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ORIGINAL ARTICLE

A co-culturing/metabolomics approach to investigate chemically mediated interactions of planktonic organisms reveals influence of bacteria on diatom metabolism

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Abstract Chemically mediated interactions are hypothesized to be essential for ecosystem functioning as co-occurring organisms can influence the performance of each other by metabolic means. A metabolomics approach can support a better understanding of such processes but many problems cannot be addressed due to a lack of appropriate co-culturing and sampling strategies. This is particularly true for planktonic organisms that live in complex but very dilute communities in the open water. Here we present a co-culturing device that allows culturing of microalgae and bacteria that are physically separated but can exchange dissolved or colloidal chemical signals. Identical growth conditions for both partners as well as high metabolite diffusion rates between the culturing chambers are ensured. This setup allowed us to perform a metabolomic survey of the effect of the bacterium *Dinoroseobacter shibae* on the diatom *Thalassiosira pseudonana*. GC–MS measurements revealed a pronounced influence of the bacterium on the metabolic profile of *T. pseudonana* cells with especially intracellular amino acids being up-regulated in co-cultures. Despite the influence on diatom metabolism, the bacterium has little influence on the growth of the algae. This might indicate that the observed metabolic changes represent an adaptive response of the diatoms. Such interactions might be crucial for metabolic fluxes within plankton communities.

Keywords Diatom · Metabolomics · Plankton interactions · Co-culture · *Thalassiosira pseudonana*

1 Introduction

Planktonic ecosystems harbor a variety of different microscopic organisms sharing the same habitat. Among these, unicellular algae contribute significantly to the global carbon cycle by performing up to 50 % of the world wide carbon fixation (Field et al. 1998). Nowadays, there is an increasing recognition that chemically mediated interactions between planktonic organisms are essential for the structure and function of the ecosystem (Ianora et al. 2011; Pohnert et al. 2007; Sieg et al. 2011; Van Donk et al. 2011). Planktonic microalgae, such as diatoms, dinoflagellates and prymnesiophytes can detect chemical signals that trigger chemical defense (Ianora et al. 2004; Pohnert 2000), intraspecific cell to cell communication (Vardi et al. 2006; Vidoudez and Pohnert 2008) and allelopathic interactions (Legrand et al. 2003; Paul et al. 2009; Yamasaki et al. 2009). Metabolites regulating these interactions are very diverse in their chemical nature. Indeed gaseous metabolites such as dimethylsulfide (DMS) (Wolfe et al. 1997) and cyanogen bromide (Vanelslander et al. 2012), complex secondary metabolites such as saxitoxins (Selander et al. 2006) and even high molecular mass protein complexes (Yamasaki et al. 2009) can cause responses in phytoplankton. This diversity makes the elucidation of chemical communication highly challenging. Chemically mediated interactions in pelagic organisms can be stimulated by direct cell contact or feeding activity. In these cases mechanical contact of interacting partners is required to trigger physiological or chemical responses (see e.g. Ianora et al. 2004; Wolfe et al. 1997; Pohnert 2000). Such interactions are local but can influence e.g. predator prey dynamics. In

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contrast, interactions that are mediated by diffusible signals have the potential to influence plankton communities by triggering responses in entire populations or in patches in the open ocean. These interactions require the release and perception of chemical signals that are active within the open water of the ocean (Pohnert et al. 2007). Investigation of plankton interactions have thus to distinguish between these fundamentally different chemically mediated processes but available methods are limited.

Several studies dealing with the influence of co-existing organisms on the metabolism of phytoplankton focused on the effect of grazers on the production of specific metabolites (Selander et al. 2011). However, from an ecological perspective the interactions of diatoms with bacteria also have a substantial influence on algal abundance and performance (Mayali and Azam 2004; Paul and Pohnert 2011; Teeling et al. 2012) even though only few studies document the influence of bacteria on diatom metabolism. For example, the production of the toxic domoic acid by the diatom *Pseudo-nitzschia multiseries* could be stimulated by the addition of bacteria (Bates et al. 1995). In a non-contact co-culturing experiment where axenic *P. multiseries* was separated by a cellophane tubing from non-axenic cultures, domoic acid concentrations in axenic cells were lower than in non-axenic ones suggesting that a direct cell contact between diatom and bacteria supports domoic acid production (Kobayashi et al. 2009). The importance of chemical communication was recently also emphasized for the interaction between the coccolithophore *Emiliania huxleyi* and the bacterium *Phaeobacter gallaecienis*. Bacteria either promoted or inhibited growth of *E. huxleyi* depending on the nature of released algal metabolites (Seyedsayamdost et al. 2011).

A major bottleneck in the identification of chemically mediated plankton interaction is the availability of suitable co-culture setups. Ideally, such devices should separate interaction partners but still allow diffusion of potential infochemicals. Previous setups using dialysis bags or cellophane tubing placed in a glass pot could not ensure identical growth conditions for the interacting partners or a sufficient diffusion between both culturing chambers (Jensen et al. 1972; Kobayashi et al. 2009; McVeigh and Brown 1954; Sieg et al. 2011). Commercial setups where membrane coated inserts are used to separate interaction partners have been employed in studies using marine organisms (Yamasaki et al. 2007). These setups allow only the investigation of small volumes of up to 5 mL. Since phytoplankton grows often only in low cell abundance such setups do not allow chemical analysis due to lack of sufficient extractable biomass.

Here we introduce a co-culture setup that overcomes these limitations. A fully replicated co-culturing experiment of the diatom *Thalassiosira pseudonana* and the bacterium *Dinoroseobacter shibae* was carried out and sufficient biomass for GC–MS based metabolomics according to Vidoudez and Pohnert (2012) was obtained. Since genome data for *T. pseudonana* as well as for the universal algal symbiont *D. shibae* are available, this study gives substantial insight into the interaction of well established model species (Armbrust et al. 2004; Wagner-Döbler et al. 2010). We observed that bacteria substantially influence specific pathways in the alga and we propose that such metabolically mediated interactions might play a fundamental role in ecosystem functioning.

2 Materials and methods

2.1 Co-culture setup

The co-culture setup consists of two modified glass vessels each holding ca. 500 mL with a 100 mm flat edge opening (Fig. 1). Modification of the commercially available Duran® flask (VWR, Dresden, Germany) included the generation of an opening and the addition of a 29 mm neck by a glass blower. Both vessels can be fitted together by a holding clamp and a 0.22 µm hydrophilic polyvinylidene fluoride (PVDF) membrane filter (Durapore, Millipore, Billerica, MA, USA) can serve to separate two culturing chambers. An O-ring made out of silicone between both vessels ensures a leak proof sealing. Each culture vessel has one 29 mm opening for filling and sampling purposes that can be covered with aluminum foil during culturing. Culture vessel, O-ring, aluminum foil, and membrane filter can be autoclaved separately and assembled under a sterile hood.

2.2 Diffusion assay

Each partition of the co-culture setup was filled with 300 mL artificial seawater (Maier and Calenberg 1994) and one chamber was supplemented with 15 µL of heptadienal (Sigma-Aldrich, Munich, Germany, 100 mM in methanol), 300 µL dimethylsulfoniopropionat (DMSP) (100 mM in water) and 177 µL of a 1.05 M sodium nitrate solution. The setup was kept at 15 °C and shaken at approximately 90 rpm during the entire experiment. To follow the kinetics of diffusion through the membrane, samples were taken from both compartments and analyzed as described below. Results are based on three independent replicates.

2.3 Instrumentation

LC–MS measurements were performed on an Acquity ultra performance liquid chromatography (UPLC) equipped with an Acquity BEH HILIC column (1.7 µm, 2.1 mm × 50 mm) coupled to a Q-ToF Micro mass spectrometer

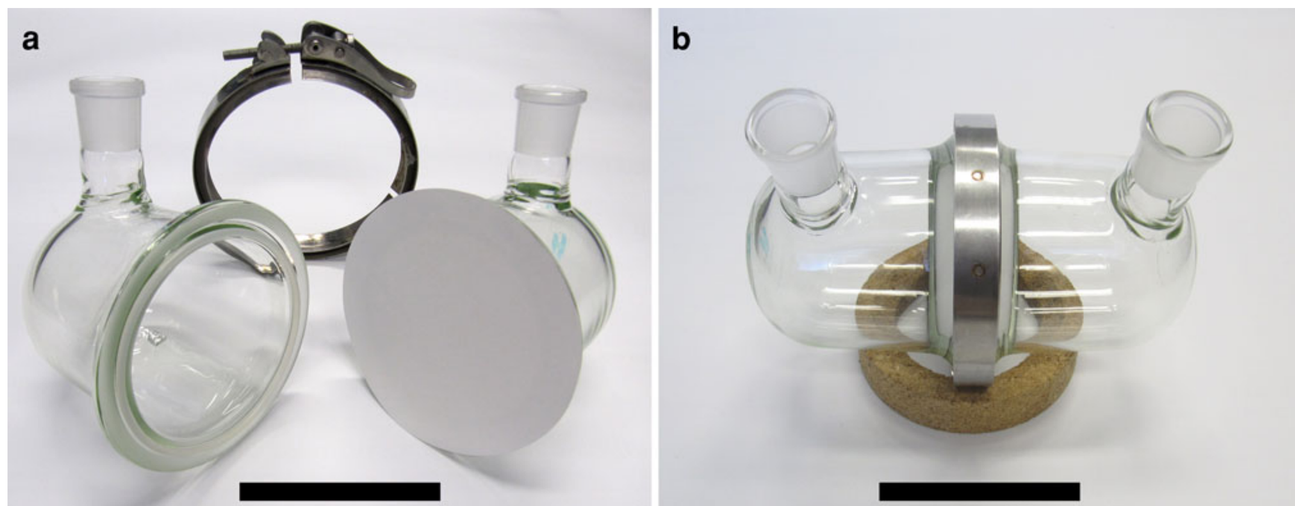

Fig. 1 Co-culture setup to grow organisms without direct contact but ensuring the exchange of metabolites. **a** Disassembled setup with all parts required for the setup and **b** assembled co-culture device. A 0.22 µm membrane separates both chambers that can each be filled with up to 500 mL medium. The black bar represents 10 cm

(Waters, Manchester, UK). Heptadienal and metabolomics samples were measured in random order with an Agilent 6890N gas chromatograph (Waldbronn, Germany) equipped with a 30 m DB-5 ms column (internal diameter 0.25 mm, film thickness 0.25 µm) protected by a 10 m Duraguard pre-column (Agilent, Waldbronn, Germany). The GC was coupled to a GCT premier mass spectrometer (Waters, Manchester, UK). He 5.0 was used as carrier gas with a constant flow rate of 1 mL min^{-1}. The MS scan rate was set to 2 and 5 scans s^{-1} for heptadienal and metabolomics samples respectively in dynamic range extension mode with the electron impact source at 70 eV. The GC oven temperature for the heptadienal analysis was held initially at 60 °C for 2 min followed by an increase of 8 °C min^{-1} to 240 °C and of 15 °C min^{-1} to 280 °C. For metabolomic samples the initial temperature of 60 °C was held for 1 min followed by an increase with a rate of 15 °C min^{-1} to 310 °C which was held for 10 min.

2.4 Solvents for extraction and derivatization

The following solvents were used for the extraction and derivatization: methanol (ChromasolvPlus, Sigma-Aldrich, Munich, Germany), chloroform (HiPerSolv, VWR, Dresden, Germany), ethanol (LiChrosolv, Merck, Darmstadt, Germany) and pyridine (ChromasolvPlus, Sigma-Aldrich, Munich, Germany).

2.5 Heptadienal analysis

For the analysis of dissolved heptadienal 10 mL water samples were derivatized with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (Fluka, Basel, Switzerland) and measured using GC–MS as previously described (Vidoudez and Pohnert 2008). The quantification was based on the ratio of the peak area of heptadienal and benzaldehyde that was added as internal standard.

2.6 DMSP analysis

The quantification is based on a published protocol (Spielmeyer and Pohnert 2010). Briefly, 1 mL water samples were supplemented with 100 µL of a 200 µM [$^{2}H_6$]-DMSP solution as internal standard and kept at −80 °C until further analysis. The sample was diluted 1:10 (v:v) with acetonitrile before measuring with UPLC–MS.

2.7 Nitrate analysis

Nitrate concentrations were determined spectrophotometrically using a Specord M42 UV–Vis spectrophotometer (Carl Zeiss, Jena, Germany) according to a protocol developed by Zhang and Fisher (Zhang and Fischer 2006) that is based on the conversion of resorcinol.

2.8 Diatom-bacteria co-cultures

An axenic *T. pseudonana* culture was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (CCMP 1335) and grown subsequently in autoclaved artificial seawater (Maier and Calenberg 1994) at a constant temperature of 15 °C under a 14/10 h light/dark regime with 40 µmol photons m^{-2} s^{-1} illumination. The axenity of the culture was regularly checked by plating 100 µL of the culture on marine broth agar plates. *D. shibae* DFL 12 was obtained from the German

Collection of Microorganism and Cell Cultures (Strain No. 16493) and grown in artificial seawater supplemented with 10 mM succinic acid (Roth, Karlsruhe, Germany) (SW+) as sole carbon source at 15 °C on an orbital shaker (90–100 rpm). To prepare the co-culture experiments both *T. pseudonana* and *D. shibae* were inoculated in SW+ medium and kept in exponential growth phase by repeated dilution with fresh culture medium. Co-culturing (Tp/Ds) was started by inoculation of one chamber with exponentially growing *T. pseudonana* to reach 50×10^3 cells mL^{-1} in a final volume of 350 mL. The other culturing chamber was inoculated with *D. shibae* resulting in 1.8×10^6 cells mL^{-1} in 350 mL. As controls cultures of *T. pseudonana* were inoculated into both chambers with identical medium and concentrations as above (Tp/Tp). All setups were placed on an orbital shaker (90 rpm) at 15 °C with a light/dark regime of 14/10 h using fluorescent tube Osram T8 36 W 840 with approximately 40 µmol photons s^{-1} m^{-2}. Samples (1 mL) were taken daily during the course of the experiment under sterile conditions to estimate cell growth, chlorophyll a fluorescence and photosystem II (PSII) efficiency. Tp/Tp and Tp/Ds were both replicated 5 times. All data including cell growth, PSII efficiency and metabolic profiling in *T. pseudonana* control cultures are based on the culture in only one culture chamber. One replicate of the mono-cultures was discarded from the metabolic profiling evaluation due to high bacterial contamination.

2.9 Monitoring algal and bacterial growth parameters

Algal growth was estimated by counting approximately 300 cells using a Fuchs-Rosenthal hematocytometer with an upright microscope (Leica DM 2000, Heerbrugg, Switzerland). Bacterial cell growth was determined by flow cytometry. Therefore, 990 µL of the sample were fixed with 10 µL of a 25 % glutaraldehyde solution (electron microscopy grade, Sigma Aldrich, Munich, Germany) reaching a final concentration of 0.25 %. The sample was then vortexed for approximately 1 min before keeping it in the dark at 4 °C for 15 min. After freezing with liquid nitrogen, samples were stored at −80 °C before further analysis. Samples were thawed at room temperature and, to avoid coincidence (two or more particles being simultaneously present in the sensor zone), diluted in ultra-pure water one, five or tenfold depending on the growth stage. Aliquots of 100 µL diluted samples were diluted with 250 µL ultra-pure water and stained for 10 min in the dark with 5 µL of SYBR Gold (Invitrogen, Carlsbad, CA, USA) in 45 µL of PCR buffer (Fermentas, St. Leon-Rot, Germany). After addition of 100 µL of a calibration standard (see below) the final concentration of SYBR Gold for the measurement was 10^{-4} lower compared to the commercial stock. Samples were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with CXP-software, a 20 mW 488 nm air-cooled argon-ion laser and standard filters. The discriminator was set to green fluorescence and the samples were analyzed for 1 min at a flow rate of 30 µL min^{-1}. Data were calibrated to polystyrene fluorospheres (3.6 µm in diameter; Beckman Coulter) measured at 620 nm using CXP analysis software and the mean of three repetitive measurements per sample was used.

For PSII efficiency measurements 200 µL of the diatom culture were added to black 96 well plates. After storing the samples in the dark at 15 °C for 30 min the initial fluorescence (F0) was measured using a Mithras LB 940 plate reader with an excitation filter of 430 nm and an emission filter of 665 nm (Berthold Technologies, Bad Wildbad, Germany). After addition of 15 µL of 3-(3,4-dichlorphenyl)-1,1-dimethylurea (71.7 µM in water) (Sigma-Aldrich, Munich, Germany) to a final concentration of 5 µM the fluorescence was measured again resulting in the maximal fluorescence (Fm). The PSII efficiency was calculated according to (Fm − F0)/Fm (Roy and Legendre 1979).

2.10 Sampling, extraction and derivatization for metabolomics

At the end of the experiment (day 5) 250 mL out of the *T. pseudonana* containing chambers (only one chamber in the Tp/Tp experiments) were concentrated on 47 mm GF/C filters (Whatman, Kent, UK) under reduced pressure (~500 mbar). The wet filters containing the cells were transferred to 25 mL beakers and immediately extracted with 2 mL of cold (−20 °C) extraction solvent mix (methanol:ethanol:chloroform, 2:6:2, v:v). Extracts were transferred to 2 mL microcentrifuge tubes. The extracts were kept at −80 °C until further work up, approximately 1 month after extraction. Immediately before measurement all samples were derivatized simultaneously. Therefore, samples were adjusted to room temperature, treated in an ultrasound bath for 10 min and centrifuged for 15 min at 4 °C with 17,000 rpm. A volume corresponding to the extract of 10^8 extracted cells of the supernatant was transferred into 1.5 mL glass vials and evaporated to dryness under reduced pressure. For derivatization (Vidoudez and Pohnert 2012) 50 µL of a methoxyamine hydrochloride (Sigma-Aldrich, Munich, Germany) solution (20 mg mL^{-1}) in pyridine were added and incubated at 60 °C for 1 h and at room temperature for additional 9 h. Subsequently, 50 µL of N-methyl-N-trifluoroacetamide (MSTFA) (Macherey–Nagel, Düren, Germany) supplemented with decane, pentadecane, nonadecane, octacosane, dotriacontane (final concentration for all 40 µM) and hexatriacontane (final concentration 20 µM) (all Sigma-Aldrich, Munich, Germany) as retention time index were

added to each sample and incubated at 40 °C for 1 h. The samples were transferred into 100 µL glass inserts of 1.5 mL vials and directly measured with GC–MS.

2.11 Data processing

Data processing was adapted from a previously published protocol (Vidoudez and Pohnert 2012). Briefly, MassLynx (version 4.1, Waters) was used to correct the chromatograms for background noise applying the Component Detection Algorithm. The chromatograms were converted to netCDF files using MassLynx DataBridge prior to running them as one batch job in AMDIS (version 2.65, NIST, http://www.nist.gov). The parameters were: Minimum match factor: 30; type of analysis: simple; component width: 12; excluded *m/z*: 147, 176, 193, 207, 219; adjacent peak subtraction: 2; resolution: low; sensitivity: medium; shape requirement: low. The AMDIS output files were used to quantify the identified metabolites using MET-IDEA (version 2.05, http://bioinfo.noble.org) with the following parameters: average peak width: 0.08; AMDIS transfer: 0.5; maximum peak width: 2; peak start/stop slope: 1.5; adjusted retention time accuracy: 0.25; peak overload factor: 0.9; MS type: TOF; mass accuracy: 0.1; mass range: 0.3; lower mass limit: 100; ion per component: 1. The resulting data matrix consisting of metabolite identity and corresponding peak areas was copied into excel 2007 (Microsoft, Redmont, USA). Signals resulting of the retention time index were deleted prior to statistical evaluation.

2.12 Statistical analysis

Differences in growth and PSII efficiency were evaluated using two way repeated measure analysis of variance (RM-ANOVA) implemented in SigmaPlot 11 (Systat Software, San Jose, CA, USA). To evaluate differences between Tp/Ds and Tp/Tp metabolic profiles unconstrained (principal coordinate analysis, PCO) and constrained methods (canonical analysis of principle coordinates, CAP) were used. Therefore, the data matrix was converted to a *.txt file and fed to the software CAP 12 (http://www.stat.auckland.ac.nz/~mja/Programs.htm). The following parameters were used: transformation: none; standardization: none; similarity measure: Bray–Curtis. The data of the resulting output file was used to graph the PCO and CAP plot using SigmaPlot 11. Correlation of metabolites with the CAP axis was considered if the correlation coefficient was above or below 0.75 or −0.75, respectively.

2.13 Identification of metabolites

The identity of all metabolites recognized as significant for the separation of both treatments in CAP analysis was manually examined based on their mass spectra and retention time. Therefore, the chemical structure suggested by AMDIS was reviewed of one exemplary chromatogram using the following mass spectral libraries: NIST library version 2005, T_MSRI_ID 2004-03-01 Golm metabolome database (Wagner et al. 2003) and an in-house library implemented in the software MS search (version 2.0 d, NIST).

The structure was accepted if the following criteria were met. First, the structure as suggested by AMDIS had to be confirmed by the used libraries. Second, the reverse match factor had to be >800, structures with a reverse match between 800 and 700 are tagged with a "?" and structures with a reverse match below 700 are marked with "??". Reverse match factors below 600 were not accepted. Structures that fulfilled these criteria needed additionally to have a retention index within the range of the retention index provided by the mass spectral libraries. To verify the identity of amino acids, retention times and mass spectra of authentic standards had to be identical with respective signal.

3 Results and discussion

3.1 Development of the co-culture setup

We developed a setup enabling non-contact co-culturing of planktonic organisms in which two species grow in separated compartments under identical conditions. This set-up allows diffusion of potential infochemicals between the separated compartments and thus a chemical communication between the interaction partners. The vessels were constructed so that regular sampling for standard growth parameters as well as metabolomic investigation of the cells is possible under sterile conditions. We constructed a custom made setup with two glass chambers separated by a membrane (Fig. 1) in which each chamber can be sterilized, filled and manipulated independently. As culturing chamber we used 500 mL flat edged glass ware that was modified with an opening for filling and sampling purposes. After autoclaving membrane, sealing, and glassware the setup was assembled under sterile conditions, which took about 3 min per co-culturing device. As biocompatible membrane we used a 0.22 µm PVDF filter which proved to be suitable to separate microalgae as well as bacteria (data not shown). In contrast to co-culture setups used previously where separation went ahead with unequal growth conditions for both partners (Paul et al. 2009), both compartments in our setup supported equal growth. This was verified by inoculation of both chambers with 50×10^3 cells mL^{-1} *T. pseudonana*. The algae started to grow exponentially and their cell counts did not differ significantly in the compartments ($P > 0.3$ for comparisons

at day 1, 2 and 3). An advantage over commercially available setups (Yamasaki et al. 2007) is the large culture volume of up to 500 mL that allows sampling for metabolomic investigations even in dilute growth situations that match cell counts in the sea (Vidoudez et al. 2011).

3.2 Diffusion of relevant metabolites

To allow planktonic organisms to interact via chemical signals the diffusion within such a co-culturing setup has to be quick and ideally result in equilibrium conditions. The nature of signal molecules that have hitherto been identified in the plankton is very heterogeneous and ranges from small ionic to larger more lipophilic compounds (Pohnert 2012). We therefore monitored the diffusion of DMSP, a zwitterionic low molecular weight metabolite with multiple functions including osmolytic regulation, antioxidant properties as well as a potential involvement in chemical defense (Groene 1995; Pohnert et al. 2007; Steinke et al. 2002). If added to one compartment diffusion readily occurred and within 24 h the concentrations in both compartments were nearly at equilibrium. After 48 h no differences were detected (Fig. 2b). Additionally, we tested the distribution of the less polar heptadienal after inoculation in one of both chambers. Heptadienal plays a role in the chemical defense of diatoms (Ianora et al. 2004) and might also be involved in the cell to cell communication (Vidoudez and Pohnert 2008) of diatoms. The diffusion of ca. 3.5 μM heptadienal resulted in nearly identical concentrations in both chambers after 24 h (Fig. 2c). In contrast to DMSP, the final concentration was only around one-third of the initial concentration, which can be explained by low stability and volatility of the compound. Further, heptadienal might be adsorbed by the glass or the membrane reducing the concentration of dissolved heptadienal. We also tested the diffusion of nitrate, an essential macronutrient supporting algal growth, added to one growth chamber (Fig. 2a). Nitrate diffusion leads also to equal concentrations in both chambers within 24 h. The same diffusion assays were performed with a 10 kDa dialysis membrane separating the culturing chambers and the results revealed very similar diffusion kinetics of the relevant metabolites (data not shown). Due to their ease of handling we decided to use 0.22 μm membranes in further experiments. We thus demonstrated that the set-up allows the testing of the influence of nutrients as well as common infochemicals. In certain cases of high molecular weight or highly lipophilic metabolites diffusion would have to be evaluated in a preliminary experiment.

In all cases the diffusion processes observed lead already to a significant exchange of metabolites between the chambers within the first hours after addition. This is definitely sufficient for the observation of most chemically mediated interactions, that often require prolonged times for an interaction to manifest. For example, the inhibitory effect of polyunsaturated aldehydes such as 2,4-heptadienal is known to take several days (Ianora et al. 2004). If however faster diffusion would be required the experimental set-up could be optimized by introducing a larger membrane in modified vessels.

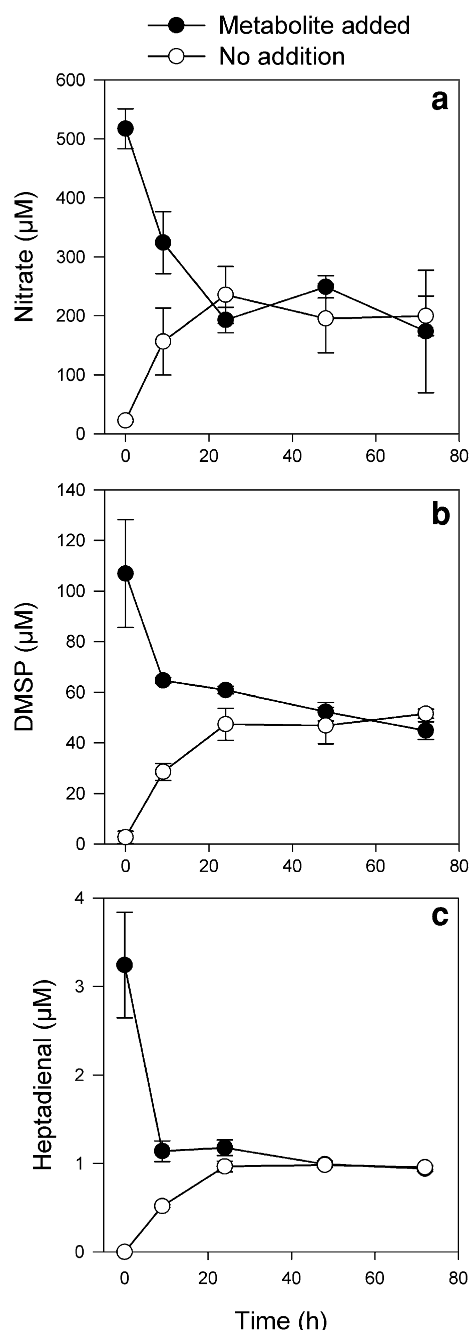

Fig. 2 Diffusion of **a** nitrate, **b** DMSP and **c** heptadienal trough a 0.22 μm membrane in co-culture devices. The respective compounds were added to one compartment and their concentrations were monitored in both compartments over time

3.3 Growth in co-cultures

Growth of *T. pseudonana* developed similarly in both treatments within the first days of the co-culturing experiments. From day 4 onwards the cell abundance in Tp/Ds was higher compared to Tp/Tp ($P = 0.002$ and $P < 0.001$ for day 4 and 5, respectively, $n = 5$). While *T. pseudonana* in Tp/Ds reached a final cell abundance of 3.1×10^6 cells mL^{-1}, Tp/Tp cultures had a cell abundance of 2.3×10^6 cells mL^{-1}. *D. shibae* was inoculated to an initial abundance of ca. 2×10^6 cells mL^{-1}. During the course of the experiment the cell abundance increased reaching a final bacterial abundance of 70×10^6 cells mL^{-1} (Fig. 3a). By plating out 100 µL culture of the Tp compartment axcenity could be verified on day 1. During the further course of the experiment bacteria with different morphology and in significantly lower amounts compared to *D. shibae* could be detected. These traces of contaminant bacteria might affect the overall metabolism of *T. pseudonana* but since they were found in both co-culture setups (Tp/Tp and Tp/Ds) in comparable amounts we conclude that they did probably not influence the outcome of the comparative metabolic profiling. This will rather reflect the effect of additional *D. shibae* bacteria in high abundance. Medium effects can be excluded since conditions were identical in both setups. The stimulated growth in Tp/Ds can be caused by several factors. Certain bacteria are known to increase algal growth by supplying vitamins to algae (Cole 1982; Croft et al. 2005; Seyedsayamdost et al. 2011). Especially vitamin B1 and B12 from *D. shibae* can support algal growth as demonstrated in co-cultures of the bacterium with *Prorocentrum minimum* (Wagner-Döbler et al. 2010). Alternatively, essential nutrients might be used up quicker in Tp/Tp since twice the cell counts with respect to the total volume were reached. However, this possibility is less likely since the experiment was conducted only during the first days of the exponential growth phase where sufficient nutrients should be available.

We estimated the health and nutrient status of the cultures by monitoring the PSII efficiency for Tp/Tp and Tp/Ds. The PSII efficiency was similar in both treatments during the entire experiment ($P > 0.088$ for all comparisons, Fig. 3b) suggesting an equal performance of the diatoms. The photosystem II efficiency allows drawing conclusions on the nutrient and health status of a culture. PSII efficiency measurements are a valid diagnostic tool for nitrogen limitation in *T. pseudonana* batch cultures (Kolber et al. 1988). This suggests no nitrate limitation, which was also supported by the fact that nitrate concentrations in both treatments did not differ (average 380 µM, $P = 0.455$). A limitation in available phosphorous leads also to a decreased quantum yield efficiency in algae (Beardall et al. 2001; Liu et al. 2011). Furthermore silica

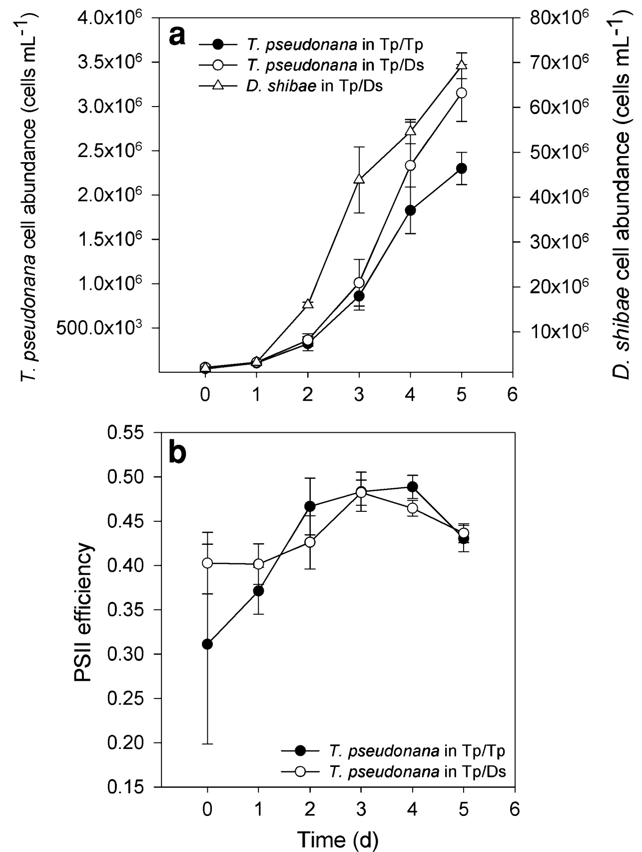

Fig. 3 Growth parameters of *T. pseudonana* and *D. shibae*. **a** Cell abundance of *T. pseudonana* in mono- and co-cultures and the cell abundance of co-cultured *D. shibae*. **b** Photosystem II (PSII) efficiency of *T. pseudonana* in mono- and co-culture

limited cells of *Thalassiosira weissflogii* had a significant reduced PSII efficiency which could be recovered after the addition of silicate (Lippemeier et al. 1999). Since the PSII efficiency did not differ between *T. pseudonana* in Tp/Tp and in Tp/Ds we conclude that *T. pseudonana* cells were not nutrient limited or did at least not have a significant different health status that could influence the metabolic profile.

3.4 Metabolic profiling

At the end of the experiment (day 5) cellular metabolites of *T. pseudonana* were extracted to evaluate the effect of exudates from bacteria on the metabolism of these diatoms. After extraction, the equivalent of 10^8 diatom cells was derivatized using methoxyamine hydrochloride followed by a silylation employing MSTFA. Analysis with GC–MS and AMDIS evaluation of the data allowed the identification of 510 ± 22 and 544 ± 30 compounds for Tp/Tp and Tp/Ds cells respectively being not significantly different from each other ($P = 0.1$). The peak areas were integrated using the MET-IDEA software and the data set consisting

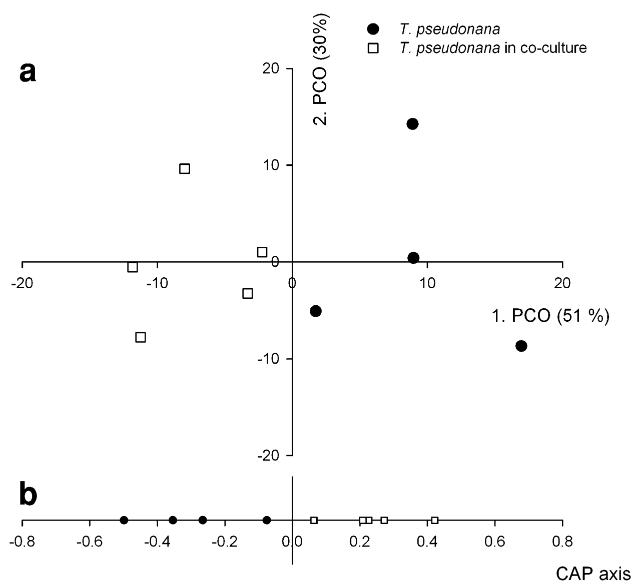

Fig. 4 Illustration of the multivariate separation of cellular metabolites of *T. pseudonana*. Separation using **a** the unconstrained method of analysis of principal coordinates (PCO) and **b** the constrained method of canonical analysis of principle coordinates (CAP). Diagnostic correlation coefficients were obtained for the correlation of each metabolite with the canonical axes

of tentatively identified substances with their corresponding peak areas was used to perform principal coordinate analysis (PCO) and canonical analysis of principal coordinates (CAP). Both protocols were previously successfully applied to discover patterns in large ecological data sets (Anderson and Willis 2003; Nylund et al. 2010) and in metabolomics experiments (Nylund et al. 2011; Vidoudez and Pohnert 2012). The unconstrained PCO as well as the constrained CAP gave a visual separation of both treatments (Fig. 4). Statistical evaluation gave a high eigenvalue of 0.9 and a P value of 0.046 for the permutation test. The misclassification was 11.1 % representing one out of nine samples to be misclassified. The correlation of each variable with the canonical axis allowed identifying variables that contribute to the separation of the two groups and that are thus potential candidates for metabolites regulated in bacteria-phytoplankton interaction. Metabolites were tentatively identified using MS libraries and the retention index, and the peak areas were plotted resulting in the heat map shown in Table 1.

The metabolic profiling procedure applied here is adapted from a protocol optimized for the diatom *Skeletonema marinoi* (Vidoudez and Pohnert 2012). As with *S. marinoi* we were able to obtain complex chromatograms with hundreds of metabolites (unedited list including substance identification according to AMDIS and peak area obtained with MET-IDEA can be found in a supplementary excel file). Interestingly, the metabolites exuded from bacteria generally stimulate the metabolism of *T. pseudonana* since nearly all observed differences are due to an increased abundance of metabolites in Tp/Ds (Table 1). We identified no substance that was negatively correlated with the canonical axis and thus down regulated in the presence of bacteria ($R < -0.75$). Since extracts were derived from a normalized amount of cells and since the cell volume did not differ between both treatments, this observed effect of the co-culturing is not simply caused by a higher amount of biomass extracted. Moreover, the activation of metabolism is not uniformly found for all metabolites, but rather specific pathways are up-regulated in the presence of bacteria. Especially amino acids including serine, proline, phenylalanine and the glutamic acid derivatives acetylglutamic acid and pyroglutamic acid are found in higher concentration in Tp/Ds compared to Tp/Tp. Pyroglutamic acid, which is not an algal metabolite, can be formed from glutamate during the analytic procedure (Gehrke and Leimer 1971; Leimer et al. 1977). The importance of amino acids for diatom-bacteria interactions was recently also recognized in biofilms. In the benthic freshwater diatom *Fragilaria pinnata* the pool of extracellular dissolved free amino acids is significantly altered if diatom-bacteria co-cultures were compared with axenic diatoms. For example, the concentration of dissolved isoleucine was significantly higher in co-cultures while the concentration of histidine was significantly reduced (Bruckner et al. 2011). However, the reason for that observation could not be elucidated in detail. Besides amino acids we detected several short and medium length acids such as glutaric acid and C16 and C18 fatty acids that were found in higher concentrations in Tp/Ds cells. Additionally, several sugars of which the identity was not elucidated in detail were strongly up-regulated in the presence of bacteria.

We cannot conclude if any of these metabolites is up regulated in response to signals from co-cultured bacteria or if diatoms can take up metabolites released by bacteria. It is known that benthic diatoms such as *Cylindrotheca closterium* (Nilsson and Sundback 1996) and planktonic diatoms such as *Thalassiosira* sp. and *Phaeodactylum* sp. (Admiraal et al. 1984; Admiraal et al. 1986; Flynn and Wright 1986) can take up dissolved free amino acids from the culture medium. Interestingly, it was also demonstrated that free amino acids can enhance primary production in pelagic and benthic diatoms (Flynn and Syrett 1986; Linares 2006), which might explain the stimulated algal growth in our co-culturing. However, cellular concentrations of free amino acids depend on a multitude of additional factors. For example, it was found that amino acid concentration varies depending on the culture growth stage in targeted (Myklestad et al. 1989) and non targeted analysis (Vidoudez and Pohnert 2012). The observed effect could also be explained with an uptake of vitamins released from the bacteria that could influence specific biosynthetic pathways of the algae (Cole 1982; Croft et al. 2005).

Table 1 Heatmap of intensities of cellular metabolites positively correlated with the CAP axis ($R > 0.75$) from *T. pseudonana* in mono- and co-cultures

Metabolite	RT (min)	Mono-cultures				Co-cultures					R
Monomethylphosphate ?	6.58	151	385	1332	468	1745	1334	1070	2446	1651	0.77
Glyceric acid	8.01	1500	2638	9483	1910	30180	12455	13380	30094	18717	0.85
Picolinic acid ?	8.05	5010	6452	11680	6101	24703	12189	14292	25658	13067	0.84
Serine *	8.29	15784	25122	23085	15992	32164	29821	40887	34010	24809	0.83
Threonic acid-1,4-lactone	8.42	2046	2881	2469	1652	3555	2431	3465	3306	2552	0.78
Glutaric acid ??	8.73	519	425	386	366	1200	849	753	588	460	0.79
Maleic acid ??	9.03	1542	1209	1180	781	2602	1725	2443	3449	2177	0.81
Pyroglutamic acid *	9.75	177925	322289	620549	289735	709794	745946	635337	930276	536875	0.87
N-acetylglutam acid ?	9.82	16235	46012	79388	22005	253266	242190	343970	422207	233094	0.86
Phenylalanine *	10.03	1092	4364	5390	1883	10400	5958	17097	12335	13194	0.77
Proline *	10.24	154271	299905	335246	180343	422092	355007	428996	373481	286540	0.86
Pentonic acid-1,4-lactone	10.76	452	515	610	324	928	592	1329	1078	801	0.8
Pentonic acid-1,4-lactone	11.41	3551	3729	3496	1449	3875	4183	5229	4633	3847	0.76
C4 sugar ?	12.11	4943	5631	6853	4919	13452	8078	8200	11694	6901	0.86
C4 sugar	12.34	13072	15747	19597	13398	23516	19251	26090	31259	15933	0.77
C4 sugar	12.42	9612	17911	19902	14319	29140	20610	18576	28631	19817	0.76
Hexadecenoic acid	13.51	311648	19096	352998	16322	312906	261116	403752	413417	342359	0.75
Octadecenoic acid ?	14.68	21914	16361	26675	14033	29900	26950	37148	34691	19123	0.83
Octadecatrienoic acid ?	15.56	49658	42673	45053	23870	64219	49337	75074	74334	54889	0.84
Monohexadecanoylglycerol	16.65	10075	19666	28349	10033	28171	20502	36361	38430	23327	0.76

The color reflects the intensities of the metabolites with bright coloring for low intensities and dark coloring for high intensities. The identity of metabolites indicated with an "*" was verified with authentic standards. Metabolites marked with a "?" had reverse match between 800 and 700 and marked with "??" a reverse match of below 700

The metabolite picolinic acid, a tryptophan catabolite, was also found in higher concentration in Tp/Ds diatom cells compared to Tp/Tp. Picolinic acid can potentially form metal complexes with limiting trace elements such as iron. Assuming a synthesis of this metabolite by the co-cultured bacteria and an uptake of iron complexes by the aglae this would be a way to establish a mutualistic interaction. Notably, a derivative of picolinic acid named thallusin with a 2,6-dicarboxy pyridine moiety was isolated from marine bacteria in symbiosis with the macroalga *Monostroma oxyspermum*. In this symbiosis the substance is essential since it induces cell differentiation in the alga (Matsuo et al. 2005). Detailed further investigations using e.g. the co-culture set-up introduced here would however be required to support such considerations.

4 Concluding remarks

We describe a co-culturing device, which enables a contact free co-culturing of both planktonic microalgae and bacteria. This setup facilitates high diffusion rates for several plankton relevant metabolites and secures identical growth conditions for both co-cultured partners. It was successfully utilized to co-culture *T. pseudonana* with *D. shibae*. While control and co-cultured *T. pseudonana* had an identical health status during the experiment as indicated by similar PSII efficiencies, the cell abundance in diatom/bacteria co-cultures was higher at the end of the experiment in comparison to mono-culture controls. Metabolic profiling of diatom cellular metabolites revealed a higher metabolic activity of co-cultured *T. pseudonana* with especially amino acids and amino acid derivatives being up-regulated. These findings might have significant ecological significance since, for example the food quality of algae might change in the presence of bacteria thereby influencing energy transfer between different trophic levels of the plankton. Further, bacteria might also influence the excretion of algal metabolites, which can in turn influence chemically mediated interactions and element cycling. Even though not addressed in our study, the presented co-culture device and the metabolomic routine will facilitate the investigation of a multitude of other plankton interactions in the future.

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