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Preliminary study on the response of marine fungoid protists, the thraustochytrids, to lipid extracts of diatoms

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Abstract Thraustochytrids are particle-associated protists, ubiquitous in the marine environment. They are known to display a negative relationship with phytoplankton cells but a positive one with their exudates and detritus. Phytoplankton are known to express allelopathic effects against other organisms, but the exact mechanism by which phytoplankton cause inhibition to thraustochytrids is understudied. This is the first report of examination of bioactivity of lipid extracts of diatom cultures on thraustochytrids. Lipid extracts from four diatom cultures, viz. *Cylindrotheca closterium* (JB2), *Skeletonema* sp.1 (JB3), *Thalassiosira* sp. (JB4) and *Skeletonema* sp.2 (JB5), were tested against the growth of thraustochytrids: *Oblongichytrium* spp. (isolates VD4 and VD6), *Parietichytrium* sp. (isolate VD12) and *Schizochytrium* sp. (isolate VDC23b). Among the thraustochytrid isolates, *Parietichytrium* sp. was the most sensitive, whereas *Schizochytrium* sp. appeared to be the most resistant to the lipid extracts. The lipid extract from *C. closterium* possessed high amounts of C16:0, C16:1, C18:0, C18:1 and C20:5 fatty acids and bioactivity against the thraustochytrids. Lipids extracted during the late stationary phase of diatoms were inhibitory to thraustochytrid growth. Overall, these observations not only enhance our understanding of the diversity of allelopathic interactions between thraustochytrids and diatoms in the marine ecosystem, but also reveal the pivotal role of diatom lipids in such interactions across trophic levels.

Keywords Bioactivity · Lipids · Diatoms · Protists · Thraustochytrids

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Introduction

Thraustochytrids are fungoid protists ubiquitously found in the marine environment (Raghukumar 2002). They belong to phylum Labyrinthulomycetes of the Kingdom Stramenopila, of the SAR domain (Damare 2019). Till the last decade, thraustochytrids were comparatively less explored than the rest of the protists. However, presently, their role as a food source for marine organisms as well as decomposers

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of organic matter in the marine environment is well recognized (Raghukumar and Damare 2011; Damare et al. 2013; Damare and Raghukumar 2015). Thraustochytrids have extensive intracellular lipid reserves, with a high proportion of omega-3 fatty acids. Thus, they provide nutritional supplements to higher eukaryotes such as microzooplankton, mesozooplankton and fishes (Raghukumar 2017; Damare and Raghukumar 2015; Raghukumar 2002). Of late, thraustochytrids are being exploited commercially for omega-3 fatty acids, which are of nutraceutical and pharmaceutical importance to human health (Marchan et al. 2018; Raghukumar 2008).

Thraustochytrids are significant remineralizers in the ecosystem, similar to bacteria, and play an important role in the carbon cycle and microbial food web (Duan et al. 2018; Raghukumar 2017; Raghukumar and Damare 2011). They feed on dissolved organic carbon (DOC) and particulate organic carbon (POC) and exhibit a positive correlation with POC and transparent exopolymeric particles (TEPs) in pelagic waters (Ueda et al. 2015; Damare and Raghukumar 2008; Raghukumar et al. 2001; Naganuma et al. 1998). The population densities of thraustochytrids in the water column vary between < 1 and 230×10^3 cells L^{-1} but may spike up to 1313 x 10^3 cells L^{-1}, particularly during conditions of high temperature and low salinity (Raghukumar 2017; Ueda et al. 2015), a pattern termed the 'boom or bust' phenomenon by Raghukumar (2017). Thraustochytrids, reported to be detritus or particle loving in nature (Raghukumar 2002), occur abundantly in biofilms (Raghukumar et al. 2000) and marine aggregates (Damare and Raghukumar 2010; Lyons et al. 2005). Considering their occurrence on marine aggregates, thraustochytrid biomass may be at par with or even exceed bacterial biomass (Duan et al. 2018; Damare and Raghukumar 2008; Raghukumar et al. 2001). Marine aggregates, usually formed from the sinking of dead cellular debris, and their plumes, are rich sources of nutrients and shelter in the heterogeneous/patchy marine environment (Azam and Long 2001). Thus, these aggregates are hot spots of biological activity, i.e. they harbour a wide range of organisms including thraustochytrids, phytoplankton, bacteria, etc. Marine aggregates also serve as vectors for transport of thraustochytrids and other associated organisms to the deeper regions of the oceans (Lyons et al. 2005, Damare and Raghukumar 2010). Thraustochytrids have been reported in bathypelagic marine snow, where they contribute to organic carbon sequestration from the surface waters to the deep sea and also bring about the degradation of semi-labile or recalcitrant organic matter that has reached the ocean bottom (Bochdansky et al. 2017; Raghukumar and Damare 2011).

Thraustochytrids promote the formation of marine aggregates, as has been experimentally demonstrated by Damare and Raghukumar (2012), due to their unique physiological features. (1) Thraustochytrids reproduce using motile biflagellated zoospores which settle on a suitable substrate and grow (Raghukumar 2002; Raghukumar et al. 2000). (2) They produce ectoplasmic net (EN) elements which facilitate their growth by clinging on to the substrates and releasing different hydrolytic enzymes for substrate breakdown. Thus, these EN elements support the clumpy or particle-associated growth of thraustochytrids (Leaño and Damare 2012; Damare and Raghukumar 2006, 2012). (3) They produce extracellular polysaccharides which are the main backbone for the production of aggregates (Liu et al. 2014; Jain et al. 2005). (4) They possess hydrophobic cell wall that helps them bind to hydrocarbons in sediments (Raikar et al. 2001), and are thus likely to promote aggregation in hydrocarbon-contaminated environments (Daly et al. 2016, Decho and Gutierrez 2017).

Thraustochytrids are closely associated with diatoms in marine aggregates (Rapp et al. 2018; Raghukumar 1986). Both groups occupy different trophic levels in the marine food web, that of remineralizers and primary producers, respectively. Additionally, thraustochytrids could be among the many zoosporic parasites of marine diatoms as some of these are known to shrink the diatom protoplasm while growing on them (Gaertner 1979). However, zoosporic organisms parasitizing marine diatoms are not well described from the environment due to complex taxonomic characterization of the zoospores (Scholz et al. 2016). Nonetheless, the interactions between these two interesting groups, whether within marine aggregates or in the water column, are scarcely documented. Till date, there are only two studies addressing the association of thraustochytrids (especially in culture) with diatoms (Raghukumar 1986; Gaertner 1979). Thraustochytrids did not exhibit a correlation with chlorophyll concentration in the Arabian Sea and Seto Inland Sea (Ueda et al. 2015;

Raghukumar et al. 2001). In addition, they were abundant above and below the chlorophyll maxima but very rarely at the same depth in Hawaiian waters (Li et al. 2013). However, a few studies displayed a positive correlation of thraustochytrids with chlorophyll (Naganuma et al. 1998; Kimura et al. 2001). This was attributed to the growth of thraustochytrids on the dissolved organic matter and particulate detritus from phytoplankton. Such a variable relationship, therefore, suggests that thraustochytrid abundance is dependent on the type of substrate available at any particular time and the absence of growth-inhibiting factors. Generally, thraustochytrid growth is restricted during conditions favourable for optimum phytoplankton growth (Raghukumar et al. 2001). In studies on macroalgae, thraustochytrid abundance was higher during the senescence stage of growth (Raghukumar et al. 1992). Similarly, Damare and Raghukumar (2012) have reported lower thraustochytrid abundance in the presence of diatom *Chaetoceros* sp. Given that diatom exopolysaccharides (EPS), which comprise of rhamnose, mannose, xylose, fucose and uronic acids, support the exuberant growth of thraustochytrids (Damare and Raghukumar 2012), it is apparent that some factor, other than the diatom EPS, restricts the growth of thraustochytrids. However, the nature of this factor is still ambiguous.

Diatoms are known to store carbon in their cells, mainly in the form of lipids, especially triglycerides, which constitutes 23–45% of the dry weight of diatom cells (Hu et al. 2008). Diatom lipids are not only storage molecules but also possess various bioactive properties such as antibacterial, antimicrobial, antimalarial and antiviral (Yi et al. 2017). This gives rise to the question of whether the intracellular lipids of diatoms could contribute to the inhibitory effects of diatoms on thraustochytrids. Considering that thraustochytrids and diatoms are associated with each other, particularly in marine aggregates, how thraustochytrids react to diatom lipids is still not reported to the best of our knowledge. The present study was therefore undertaken to determine the sensitivity of thraustochytrids to diatom lipids and addresses the following questions. (1) Are thraustochytrids inhibited by diatom lipid extracts? (2) How do thraustochytrids react to lipid extracted from different diatom growth phases? (3) Do thraustochytrids exhibit species-specific sensitivity profiles to diatom lipids?

Materials and methods

Experimental approach

This study examines the influence of diatom lipids, extracted from the logarithmic and late stationary phases, on the growth of thraustochytrids. The bioactivity of lipid extracts from four diatom cultures against four thraustochytrid isolates was determined by the cross-streak method.

Source of thraustochytrids

Four thraustochytrid isolates were used in this study. These were obtained from seawater and seaweeds from two locations in Goa, along the west coast of India, viz. Anjuna Beach and Divar Island (Table 1). Thraustochytrid cultures were isolated by incubating respective substrates in sterile seawater baited with sterile pine pollen for 5 days and purified on modified Vishniac (MV) agar (Damare and Raghukumar 2006) supplemented with 10% Ampilox-c (500 mg of ampicillin and 500 mg cloxacillin).

Maintenance and identification of thraustochytrid isolates

All isolates were cultured and maintained on plain MV agar without any antibiotics and subcultured every 10 days. Initial characterization was based on cell morphology and life cycle features. This was followed by molecular identification of three isolates, viz. VD4, VD6 and VDC23b, by 18S rRNA gene sequencing, and characterization of their signature fatty acid profiles by fatty acid methyl ester (FAME) analysis.

18S rRNA gene sequencing

The 18S rRNA gene of the isolates was amplified using universal 18S external primers NS1 and NS8 and two internal primers NS3 and NS4 (White et al. 1990). The isolates were grown in MV broth for 5–7 days, and DNA was extracted using ZR fungal/bacterial DNA MiniPrep^{TM} kit (Cat. No. D6005, Zymo Research). The 18S rRNA gene was amplified using two sets of primers, viz. NS1–NS4 and NS3–NS8 [(according to conditions described by Damare and Raghukumar (2010) except for the annealing

Table 1 Details of isolation of thraustochytrid cultures from substrates such as seaweeds and seawater from Goa, India

Isolate number	Substrate	Location	Period of isolation
VD4	*Ulva fasciata*	Intertidal region of Anjuna Beach	January 2018
VD12	*Ulva compressa*	Intertidal region of Anjuna Beach	January 2018
VD6	Seawater	Anjuna Beach	January 2018
VDC23b	Seawater	Divar Island	November 2017

temperature of 59 °C for NS1–NS4 and 62 °C for NS3–NS8)]. The PCR products were purified using Wizard® SV Gel and PCR Clean Up kit (Promega, Cat. No. A9282) and sequenced using a 3130XL Genetic Analyser (ABI Sequencer 2200), Applied Biosystems.

FAME analysis by gas chromatography and mass spectrometry

Lipids were extracted from thraustochytrids using the Bligh and Dyer (1959) method. The thraustochytrid isolates were grown in MV broth for 7 days under aeration (125 rpm), and their biomass harvested by centrifugation. The biomass (approximately 100–200 mg wet weight) was transferred into tubes containing sterile zirconium beads, mixed with 8 mL of 2:1 (v/v) chloroform/methanol mixture and vortexed well. The tubes were centrifuged at 1000 rpm for 5 min at RT. The supernatant was separated, and the above step was repeated twice. The final supernatant was mixed with 1:1 chloroform/distilled water, vortexed and centrifuged at 1000 rpm for 5 min at RT. The bottom phase was recovered by inserting a Pasteur pipette through the upper phase with gentle positive pressure. The recovered bottom phase was washed with 'authentic upper phase' which was prepared similarly as the sample except using distilled water in place of the thraustochytrid sample. The bottom phase was recovered after this washing step into a pre-weighed rotary evaporator flask and dried completely under nitrogen. The total lipid content was determined gravimetrically (Mamatha et al. 2010).

Transesterification of the fatty acid components of the total lipids was carried out following the method described by Mamatha et al. (2010). Fatty acid methyl esters (FAMEs) were analysed on a gas chromatography–mass spectrometer (GCMS) system (Shimadzu-2010 ultra) equipped with a DB-23 capillary column (60 m × 0.25 µm ID, 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. The mass selective detector was operated by electronic impact (70 eV). Injector and interface temperature was 250 °C. The column oven temperature programmed was as follows: 60 °C for 1 min hold and then raised to 150 °C at the rate of 20 °C min^{-1} and then raised to 245 °C at the rate of 5 °C min^{-1} and held for 10 min at 245 °C. Each fatty acid concentration was calculated from the total identified fatty acids, and values were presented as relative percentage (%).

Growth and maintenance of diatom cultures

Four different isolates of diatoms used in this study were isolated previously from Dona Paula, Goa, India, during July–September 2014. These were named JB2, JB3, JB4 and JB5 and were identified morphologically as *Cylindrotheca closterium*, *Skeletonema* sp.1, *Thalassiosira* sp. and *Skeletonema* sp.2, respectively. The isolates mentioned above were maintained in f/2 medium (Guillard and Ryther 1962) grown under 8 h:16 h light–dark cycle at temperature between 25 and 32 °C. These were subcultured in fresh *f*/2 medium every 14 days.

Based on previous experiments (unpublished data), the onset of logarithmic growth (12 days), late stationary (20 days) and decomposition phase (25 days) of the diatom cultures were known. To compare bioactivity, logarithmic phase and late stationary phases (12 and 20 days, respectively) were selected for lipid extraction. Simultaneously, the diatom abundance and chlorophyll concentration were determined during both these periods. Diatom abundance (cells mL^{-1}) was quantified microscopically; chlorophyll *a* concentration (mg L^{-1}) was determined spectrophotometrically following the 90% acetone extraction method (Parsons et al. 1984). These

analyses were carried out in triplicates. The results are provided in Supplementary Figures S1 and S2.

Extraction and analysis of diatom lipids

Approximately 200 mL of 12- and 20-day-old diatom cultures (logarithmic and late stationary phases, respectively) was filtered through 0.22-μm filter paper, and the biomass obtained (approximately 100 mg) was used for lipid extraction, following the procedure detailed above for thraustochytrids. The total lipids extracted were transferred to pre-weighed rotary evaporator flasks, dried completely and determined gravimetrically (hereafter referred to as 'diatom lipid extracts'). FAME analysis was done as mentioned above for thraustochytrids.

Bioactivity of diatom lipids against thraustochytrids

Thraustochytrid isolates—VD4, VD6, VD12 and VDC23b, were grown in MV broth for 5 days at 28 ± 2 °C under aeration (125 rpm). Subsequently, these isolates were streaked in a parallel fashion on MV agar plates in triplicates. Sterile filter paper strips (8 × 5 mm) were wetted with 20–30 μL diatom lipid extracts from logarithmic as well as late stationary phases separately, amounting to approximately 0.1 g per strip. Each strip was then placed in the centre of the inoculated MV agar plate. The plates were incubated at RT for 48–72 h and observed for a zone of clearance. Two controls, viz. chloroform/methanol (2:1) and distilled water, were maintained separately, by dipping filter paper strips in respective controls instead of diatom lipid extracts. Controls were also maintained in triplicates.

The bioactivity of late stationary phase diatom lipid extracts against thraustochytrid isolate VD6 was also examined in broth. One mL of the 5-day-old thraustochytrid culture broth (MV) was transferred to a sterile 24-well culture plate. Aliquots of 50 μL of late stationary phase diatom lipid extracts of the four different diatoms were added separately to the wells and incubated at RT for 5 days. One control consisted of 1 mL VD6 culture broth and 50 μL of 2:1 chloroform/methanol mixture, whereas an additional control consisted of only VD6 culture broth without any test sample. After incubation, the thraustochytrid cells in the wells were counted using a haemocytometer. Cells were also counted immediately after inoculation to obtain day 0 reading.

Statistical analysis

The bioactivity data (from agar medium) were analysed statistically to determine whether the sensitivity of different thraustochytrid isolates to individual lipid extracts differed significantly. The variation across growth phases (logarithmic vs. late stationary) was also assessed. These analyses were carried out using the STATISTICA 8 software. Prior to analysis, the data were checked for normality and homogeneity of variances using Shapiro–Wilk's and Levene's tests, respectively. The data were not normally distributed, even after transformation, and were therefore analysed using nonparametric tests. The variation across thraustochytrid isolates was analysed using Kruskal–Wallis tests, separately for lipid extracts from each diatom. The variation across logarithmic and late stationary phases was determined separately for each thraustochytrid isolate against each diatom using Wilcoxon matched pairs test. All statistical analyses were considered at a significance level of 0.05.

Results

Identification of thraustochytrids

Isolates VD4 and VD6 were identified as species of *Oblongichytrium*, and VDC23b was *Schizochytrium* sp. based on their cellular morphology, life cycle features and 18S rRNA gene sequencing (Supplementary Fig. S3). The sequences of thraustochytrid isolates obtained were submitted to the NCBI database and bear accession numbers MK234637-39. Isolate VD12 was identified as *Parietichytrium* sp. based on its morphological and life cycle characteristics (Supplementary Fig. S4).

Fatty acid composition of thraustochytrids

The amount of lipid extracted was 0.093, 0.107, 0.121 and 0.152 g g^{-1} biomass (dry weight) in *Oblongichytrium* sp.VD4, *Oblongichytrium* sp. VD6, *Parietichytrium* sp. VD12 and *Schizochytrium* sp. VDC23b, respectively. The FAME analysis showed distinct fatty acid profiles for each of the isolates

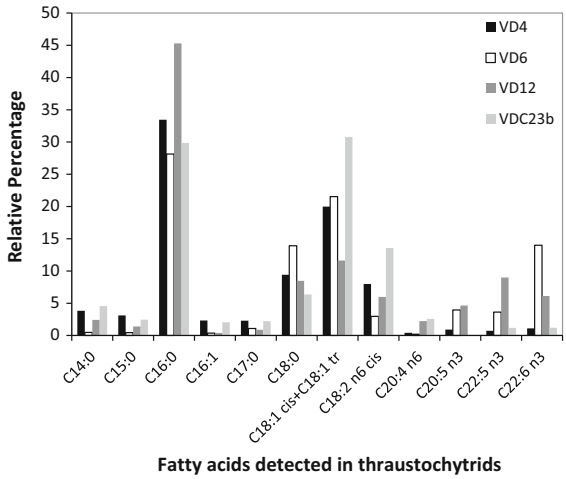

Fig. 1 Fatty acid profiles of the four thraustochytrid isolates

(Fig. 1). *Oblongichytrium* sp. VD4 showed higher proportions of saturated fatty acids (C14:0-C17:0) than *Oblongichytrium* sp. VD6. The latter showed higher proportions of unsaturated fatty acids (mono—i.e. MUFA and poly—i.e. PUFA) except C16:1 and C20:4 than the former, indicating that they are different species of *Oblongichytrium*. Overall, among all the four isolates, palmitic acid (C16:0) was maximum, followed by oleic acid (C18:1). Significant differences were found in eicosapentaenoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA) concentrations between different species studied. EPA was highest in *Parietichytrium* sp. VD12 (4.65%) followed by *Oblongichytrium* sp. VD6 (3.96%), whereas DHA was highest in *Oblongichytrium* sp. VD6 (14%) followed by *Parietichytrium* sp. VD12 (6.13%).

Fatty acid composition of diatoms

The amount of lipids extracted from diatoms ranged from 0.269 to 0.309 g g^{-1} during the logarithmic growth phase and 0.361–0.725 g g^{-1} during the late stationary phase. Among the four diatoms, *Thalassiosira* sp. JB4 produced the lowest amount (0.269 g g^{-1}) during the logarithmic phase and the highest amount (0.725 g g^{-1}) during late stationary phase (Supplementary Table S1). The other three diatom cultures also produced higher amount of lipids during late stationary phase (0.361–0.395 g g^{-1}) than the logarithmic phase (0.299–0.309 g g^{-1}) (Supplementary Table S1).

Fatty acids in the four diatom cultures varied from C12:0 to C22:6 (Fig. 2; Supplementary Table S2). The most common fatty acids were myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:0), palmitoleic acid (C16:1n-7) and EPA (C20:5n-3). Low levels of tetracosanoic acid (C24:0) and cis-15-tetracosenoic acid (C24:1) were also observed. The concentration of saturated fatty acids (SFAs) was higher than unsaturated fatty acids (UFAs) in all the diatom cultures. Overall, the logarithmic phase extracts had higher SFAs than late stationary phase extracts except in *C. closterium* JB2. MUFA concentration was higher in late stationary than logarithmic phase except in case of *Thalassiosira* sp. JB4. PUFAs varied between logarithmic and stationary phases in *C. closterium* JB2. EPA was higher in logarithmic phase than late stationary phase, and docosapentaenoic acid (DPA, C22:5) and DHA (C22:6), though reported in the logarithmic phase, was below detectable levels in the late stationary phase of *C. closterium* JB2 (Supplementary Table S2).

Bioactivity of diatom lipids against thraustochytrids

The diatom lipid extracts exhibited varying levels of inhibition against the thraustochytrid isolates (Fig. 3, Supplementary Table S3). Overall, late stationary phase extracts were more inhibitory towards the thraustochytrids than the logarithmic phase extracts (Fig. 3). The late stationary phase extracts showed inhibition against all the four thraustochytrid isolates, whereas the logarithmic phase extracts of *Thalassiosira* sp. JB4 and *Skeletonema* sp. JB3 and JB5 showed inhibitory activity against only one thraustochytrid *Parietichytrium* sp. VD12 (Fig. 3). Among all the four diatoms, the logarithmic and stationary phase extracts of *C. closterium* sp. JB2 showed the highest zones of inhibition. The late stationary phase extract of JB2 consistently produced larger zones of inhibition (45–55 mm) against thraustochytrids compared to the corresponding logarithmic phase extract (0.6–2.8 mm) (Table 2, Supplementary Table S3). The zone of inhibition of the late stationary phase extracts of other diatom isolates was in the range of 0.5–14 mm. The sensitivity of all the four thraustochytrid isolates to the diatom lipid extracts varied

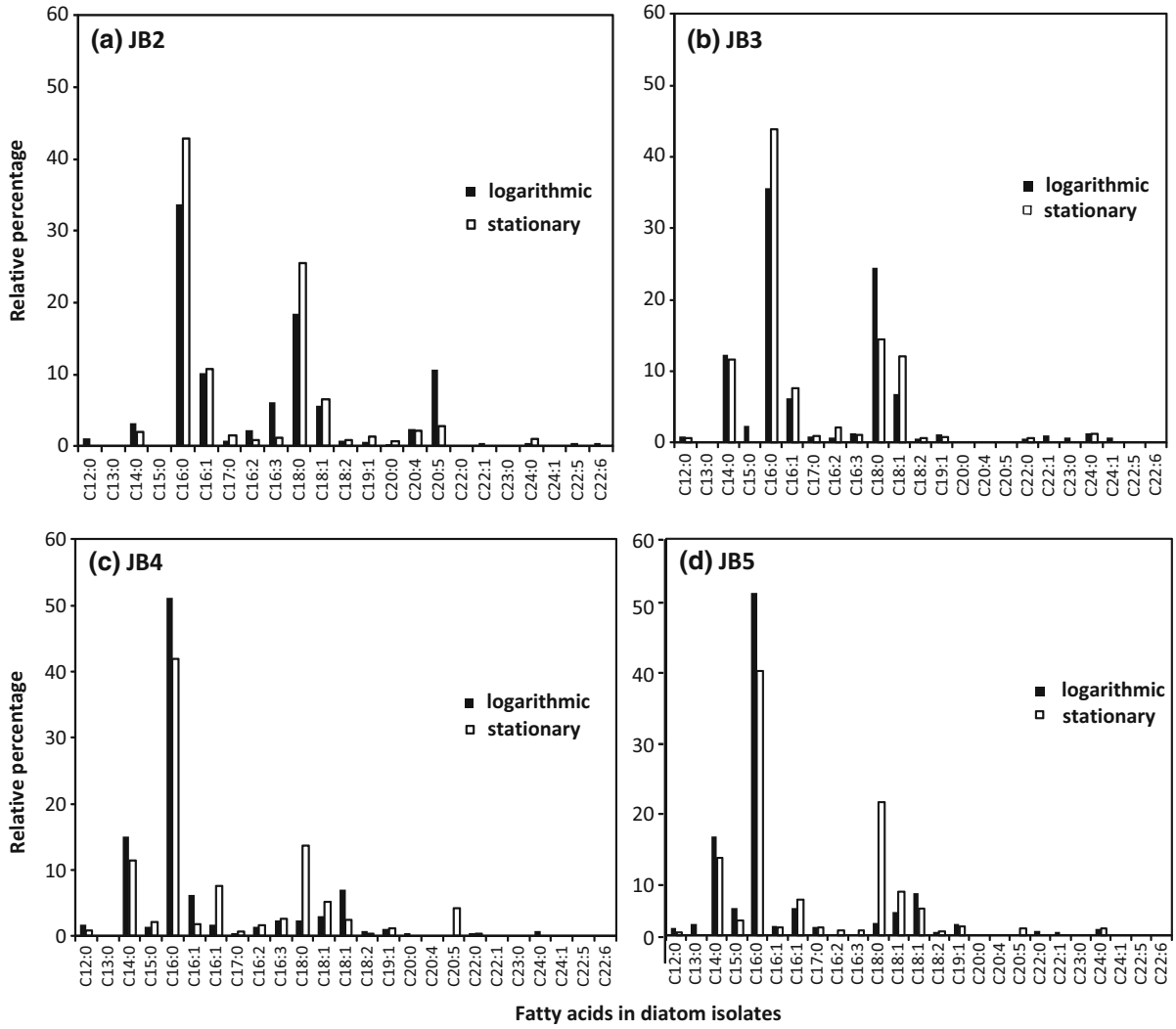

Fig. 2 Fatty acid profiles of the four diatom isolates. **a** JB2: *C. closterium*, **b** JB3: *Skeletonema* sp.1, **c** JB4: *Thalassiosira* sp. and **d** JB5: *Skeletonema* sp.2

significantly with respect to the growth phase (logarithmic and stationary phase) of the diatom *Cylindrotheca closterium* JB2, whereas thraustochytrid isolate VD6 showed a similar significant variation in case of *Thalassiosira* sp. JB4 and *Skeletonema* sp. JB5, also (Wilcoxon matched pairs test, Table 3).

Comparison between the four thraustochytrid isolates showed that *Parietichytrium* sp. VD12 was the only thraustochytrid isolate that was sensitive to the logarithmic phase diatom lipid extracts of all the four diatoms used in this study (Fig. 3). On the contrary, *Schizochytrium* sp. VDC23b was the most resistant to the logarithmic phase lipid extracts of all the diatoms tested. All the four thraustochytrid isolates grew in the presence of both the controls. The thraustochytrid isolates differed with respect to their sensitivity to diatom lipid extracts (Fig. 3), with statistically relevant differences observed in all the experimental treatments, except late stationary phase of *Cylindrotheca closterium* JB2 and *Thalassiosira* sp. JB4 (Kruskal–Wallis *H* test, Table 2).

Since *Oblongichytrium* sp. VD6 showed significant variation in sensitivity towards diatom lipid extracts with respect to growth phases (Table 3), it was selected for re-examining the bioactivity of late stationary phase diatom lipid extracts in liquid

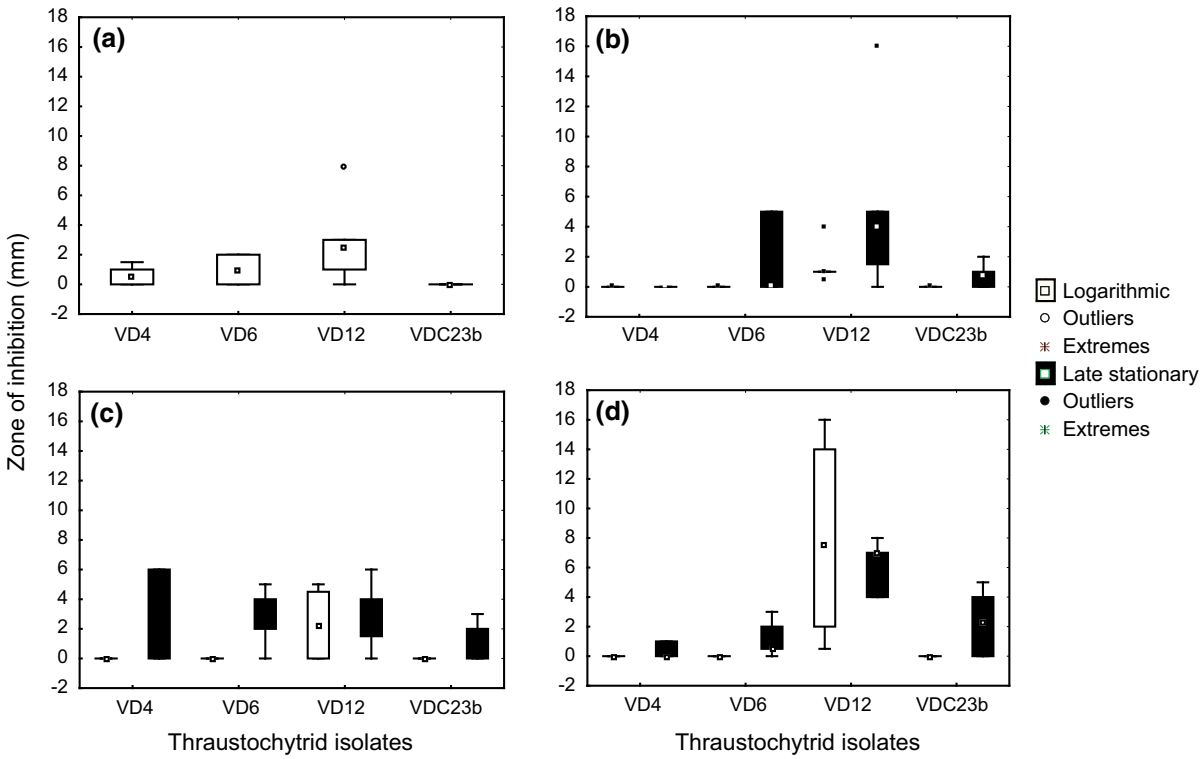

Fig. 3 Box–Whisker plots depicting the sensitivity of thraustochytrid isolates to lipid extracts of diatoms from logarithmic and late stationary phases. **a** JB2: *C. closterium*, **b** JB3: *Skeletonema* sp.1, **c** JB4: *Thalassiosira* sp. and **d** JB5: *Skeletonema* sp.2. In **a**, the trend for late stationary phase is not included, since the zones of inhibition were in the range of 45–55 mm for all the late stationary phase extracts of *C. closterium*

Table 2 Results of the Kruskal–Wallis test to analyse the sensitivity of thraustochytrid isolates to lipid extracts of diatoms

Experimental treatments	*H*	*p* value	Chi-square	*p* value
JB2/logarithmic phase	**10.31666**	**0.0161**	**9.33333**	**0.0252**
JB2/late stationary phase	0	1	0	1
JB3/logarithmic phase	**22.56472**	**0.00005**	**24**	**0.00001**
JB3/late stationary phase	**8.969603**	**0.0297**	**9.902098**	**0.0194**
JB4/logarithmic phase	**13.65773**	**0.0034**	**14.4**	**0.0024**
JB4/late stationary phase	1.648985	0.6483	1.5	0.6823
JB5/logarithmic phase	**22.39519**	**0.0001**	**24**	**0.00001**
JB5/late stationary phase	**14.12145**	**0.0027**	**13.3333**	**0.004**

p values of H and Chi-square values are mentioned at their right, respectively

Significant *p* values are marked in bold

ND not derived

medium, i.e. MV broth. It showed sensitivity to late stationary phase extracts of all diatoms on agar medium, and the same was observed in broth. *Oblongichytrium* sp. VD6 cell counts in the test broth were less than that in the control broth devoid of any diatom extract (Fig. 4). The decrease in cell numbers was 48–71% and 41–58% (compared to control) on days 2 and 5, respectively.

Table 3 Results of the nonparametric Wilcoxon matched pairs test to determine the sensitivity of thraustochytrid isolates to lipid extracts of diatoms with respect to the growth phase (logarithmic vs. late stationary)

Experimental treatments	*Z*	*p* value
JB2/Thrausto#VD4	**2.201398**	**0.027709**
JB3/Thrausto#VD4	ND	ND
JB4/Thrausto#VD4	1.825742	0.067890
JB5/Thrausto# VD4	ND	ND
JB2/Thrausto# VD6	**2.201398**	**0.027709**
JB3/Thrausto# VD6	ND	ND
JB4/Thrausto# VD6	**2.022600**	**0.043115**
JB5/Thrausto# VD6	**2.022600**	**0.043115**
JB2/Thrausto# VD12	**2.201398**	**0.027709**
JB3/Thrausto# VD12	1.483240	0.138012
JB4/Thrausto# VD12	0.104828	0.916512
JB5/Thrausto# VD12	0.674200	0.500185
JB2/Thrausto# VDC23b	**2.201398**	**0.027709**
JB3/Thrausto# VDC23b	1.825742	0.067890
JB4/Thrausto# VDC23b	1.825742	0.067890
JB5/Thrausto# VDC23b	1.825742	0.067890

Significant *p* values are marked in bold

ND not derived

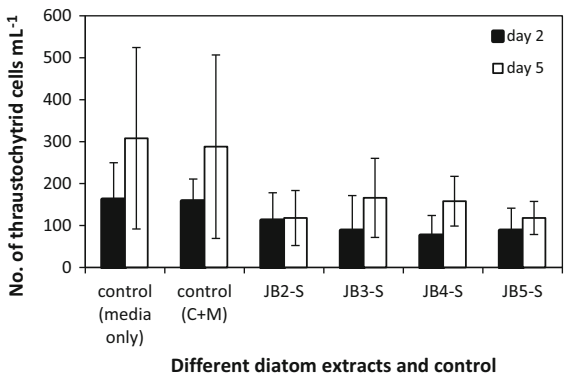

Fig. 4 Thraustochytrid *Oblongichytrium* sp. VD6 cell counts (cells mL^{-1}) in the presence of late stationary phase extracts of different diatoms along with controls. JB2: *C. closterium*, JB3: *Skeletonema* sp.1, JB4: *Thalassiosira* sp., JB5: *Skeletonema* sp.2. C + M in the control represents chloroform + methanol. S stands for late stationary phase

Discussion

Interactions between thraustochytrids and diatoms have not been extensively studied, though their coexistence in coastal and oceanic waters has been reported (Berdjeb et al. 2018; Damare and Raghukumar 2015; Georges et al. 2014; López-García et al. 2001; Raghukumar 1986). Damare and Raghukumar (2012) were the first to investigate the interaction between thraustochytrids and diatom polysaccharides using an experimental approach. The present study substantiates interactions between thraustochytrids and diatoms, based on experiments on the bioactivity of diatom lipid extracts against thraustochytrid isolates. To our knowledge, this study is the first investigation of the bioactivity of diatom lipids against thraustochytrids.

Lipids extracted from all diatoms showed bioactivity against thraustochytrids, in a species-specific manner (Fig. 3). The fatty acid composition of diatoms is known to differ with species (Yi et al. 2017; Prartono et al. 2013). *Cylindrotheca* species are usually lipid rich with palmitoleic acid (C16:1) and oleic acid (C18:1) as principal MUFAs, pentadecanoic acid (C15:0) and palmitic acid (C16:0) as SFA and EPA (C20:5) as PUFA (Demirel et al. 2015), also observed in the present study. *Thalassiosira* sp. possesses C14:0, C16:0, C16:1, C16:3 and C20:5 as major fatty acids (Zhukova 2004; Volkman and Hallegraeff 1988). A similar composition of fatty acids in *Thalassiosira* sp. (JB4) was also observed in this study. It is interesting to note that these diverse fatty acids may play a role in inhibiting thraustochytrids.

Overall, late stationary phase extracts displayed higher levels of inhibition than the corresponding logarithmic phase extracts. This could be attributed to the change in lipid composition across the different growth stages (logarithimic vs. late stationary phases) of diatoms, reported earlier by Rodríguez-Núñez and Toledo-Agüero (2017) and Pratoomyot et al. (2005). Although our study showed higher SFAs than UFAs in diatoms, the former decreased with the age of diatoms. *Skeletonema* sp. JB3 and JB5 as well as *Thalassiosira* sp. JB4 had lower SFA in late stationary phase than the logarithmic phase. Our study demonstrated less accumulation of unsaturated fatty acids (UFA) and high accumulation of SFAs during the logarithmic growth phase in all diatoms except *C. closterium* (JB2). In case of SFAs, palmitic acid (C16:0) and stearic acid (C18:0) were higher in the late stationary phase than the logarithmic phase except in the case of *Thalassiosira* sp. (JB4) for the former and *C. closterium*

(JB2) for the latter. The trend in EPA concentration in the logarithmic and late stationary phases of *Thalassiosira* sp. JB4 was similar to that reported by Zhukova (2004) and Pratoomyot et al. (2005). In cultures *Skeletonema* sp JB3 and JB5, SFA decreased with age while MUFAs and PUFAs showed a reverse trend. EPA concentrations were highest during the late stationary phase. This is consistent with the observations of Guihéneuf et al. (2008) in their study on the effect of irradiance and carbon source on the concentration of omega-3 fatty acids in *Skeletonema costatum*. Some diatoms produce PUFAs during the logarithmic phase and MUFA during the stationary phase, whereas others produce SFAs along with PUFAs during the logarithmic phase and only SFAs during stationary phase (Rodríguez-Núñez and Toledo-Agüero 2017). In our study, although MUFAs were higher in the late stationary phase as reported above, PUFAs also increased with the age of diatoms, except in the case of *C. closterium* JB2. Logarithmic phase lipids of diatoms, consisting primarily of SFAs, had no or minimum inhibitory action against thraustochytrid isolates (Fig. 3). SFAs increase the doubling time in bacteria by increasing the duration of their lag phase; this is influenced by the phase of diatom growth during which they are accumulated as well as their concentration (Fay and Farias 1975). The same holds true for MUFAs, which were higher in the late stationary phase extracts in the present study.

Thraustochytrids exhibited varying levels of sensitivity to the diatom lipid extracts tested (Fig. 3). *Schizochytrium* sp. VDC23b was the most resistant among the thraustochytrid isolates studied. *Parietichytrium* sp. VD12 was the most sensitive; it displayed sensitivity to all the lipid extracts tested (logarithmic and late stationary phase extracts). *Parietichytrium* genus was erected after emendment of the genus *Ulkenia* (Yokoyama et al. 2007); Raghukumar (1986) had observed that *Ulkenia visurgensis* which was obtained in culture from environmental samples of diatoms never grew in the presence of fresh diatom cultures. Of all the diatoms studied, *C. closterium* JB2 displayed the highest inhibitory activity against all thraustochytrid isolates (Table 3). Interestingly, it showed a high proportion of EPA along with DPA and DHA in the logarithmic phase; these PUFAs were not detected in the logarithmic phase of the other three diatom cultures.

Ecological implications of lipid-mediated interactions between thraustochytrids and diatoms

Fatty acids influence the growth of competitor micro-organisms during biofilm formation, as observed in case of bacteria (Huang et al. 2011). The cellular lipids, as well as those associated with diatom frustules, contribute to the fatty acid pool. The frustule-associated fatty acids account for 5.8% of the organic matter after 30 days of growth (Suroy et al. 2014). These frustule-associated fatty acids, which comprise C16:1 and C18:1, are less susceptible to degradation by micro-organisms. These, therefore, remain intact during senescence of diatom cells and may thus mediate allelopathic effects, if any. The same may be true for cellular lipids; their role in influencing the growth of organisms in close proximity to them cannot be ignored.

Usually, the growth phases of the diatoms influence the fatty acid composition of the whole cell and not that of the frustule-associated lipids alone (Suroy et al. 2014). In nature, the termination of diatom blooms, in response to nutrient limitation, starts with autolysis of diatom cells and release of cytoplasm. Thus, cell death starts during stationary and senescent phases, often giving rise to precursors for the formation of aggregates (Armbrecht et al. 2014). Based on the results of our study, it is possible that the fatty acids released to the environment on autolysis will restrict the numbers of thraustochytrids, due to the inhibitory action of the late stationary phase diatom lipids. Recently, it has been observed that aplanochytrids, which are the sister group to thraustochytrids, obtain nutrition from living diatom cells by absorption through EN elements (Hamamoto and Honda 2019). Thus, the predator–prey (aplanochytrid–diatom) relationship also seems to exist between the two groups of organisms. In order to avoid predation by the Labyrinthulomycetes protists, the diatoms may show allelopathy against them by means of diatom lipids.

A sudden release of intracellular contents of diatoms may also occur due to zooplankton grazing, thus exposing the surrounding organisms to the allelochemicals released from the grazed cell (Xu et al. 2015). This might restrict thraustochytrid numbers in the water column and may be one of the reasons for their patchy distribution (Damare and Raghukumar 2008). The patchy occurrence of thraustochytrids has also been attributed to their association

with particulate matter in the water column (Damare and Raghukumar 2008; Raghukumar et al. 2001). Once aggregate formation is initiated, particulate matter like cell debris gets attracted towards it (Armbrecht et al. 2014), thus increasing the size of the aggregate and enriching it with nutrients that eventually support the growth of thraustochytrids. The transparent exopolymeric particles (TEPs) or exopolysaccharides released by diatoms during aggregate formation favour the growth of thraustochytrids (Damare and Raghukumar 2012). Therefore, our study supports the perception that thraustochytrids generally dominate during the decay of the phytoplankton bloom and not during the initial stages (Raghukumar 2002; Raghukumar et al. 2001).

In conclusion, the thraustochytrid isolates were sensitive to the lipid extracts of diatoms, especially to those from the late stationary phase. Among the four thraustochytrids studied, varying extent of susceptibility was observed; *Parietichytrium* sp. VD12 was the most sensitive, whereas *Schizochytrium* sp. VDC23b was the most resistant. The lipid extract of diatom, *C. closterium* JB2, was the most inhibitory as compared to the other three diatoms extracts tested. These observations not only enhance our understanding of the diversity of allelopathic interactions between thraustochytrids and diatoms but also reveal the pivotal role of diatom lipids in such interactions across trophic levels. It must also be remembered that the diatom cultures used in these experiments were not axenic. In nature, as well, diatoms and bacteria are always found in close association (Amin et al. 2012). Thus, the possibility of associated bacteria contributing to the bioactivity of diatom lipids cannot be ruled out and has to be addressed in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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