of diatoms before they agglomerate the empty frustules to their loricae, making diatoms a valuable resource to them (Gowing and Garrison, 1992).

In this study, we focus on two tintinnid species with hard, agglomerated lorica, endemic to the Southern Ocean (Dolan and Pierce, 2013). *Laackmanniella naviculaefera* has been reported to occur between 43°S and 78°S (Dolan *et al.*, 2012). It is widely known that this tintinnid agglomerates dead frustules of the diatom species *Fragilariopsis curta*, *F. cylindrus* and *F. pseudonana* (amongst other diatoms) to their lorica (Wasik *et al.*, 1996). Similarly, *Codonellopsis gaussi* has been reported to agglomerate empty frustules of *F. cylindrus*, *F. pseudonana*, *F. rhombica* and *F. separanda* (amongst other diatoms; Wasik *et al.*, 1996).

Here, we provide the first report of living diatoms agglomerated to loricae of the tintinnids *L. naviculaefera* and *C. gaussi*. These observations were made during two independent voyages in East Antarctica, in 2014 and 2016. We present the distribution of living tintinnid–diatom associations, discuss the ecological significance of our novel observation and suggest future research objectives to be considered in this context.

METHOD

Sampling sites

Totten Polynya, East Antarctica (NBP14-02)

Sampling was conducted along a north–south transect (~56–67°S) from aboard the *RVIB Nathaniel B. Palmer* (NBP) during voyage NBP14-02 in the vicinity of the Totten Polynya, East Antarctica, in February 2014 (Fig. 1). In total, we collected 35 samples (74–100 mL) from the underway water intake at ~7 m subsurface (Supplementary Material Table 1), which were immediately fixed with Lugol's solution (~0.5 mL) for subsequent laboratory-based analyses. Live samples concentrated from the seawater intake line were examined on-board using inverted light microscopy (Olympus IMT-2, Japan), where the first observations of the living tintinnid–diatom association were noted. The preserved water samples were concentrated into Utermöhl chambers in the home laboratory to a final volume of 3 mL by sedimentation (48 h). Using the Utermöhl method (Utermöhl, 1958), a minimum total of 400 microphytoplankton and microzooplankton cells and specimens, respectively, were identified and counted at a magnification of 400× under the same inverted microscope used previously on-board. At the furthest offshore stations (NBP48-50), microplankton concentrations were low and a total raw cell/specimen count of 254, 135 and 317, respectively, could only be determined. All live microphyto- and microzooplankton (i.e. alive at the time of preservation with Lugol's solution, which, in the case of

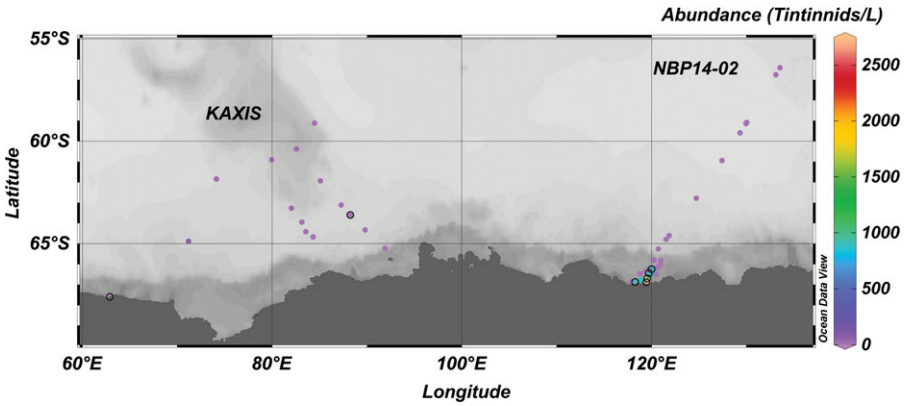

Fig. 1. Sampling locations and abundance of tintinnid–diatom associations. Map showing the sampling locations of the two voyages KAXIS (2016) and NBP14-02 (2014) along the East Antarctic coast. Also shown is total tintinnid abundance including both *Laackmanniella naviculaefera* and *Codonellopsis gaussi* (total tintinnids/L). Locations where living diatoms (*Fragilariopsis* spp.) were found agglomerated to either *L. naviculaefera* or *C. gaussi* are indicated by black circles. For details on the abundance of the individual tintinnids counted separately during the KAXIS voyage see Supplementary Material Table 1. Map created in Ocean Data View 4 (Schlitzer, R., Ocean Data View, http://www.awi-bremerhaven.de/GEO/ODV, 2005) with default bathymetry background of 0–6500 m depth.

tintinnids, means the cell was inside the lorica) were counted. However, as we did not separate tintinnid species during these counts we only present total tintinnid abundance data from the NBP samples. We also noted whether live diatoms or empty diatom frustules were attached to their loricae. Photos of tintinnid–diatom associations were taken using a Leica MC170 HD camera attached to the inverted microscope and LAS V4.2 software.

To accurately identify diatoms agglomerated to tintinnid loricae, selected samples were prepared for Scanning Electron Microscopy (SEM; Supplementary Material Table 1). About 200 µL were removed from the bottom of the Lugol's preserved sample used previously for the counts and washed three times with 1 mL H_2O after a settling time of ≥8 h in between each wash. The bottom of the sample (30 µL) was transferred onto a cover glass mounted on a carbon tab (ProSciTech, Australia) covered SEM stub (12.6 mm diameter) and air dried under a fume hood for 36 h. After gold-coating, we examined and photographed individual tintinnids with agglomerated diatoms using a JSM 6480LV SEM (JEOL, USA).

Tintinnids and diatoms were identified to species level using appropriate taxonomic literature (Hasle, 1965; Tomas, 1997; Scott and Marchant, 2005; Cefarelli *et al.*, 2010; Kim *et al.*, 2013; Santoferrara *et al.*, 2016). Light microscopy and SEM photographs were used to measure size ranges of tintinnids and diatoms found in association (measuring the smallest and largest individual for each species per sample) in Adobe Illustrator, and to note prevalence (rare, present, dominant) of diatom species attached to the tintinnids.

Kerguelen Plateau and Mawson Station, East Antarctica (KAXIS)

Surface water sampling was undertaken over the Kerguelen Plateau, within the Indian Ocean Sector of the Southern Ocean, East Antarctica, across the sea-ice zone and within polynyas near Mawson Station (~59–68°S) from aboard the *Aurora Australis* (January–March, 2016; Fig. 1). A total of 15 samples were collected using a combination of (i) phytoplankton net tows (20 µm mesh size) and (ii) a prototype "basket sampler" (a 5 × 10 cm plastic pre-filter housing, customized with the addition of 20 µm mesh to the inner basket attached to the underway seawater supply taking in water from ~7 m depth, gently concentrating microplankton). Samples were either preserved in glutaraldehyde (at a final concentration of ~1–2%) or examined live on-board using light and fluorescence microscopy (Leica DMLB2) with image capture using a Leica ICC50 in-body camera. During shipboard observations, we noted living diatoms on tintinnids, even when the lorica did not contain the ciliate cell. On land, 5 mL subsamples were counted for all tintinnids and concentrations were corrected for the volume of water sampled by either the plankton net or the basket sampler. *Laackmanniella naviculaefera* and *C. gaussi* were counted separately in each sample and we recorded whether each individual tintinnid had living or empty diatom frustules agglomerated to them. Measurements of tintinnids and diatoms were conducted as for the NBP samples based on photography and taxonomic identification followed the same literature (see previous section).

RESULTS

Tintinnid–diatom complex composition and distribution

Live tintinnids were found primarily south of 63°S (Fig. 1). The majority of tintinnids observed belonged to the species *L. naviculaefera*. We rarely encountered tintinnids of the species *C. gaussi* (a maximum of 35 L^{-1} at 64.88°S, KX47), compared to maximum of 2684 L^{-1} of total tintinnids (mostly *L. naviculaefera*) at 66.88°S, NBP25 (Fig. 1). Total abundance of the sum of *L. naviculaefera* and *C. gaussi* is shown in Fig. 1. Detailed records on the abundance of *L. naviculaefera* and *C. gaussi* during the KAXIS voyage are documented in Supplementary Material Table 1.

During both voyages we found living diatoms agglomerated to tintinnid loricae (Fig. 1). These diatoms generally occurred either as single cells or in short chains (Fig. 2). During the NBP voyage, light microscopy revealed living diatoms were agglomerated to *L. naviculaefera* (Fig. 2A,D). In our land-based counts, we found these associations in samples from 66.68°S, 66.88°S, 66.41°S and 66.24°S (NBP18, NBP25, NBP27 and NBP33, respectively; Fig. 1). Additionally, living tintinnid–diatom associations were also observed during shipboard examinations of a sample taken at 66.87°S (NBP20; Fig. 1). SEM analysis clearly showed *F. curta*, *F. cylindrus* and *F. rhombica* to be attached to *L. naviculaefera* (Fig. 2D). In the rare cases of *C. gaussi* presence in our NBP samples, no live agglomerated diatoms were observed. Supplementary SEM imaging showed that empty diatom frustules agglomerated to *C. gaussi* included *F. cylindrus* and *F. pseudonana* (Supplementary Material Fig. 1F,G). Additional information on the occurrence of living tintinnid–diatom associations and their illustration can be found in Supplementary Material Table 1 and Supplementary Material Fig. 1.

During the KAXIS voyage, light microscopy revealed living diatoms on tintinnids at ~63.59 (KX24) and 67.6°S (KX48 Mawson Bay, ice-free during sampling; Fig. 1). These living associations were found in combination with both *L. naviculaefera* and *C. gaussi*. Light microscopy provided limited resolution to determine the diatoms to species level in most cases, however, on-board investigations of the KX37 sample clearly revealed one empty frustule of *F. kerguelensis* and several empty frustules of *F. curta*, *F. cylindrus*, *F. pseudonana* and *F. rhombica* agglomerated to *L. naviculaefera* (Fig. 2B) and numerous living *F. cylindrus* and *F. pseudonana* cells agglomerated to *C. gaussi* (Fig. 2E,F). Further shipboard observations from KX48 detected one valve of *Chaetoceros* cf. *atlanticus*, an empty *Chaetoceros* resting spore one empty *Asteromphalus* sp., and a living *Thalassiosira* cf. *gravida* potentially agglomerated to *L. naviculaefera* (Supplementary Material Table 2). Epifluorescence microscopy showed that the diatoms on both *L. naviculaefera* and *C. gaussi* were clearly alive (Fig. 2C,F). In the KAXIS samples, we observed very dense agglomerations of diatom frustules, chiefly on the bowl, and very rarely (one specimen) on the tintinnid collar. Additionally, there was a pattern of smaller diatoms towards the anterior end, and larger cells at the posterior end.

Summarizing size measurements of living tintinnid–diatom associations found (Supplementary Material Table 2) allowed us to establish a rough assessment of which diatom species were most prevalent on the two tintinnids. As such, *L. naviculaefera* most often had large numbers of individual living *F. cylindrus* and *F. curta*, or short chains of these two species, attached. *Fragilariopsis pseudonana* was also commonly agglomerated to *L. naviculaefera*

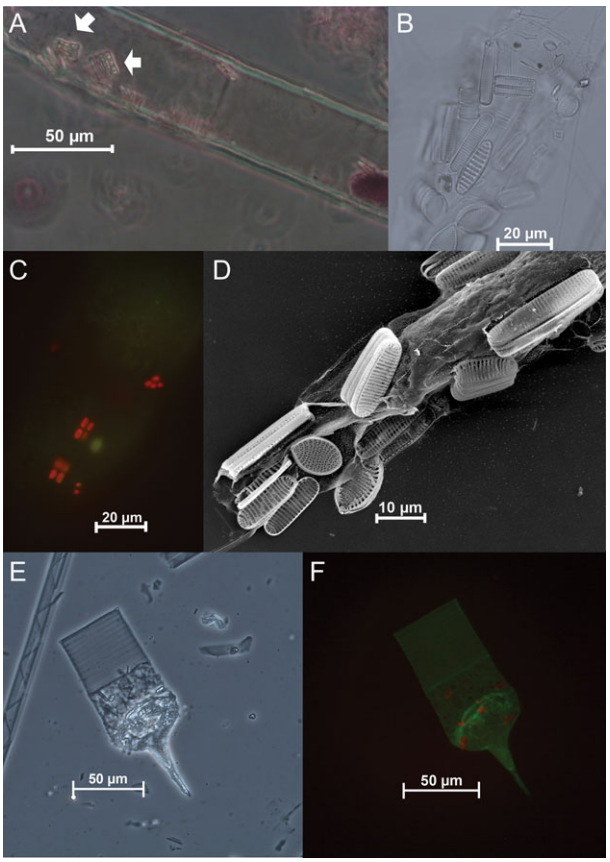

Fig. 2. Tintinnid–diatom associations. (**A**) Live *Laackmanniella naviculaefera* with short chains (indicated by arrows) of live *Fragilariopsis* spp. (NBP27). (**B**) Empty *L. naviculaefera* with empty diatom frustules including *Fragilariopsis kerguelensis*, *F. curta*, *F. pseudonana* and *F. rhombica* (KX37). (**C**) Empty *L. naviculaefera* with live *Fragilariopsis* spp. shown by red autofluorescence (KX48). (**D**) Live *L. naviculaefera* with *F. curta* and *F. rhombica* (live/dead status of diatoms indiscernible; NBP43). (**E**, **F**) Empty *Codonellopsis gaussi* with living *Fragilariopsis* spp. agglomerated using light and fluorescence microscopy (KX24).

but in very small numbers, whilst any other diatom species was rarely encountered (*F. rhombica*, small centrics). *Codonellopsis gaussi* only had the two smallest *Fragilariopsis* species, *F. cylindrus* and *F. pseudonana* (apical length <10 µm, transapical length <4 µm) attached to its lorica, with *F. cylindrus* dominating lorica coverage at both the NBP and KAXIS locations (Supplementary Material Table 2).

DISCUSSION

Our examinations indicate the agglomeration of living diatoms (as individual cells and short chains) to the tintinnids *L. naviculaefera* and *C. gaussi* in the East Antarctic region between ~62°E and 135°E. Analysis revealed *L. naviculaefera* agglomerated frustules of *F. curta, F. cylindrus, F. pseudonana* and *F. rhombica*, while *C. gaussi* was only ever observed with *F. cylindrus* and *F. pseudonana* frustules attached. Living representatives of these species agglomerated to the tintinnid's loricae were found exclusively south of 63.59°S. This observation prompts speculation on the formation, ecology and benefits of this newly found association between tintinnids and the specific sea-ice diatom taxa found attached to them.

Our observation of the presence of tintinnids south of 60°S is consistent with previous studies on the biogeographical range of *C. gaussi* and *L. naviculaefera* and their endemism to the Southern Ocean (Dolan *et al.*, 2012). *Codonellopsis gaussi* has been found to occur in relatively high abundances in open waters between Terra Nova Bay and the Ross Sea Shelf (~74–78°S; Fonda Umani *et al.*, 2005) and in the northern region of the Weddell Sea (61–70°S; Boltovskoy and Alder, 1992). *Laackmanniella naviculaefera* has frequently been reported to be highly abundant in relatively low-salinity waters in the vicinity of sea-ice, whereby high abundances appear to be associated with high sea-ice edge productivity rather than low-salinity meltwater (Garrison and Buck, 1989; Boltovskoy and Alder, 1992; Garzio and Steinberg, 2013). Generic identifications of *Laackmanniella* spp. and *Codonellopsis* spp. occurrences have been recorded within the Antarctic pack ice, near our sampling site (Terre Adélie, ~66°S, 140°E; Delille *et al.*, 2002). However, although ciliates are believed to be able to invade sea-ice brine channels when they become porous at the end of summer (Fenchel and Lee, 1972; Sullivan and Palmisano, 1984), it is unknown if this applies to *L. naviculaefera* or *C. gaussi* specifically.

On a temporal scale, our observations of *L. naviculaefera* and *C. gaussi* near the East Antarctic sea-ice edge, are also consistent with the literature on seasonal tintinnid abundance and succession. Both our samplings took place in Austral summer (and into the beginning of autumn in the case of the KAXIS voyage), a time during which Antarctic tintinnid abundances have been observed to be at their maximum (Leakey *et al.*, 1994; Wasik and Mikolajczyk, 1994; Clarke and Leakey, 1996). Numerous investigations have shown that the seasonal increase in tintinnid abundance is associated with elevated phytoplankton and chlorophyll *a* concentrations, indicating a successional pattern in tintinnid growth following phytoplankton blooms initiated by seasonal fast- or sea-ice melt (Garrison and Buck, 1989; Leakey *et al.*, 1994; Garzio and Steinberg, 2013). During sea-ice melt, overwintering sea-ice algae have been found to play an important role in acting as "seed populations" to subsequent phytoplankton blooms and to contribute to regional primary production (Garrison *et al.*, 1987; McMinn and Hodgson, 1993; Lizotte, 2001). The agglomeration of living diatoms (that are most likely able to photosynthesize) to the tintinnids might extend the period of primary production for these diatoms past peak bloom times and contribute to a previously un-recognized carbon input to the Southern Ocean.

The living *Fragilariopsis* spp. we have observed are species principally related to an existence in a sea-ice environment and in association with melt waters of the nearby open ocean. The latter raises the question as to where exactly tintinnid–diatom associations are formed, i.e. in brine channels within the sea-ice, their exits, or the vicinity of sea-ice in the open water. *Fragilariopsis curta*, *F. cylindrus* and *F. pseudonana* have been reported consistently as dominant diatom species in sea-ice (Garrison *et al.*, 1986; Lizotte, 2001). Kang and Fryxell (1992) reported a prominent abundance of all of the agglomerated *Fragilariopsis* spp. identified in our samples in near-ice open water conditions. During the NBP2014-02 voyage, *Fragilariopsis* spp., in particular, *F. cylindrus/pseudonana* (grouped) and *F. curta*, were the most abundant phytoplankton species at all stations (30 and 11% of total microplankton on average across all samples, respectively, unpublished data). Living tintinnid–diatom associations were found exclusively south of 66.24°S during the NBP14-02 voyage, suggesting a relationship between the formation of the complexes and sea-ice; however, this is not supported by our finding of living diatoms agglomerated to a tintinnid further north at 63.59°S during the KAXIS voyage. Attachments to substrates such as sea-ice might have benefits, for example, previous studies have indicated that attachment of the tintinnid *Eutintinnus inquilinus* to various substrates enhanced current flow rates around the loricae leading to an 80% feeding rate increase (Jonsson *et al.*, 2004). Whether *L. naviculaefera* and *C. gaussi* agglomerate diatoms in or near sea-ice, potentially using the ice as

attachment substrate to maximize feeding (as speculated based on Jonsson *et al.*, 2004), or in open water conditions, should be the focus of future studies, as this will provide valuable information about the ecology and life cycle of these microzooplankton grazers.

Despite the fact that the tintinnids in our study seemed to agglomerate *Fragilariopsis* spp. that were highly abundant in the water column (see above), indicating non-selectivity in particles being attached, we still speculate that *Fragilariopsis* spp. might represent an optimally sized resource while agglomerated frustules are used as protection. Gowing and Garrison (1992) and Takahashi and Ling (1984) have reported that tintinnids can draw out protoplasts and arrange particles in specific ways on their loricae. *Fragilariopsis* spp. (*F. curta*, *F. cylindrus*, *F. pseudonana*, *F. rhombica*) agglomerated to the two tintinnids in this study were ~2 µm wide and ~25 µm long. Notably, *C. gaussi* appears to only have the smallest of the *Fragilariopsis* species (*F. cylindrus* and *F. pseudonana*) attached to their loricae. However, the maximum frustule size exceeds the typical prey size being ~20–30% that of mouth diameter (Dolan, 2010), thus utilization of the protoplast and agglomeration of the frustule seems feasible in both tintinnid species. Additionally, our observation of frustule agglomeration primarily to the bowl only, and smaller (larger) frustules to the bowl (collar) are consistent with previous observations suggesting a selectivity of particles based on size (Agatha *et al.*, 2013).

The above assumes that the tintinnid is the only beneficiary, and the diatom the "victim", of this newly found association. However, it has been shown that diatoms can increase their rate of survival, in particular, through the production of sticky extrapolymer substances, providing a mechanism to attach to surfaces and prevent damage from crystal formation in the brine channel (Krembs *et al.*, 2002). Should the tintinnid–diatom associations form within, or at the exits of, brine channels, tintinnid loricae may provide an ideal substrate, on which diatoms grow actively, explaining our finding of small chains agglomerated to the tintinnids. Additionally, the tintinnid would be a rapid transport mechanism for diatoms to enter the open water once increased ice porosity and/or melting allowed it.

CONCLUSION

For the first time, we observed living tintinnid–diatom associations south of 63.59°S. This finding is a key step forward towards illuminating the ecology of Antarctic tintinnids, about which very little is known. Our study opens new perspectives on the life history of tintinnids and agglomerated diatoms. Future studies in the sea-ice zone and open Southern Ocean should focus on investigating the exact location of tintinnid–diatom complex formation, their potential influence on the carbon cycle, as well as the identification of which organism is the beneficiary in this association. The latter could be addressed using culturing approaches and the measurement of metabolite fluxes between the diatoms and tintinnids using, for example, Secondary Ion Mass Spectrometry.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Plankton Research* online.

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