Metabolomic Profiling of 13 Diatom Cultures and Their Adaptation to Nitrate-Limited Growth Conditions

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Abstract

Diatoms are very efficient in their use of available nutrients. Changes in nutrient availability influence the metabolism and the composition of the cell constituents. Since diatoms are valuable candidates to search for oil producing algae, measurements of diatom-produced compounds can be very useful for biotechnology. In order to explore the diversity of lipophilic compounds produced by diatoms, we describe the results from an analysis of 13 diatom strains. With the help of a lipidomics platform, which combines an UPLC separation with a high resolution/high mass accuracy mass spectrometer, we were able to measure and annotate 142 lipid species. Out of these, 32 were present in all 13 cultures. The annotated lipid features belong to six classes of glycerolipids. The data obtained from the measurements were used to create lipidomic profiles. The metabolomic overview of analysed cultures is amended by the measurement of 96 polar compounds. To further increase the lipid diversity and gain insight into metabolomic adaptation to nitrogen limitation, diatoms were cultured in media with high and low concentrations of nitrate. The growth in nitrogen-deplete or nitrogen-replete conditions affects metabolite accumulation but has no major influence on the species-specific metabolomic profile. Thus, the genetic component is stronger in determining metabolic patterns than nitrogen levels. Therefore, lipid profiling is powerful enough to be used as a molecular fingerprint for diatom cultures. Furthermore, an increase of triacylglycerol (TAG) accumulation was observed in low nitrogen samples, although this trend was not consistent across all 13 diatom strains. Overall, our results expand the current understanding of metabolomics diversity in diatoms and confirm their potential value for producing lipids for either bioenergy or as feed stock.
Introduction

Diatoms are eukaryotic phototrophs and are able to very efficiently sequester water-dissolved carbon dioxide. It is estimated that diatoms are responsible for up to 20% of the global primary production [1,2]. Diatoms can also efficiently use other available nutrients such as silica (main elemental component of their cell wall) and nitrogen to maintain their cell cycle [3–6]. Like in other organisms, the metabolism of carbon and nitrogen is closely coupled in diatoms. Limitation of one of these elements leads to severe changes in fluxes and utilization of the other [7].

Diatoms are very efficient at nutrient utilization and thus successful organisms in various environments [8] but can be readily adapted into prolific cultures; in these artificial conditions they often outcompete other algae in mixed cultures [9,10]. They are also relatively resistant to various pathogens [11,12]. Hence, they are highly regarded for their potential in producing feed or as a sustainable source of valuable substances, especially biofuels. Producing renewable, sustainable and carbon neutral fuels are of paramount importance to security and economic prosperity. One way of obtaining desired production is to manipulate culturing conditions such as nutrient availability. A combination of nitrogen and silicon limitation might be used to induce accumulation of lipids up to 50% of dry weight [13]. The genetic transformation of diatoms was initiated with the aim of improving oil production [14]. This approach might be very promising especially with the development of genomics tools for diatoms [15–19]. Moreover, marine algae synthesize diverse lipids, such as phosphatidylcholine, which might be important for food or feed production [20].

There are estimated 100,000 extant species of diatoms colonizing various environments [21]. With such diversity, we expect diatoms to produce a wide range of products depending on their local environment and evolutionary history. We already know diatoms produce several marketable natural products—biogenic silica and lipids—but as yet the yields have not been as cost-efficient for mass production. By bioprospecting for lipids and additional products from diatoms and other marine algae, we can increase the efficiency of farming these algae with the extraction of multiple products from cultures as well as discover novel products for future biotechnological applications [22,23]. To gain insight into the metabolism of diatoms we have selected cultures from a widest systematic range of diatom lineages ever sampled: Amphitetras antediluviana, Biddulphia biddulphiana, Cerataulina daemon, Chaetoceros simplex, Eunotogramma sp., Hemiaulus sinensis, Leptocylindrus danicus, Rhizosolenia setigera, Thalassionema frauenfeldii, Thalassiosira pseudonana (two strains CCMP1007 and CCMP1335) and Thalassiosira weissflogii (two strains CCMP1587 and CCMP1336) and studied their metabolome under nitrogen-rich and nitrogen-limiting conditions. To fully control the growth medium and nutrient levels diatoms were cultivated in artificial sea water in standard f/2 medium [24] and low-nitrate containing media. We have studied the adaptation to the applied conditions by measuring levels of 142 glycerolipids and changes in content of 96 polar compounds (mostly representing primary metabolites). Here we compare different N-limitation adaptation responses in 13 diatom cultures and describe the diversity of identified metabolites.

Materials and Methods

Algal cultures

Diatom starter cultures were obtained from National Center for Marine Algae and Microbiota in Bigelow, USA (Thalassiosira pseudonana CCMP1335, T. pseudonana CCMP1007 and T. weissflogii CCMP1587, T. weissflogii CCMP1336, Chaetoceros simplex CCMP200) and from a collection at University of Texas at Austin (Amphitetras antediluviana, ECT3627; Biddulphia
biddulphiana, ECT3902; Cerataulina daemon, AP8; Eunotogramma sp. (laeve), AP8Eunoto; Hemiaulus sinensis, 24I10-1AHemi; Leptocylindrus danicus, ECT3929araphid3; Rhizosolenia setigera, 25VI12-2ARhizo; Thalassionema frauenfeldii, ECT3929ThalXL). The cultures of diatoms were maintained in f/2 medium [24]. To control the chemical composition of the growth conditions, the medium was prepared by dissolving the f/2 salts and vitamins in artificial sea water, which was prepared from inorganic salts according to recipe for ESAW by Berges et al. [25]. The light intensity was 80 μmol/m²/s and the temperature was kept at 22°C throughout the 16 h day/8 h night regime. In order to induce nitrogen limitation and maintain high cell yield per flask, an inoculum from a stationary culture was transferred into a fresh full nitrogen f/2 medium (880 μM NO₃⁻) and to a medium containing 20% of the nitrate in f/2 concentration. Each culture was represented by five replicates in these two nutrition conditions.

To ensure induction of the nitrogen limitation response, diatom cultures were harvested two days after the concentration of nitrate in low-nitrogen medium fell below the limit of detection. Diatoms were harvested by filtration onto a glass microfibers filter GF/C (Whatman) and frozen in liquid nitrogen. Usually 40 ml were harvested, with the exception of dense cultures of Thalassiosira in which 15–20 ml were used. The harvested samples of diatoms were kept in -80°C prior to the metabolite analyses. Cell number in each flask at the point of harvest was estimated using a Neubauers’ haemocytometer. In case of Thalassiosira cultures cell density was measured with Coulter Counter Z2 (Beckman).

The nitrate levels in media were estimated using an AQUANAL-plus nitrate test kit (Sigma-Aldrich). To increase reproducibility the original protocol was adapted as follows. The kit reagents A, C, and D were dissolved in distilled water: 1 spoon per 1 ml. A 1 ml sample of the medium was spun at 21,000 g for 2 min to remove cells and debris. In the following step, 800 μl of the supernatant was mixed with 200 μl of the dissolved reagent A and 26 μl of the reagent B. After vortexing, 200 μl of each reagent C and D were added. The reaction tube was vortexed and left for 5 min before spectrophotometric (Ultrospec2000, Pharmacia Biotech) measurement at 530 nm wavelength. The artificial sea water f/2 medium without added nitrate was used as the blank sample. The lower detection level of nitrate in the medium used in these experiments was 2.5 μM.

Analytics and data analysis

Metabolite extraction from diatoms was performed using the method of Giavalisco et al. [26] and Bromke et al. [7]. In short, cells collected on filters were extracted with a cold mixture of methyl-tert-butyl-ether:methanol (3:1), spiked with 0.1 μg/ml phosphatidylethanolamine (PE 34:0 [17:0/17:0]), phosphatidylcholine PC 34:0 [17:0/17:0]) and 13C-sorbitol as internal standards. To facilitate cell disruption, samples were shaken and incubated in a cooled sonic bath for 10 min. The subsequent addition of a water:methanol (3:1) mixture to the extract resulted in the formation of two liquid phases (polar and non-polar phase). The lipid containing non-polar phase was aspirated, dried in vacuum and kept in ~20°C prior to the profiling. The fractionation of lipids was performed by a Waters Acquity UPLC system using a C₈ reversed-phase column (100 mm × 2.1 mm × 1.7 μm particles; Waters). For the UPLC gradient the mobile phases consisted of two solvents: solvent A, 1% 1 M NH₄Ac and 0.1% acetic acid in water; and solvent B, acetonitrile/isopropanol (7:3, 1% 1 M NH₄Ac, 0.1% acetic acid), with an injection volume of 2 μl. The following gradient profile was applied: 1 min, 55% B; 3 min, linear gradient from 55% B to 75% B; 8 min, linear gradient from 75% B to 89% B; 3 min, linear gradient from 89% B to 100% B. After washing the column for 4.5 min with 100% B, the mixture was set back to 55% B and the column was re-equilibrated for 4.5 min (24.5 min total run time), with a flow rate of the mobile phase of 400 μl/min. The mass spectra were acquired using an Orbitrap mass
spectrometer (Exactive, Thermo Scientific). The spectra were recorded alternating between
full-scan and all-ion fragmentation-scan modes, covering a mass range from 100 to 1500 m/z
[26]. Chromatograms from the UPLC-MS analysis were analysed and processed with
REFINER MS® 7.5 (GeneData). The software extracted analytical features (analytes described
by their mass, retention time, and associated intensity) from raw files generated by the mass
spectrometer. Further processing of the data included the removal of the fragmentation infor-
mation and chemical noise (constantly eluting non-biological compounds derived from the
column or the employed UPLC solvents) as well as the retention time alignment. The output
data were normalized to internal standard PC 34:0 due to lower number of co-eluting analytes.
Peaks were annotated with help of an in-house database of lipid compounds. Further process-
ing was performed with scripts written in R [27], which filtered aligned data matrices to keep
annotated peaks and 1200 analytes (a number based on previous experience with analysis of
lipidomic data) of highest intensity. Additionally, analytes that showed up in less than four rep-
licates were removed. The data were normalized to the cell density at the time of harvest, scaled
to the median of the data matrix and log2-transformed. The data are available in the S1 Table.
Visualization of lipid data was performed with R. The generation of Primary Component Anal-
ysis plots used the ’pca’ function with the default ”ppca” method. Heatmaps were generated
using function ’heatmap.2’ (a part of a ’gplots’ package), which utilizes Euclidean distance as a
basis for hierarchical clustering.

The polar fractions of the metabolite extracts were derivatized and prepared for the
GC-MS-based analysis. Shortly thereafter, the dried extracts were resolved in a mixture of
methoxyamine hydrochloride in pyrydine and derivatized with N-methyl-N-(trimethylsilyl)-
trifluoroacetamide (MSTFA). The samples were analyzed, coupled to an Agilent 6890 gas chro-
matographer coupled with a time-of-flight mass spectrometer (Agilent Technologies). Details
of the method are described by Lisec et al. [28]. Chromatograms were evaluated in Leco Chro-
maTof (Agilent technologies) software and exported in.cdf format. Further processing of chro-
matograms (peak detection, retention time alignment and library matching) was performed
using the TargetSearch R package from bioconductor [29]. To offset errors introduced through
sample-handling the intensity of each analyte peak was divided by the intensity of 13C-sorbitol
(the polar-phase internal standard). The data were normalized to the cell density of every cul-
ture. The statistical significance of changes between N-replete and N-limiting conditions was
tested with the T-test.

Prior to further analysis and visualization the metabolomic data were log2-transformed.
The data are available in S2 Table. The data visualization with the heatmap was performed as
described above. Changes in p-values less than 0.05 were marked with asterisks.

Results

Different species and isolates of diatoms were acquired from algal collections and grown under
laboratory conditions. Cultures were selected on the basis of their culturing properties (ease of
handling, low or no adhesion to the glass surfaces) and ability to grow fast at a relatively high
temperature of 22°C. To induce changes in lipid metabolism of diatoms, they were cultivated
in standard f/2 medium [24] as well as in this medium containing only 20% of the f/2-medi-
um’s nitrate (176 μM). Samples of each culture (full nitrate and low nitrate containing media)
were grown for the same time and harvested when nitrate was depleted in the low-nitrate
medium. Until the time point at which nitrate was depleted, no major changes in growth rate
were observed between the full f/2 and the low nitrate cultures. For each condition five biologi-
cal replicates were independently grown, harvested and analysed. By application of polar and
non-polar solvent mixtures for extraction of each sample we obtained two phases of extracts.
that were analysed separately with use of gas and liquid chromatography, respectively. The data collected in both approaches were used to describe metabolism of the diatom cultures in nitrogen-replete and nitrogen-limited conditions.

**Growth in nitrogen-deplete or nitrogen-replete conditions affects lipid accumulation but has no major influence on lipid patterns**

From the data obtained in the chromatographic analysis of the non-polar phase we selected almost 1200 high-quality features. Out of this number we were able to annotate and determine relative levels of 142 peaks representing lipid species in extracts from 13 diatom cultures (Fig 1). These lipids belong to six classes of glycerolipids: diacylglycerols (DAG; 10 analytes), phosphatidylethanolamines (PE; 7 analytes), phosphatidylcholine (PC; 31 analytes), monogalactosyldiacylglycerols (MGDG; 15 analytes), digalactosyldiacylglycerols (DGDG; 11 analytes), and triacylglycerols (TAG; 68 analytes). There were differences between analysed cultures in number of detected analytes. We measured as many as 129 and 123 analytes in nitrogen-replete and depleted cultures of *Cerataulina daemon*, respectively (Figs1 and 2). On the other hand, we were able to annotate as few as 64 and 60 lipid species in *Thalassionema frauenfeldii* cultivated in high- and low-nitrogen, respectively (Figs 1 and 2). There were 32 lipid species common to all samples and out of this number, 25 represented TAG of different total carbon atoms in their acyl chains, ranging from 48 to 66 C atoms.

One of the main aspects of our study was to assess the influence of different culturing conditions (low versus high availability of nitrate) on lipid composition of diatoms. As shown in Figs 1 and 2, different amounts of nitrate available changed the lipid profiles leading to distinct patterns; however the species remained the most important discriminating factor. This means that the lipid analytes diversity in low nitrate cultures resembled the profile of the control, the high nitrate medium.

Whereas specific lipid composition was only slightly influenced by the nitrate availability, a change in lipid concentration was observed. Fig 2 shows a comparative analysis of the lipid profiles obtained from diatoms grown under low and high nitrate conditions in growth media. In agreement with other data [30,31], an increase in lipid species mostly of the TAG type was recognizable. However, this was not true for all diatom species analysed. The accumulation of TAG was especially visible in dense cultures of *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. Surprisingly there was no specific pattern of changes in content of nitrogen-containing phospholipids, PC and PE. Also other classes of lipids showed no specific changes induced by the low-nitrogen in the growth medium.

The most abundant group of lipids were TAG with 50 carbon atoms in their acyl chains. The intra-lipid species correlation for these TAG (as well as TAG 48:X and TAG 52:X) formed a high-content block across the heatmap (Fig 2, cluster A). The cluster A contains 11 TAG species: TAG 48:4, TAG 50:1; TAG 50:2, TAG 50:3, TAG 50:4, TAG 52:2, TAG 52:4, TAG 54:2, TAG 54:6, TAG 54:7, TAG 56:2. The richest sources of these lipids were cultures of *B. biddulphiana*, *A. antediluviana*, and *R. setigera*. In most samples we detected TAGs of a high mass, which probably represent triacylglycerols with three poly-unsaturated fatty acids. We detected and annotated TAG 66:18, which was observed only in *L. danicus*, *T. weissflogii* CCMP1336, and *R. setigera*, the latter species was especially rich in this compound. There was also a high abundance of TAG 60:15 in lipid extracts.

Generally, our measurements showed that *A. antediluviana* and *B. biddulphiana* had the highest content of annotated lipids per cell but lowest cell density. The lowest amounts of lipids were observed in *Ch. simplex* and *T. frauenfeldii* extracts. Annotated lipid data are in S2 Table.
lipids in N replete conditions
PCA allows clear discrimination of diatom species

In order to obtain further insight into the diversity of the diatom lipids, the data were subjected to a principal component analysis (PCA, Fig 3). Two main conclusions were apparent: first, all 13 diatom strains could be separated from each other in a non-supervised fashion, clearly proving the diversity and species specificity of the lipid profiles; second, all five biological replicates of each species clustered together supporting the statistical relevance of our findings and the reproducibility of the culture conditions. The analysed data set contained profiles of 142 annotated analytes. The first two principal components accounted for more than 83% of the variance (Fig 3). The compound with the strongest influence on resolution was PC 40:6, being on extremes of principal component 1 and 2. This lipid compound was detected only in extracts of *T. pseudonana* CCMP1007, *T. weissflogii* CCMP1336, *T. weissflogii* CCMP1587, and *Leptocylindrus danicus*. All *T. pseudonana* profiles clustered very closely, and the two isolates CCMP1335 and CCMP1007 of *T. pseudonana* were not well discriminated. This was also evident in the heatmap (Fig 1), where we used a colour scale for visualization of individual data points in lipid profiles. The hierarchical clustering analysis applied to profiles of the other pair of related isolates of *T. weissflogii* showed a surprising difference. In consequence, the isolate *T. weissflogii* CCMP1336 seemed to be more similar to *T. pseudonana* profiles than to the *T. weissflogii* CCMP1587, which clustered together with *C. daemon*.

Primary metabolites

The polar phase of each extract was used to measure low molecular size compounds representing mostly primary metabolites. The extracts were chemically derivatized and analysed by CG-MS. Obtained spectra were compared with the Golm Metabolomics Database (gmd.mpimp-golm.mpg.de). This resulted in a list of 96 polar analytes representing primary metabolites (Fig 4). Due to the nature of the derivatization process some metabolites were represented by two analytes, representing different derivatization products of the same metabolite. The largest changes observed in the data set were accumulation of citric acid (more than 28 fold) in cells of *Ch. simplex* (slight accumulation was also observed in *H. sinensis*, *B. biddulphiana*, and *T. pseudonana* CCMP1335) and reduction of 4–hydroxy–benzoic acid levels in three of four *Thalassiosira* species (8 to 20 fold less than in controls). These three diatom cultures (*T. pseudonana* CCMP1335, *T. pseudonana* CCMP1007, *T. weissflogii* CCMP 1336) also exhibited a distinctive accumulation of many metabolites during the treatment. Even more metabolites showed elevated levels in cells of *B. biddulphiana*. In contrast, *A. antediluviana* and *H. sinensis* showed reduced levels for most of analytes measured. The remaining diatom cultures in this experiment showed mixed phenotypes, in which levels of some analytes were increased and for some these levels dropped in the low nitrogen conditions.

The hierarchical clustering of metabolite changes showed a distinctive cluster of metabolites with reduced levels (bottom of the Fig 4). This was not uniform across the studied cultures, though none of the increased levels could be confirmed by the t-test. This cluster contained 4 nitrogen-containing metabolites: tryptophan, tyrosine, proline and 4-hydroxy-benzoic acid. Also, alanine and ornithine levels were reduced in most of the analysed cultures. The exception
to this pattern was *B. biddulphiana*, which seemed to accumulate alanine and homoserine. Many other analytes showed elevated levels in cells of nitrogen-limited *B. biddulphiana*, but the p-value calculated was above the 0.05 threshold.
Contrasting responses to the N-limitation were observed in the case of xylose, glutamic acid and 2,4-diaminobutanoic acid (Fig 4). These three metabolites were found in reduced levels in cells of *L. danicus*, *Eunotogramma* sp. and *Ch. simplex*. Also other diatoms showed reduced levels of one or two of the metabolites. On the other hand three *Thalassiosira* cultures (*T.*
Discussion

Lipid profiling

One of the conclusions from these experiments is that lipid profiling is powerful enough to be used as a molecular fingerprint to characterize a wide phylogenetic diversity of diatom species. This was shown with the Pearson’s correlation-based clustering of profile data and displayed on the heatmap (Fig 1). This observation is also supported by the PCA (Fig 3), where clustering of biological replicates confirms the reproducibility of measurements and specificity of lipidomic profiles of analysed diatoms. In our experiments even the nitrogen-limited profiles of different isolates of the same species (T. pseudonana CCMP 1335 vs. T. pseudonana CCMP 1007; T. weissflogii CCMP1336 vs. T. weissflogii CCMP 1587) showed high similarity to the profiles of samples from control diatoms grown in nitrogen-replete conditions (Fig 2). This suggest an idea that nitrogen limitation may be changing the profiles (lipid and primary metabolites) within boundaries set by the genetic background of the species as displayed by the clustering of the low nitrogen profiles with respective controls.

Hu et al [30] reported an average lipid content of 22.7% dry weight for oleaginous diatoms grown under nitrogen-replete conditions, whereas the total lipid content under nutrient depletion stress reached 37.8% dry weight. The increase in total lipids of stressed diatoms is mainly explained by the shift in metabolism, which directs de novo synthesis and conversion of membrane polar lipids towards triacylglycerols. Many previous studies describe total changes in lipid content or abundance of specific classes in N-limited diatoms [22,30,32,33]. In our experiments we observed an accumulation of TAG (showing increase of specific TAG type levels) in low-nitrogen samples; however this trend was not consistent across analysed diatoms. This could be explained by the adaptation of cellular processes, which adapt carbon flux to the growth conditions such as mineral nutrient availability. The differences in accumulation of TAG under N-limitation could be explained by the different strategies of nitrogen utilization by these diatoms. For instance, the diverse response of diatoms on the molecular level might be explained by the assumption that the applied uniform nitrate concentrations (880 μM for high-nitrate control, 176 μM for low-nitrogen treatment) might fall into different specific growth optima. Further, these organisms differ in profiles of primary (especially nitrogen-containing) metabolites (Fig 4), which could be considered snapshots of metabolic steady states. Larger pools of soluble inorganic and organic N-containing compounds may be mitigating the N-limitation stress. It has been shown previously that diatoms can store nutrients in their vacuoles allowing several cell-divisions [34]. Even though there was no detectable nitrate in the culture medium for two days, diatoms in our experiments could still be utilizing nitrate that was stored in vacuoles. Moreover, the availability of nitrogen remobilized from internal structures such as ribosomal proteins and photosynthesis-related proteins, might strongly differ as much as size of the analyzed diatoms. Taken together this provides a plausible explanation of discrepancies in TAG accumulation observed between species.
The regulation of the lipid biosynthesis pathway in diatoms cells under N stress has not yet been studied in detail [35]. Previous research on T. pseudonana and Phaeodactylum tricornutum in stressed by N-limitation showed reduced cell protein content, down-regulation of photosynthesis and a significant increase of glycolytic enzymes [36–38]. This, as well as the increased expression of fatty acid synthesis genes [37,38], suggest that increased amounts of acetyl-CoA coming from degradation of storage glucans may be feeding de novo synthesis of fatty acids and subsequently the accumulation of TAG in nitrogen limited diatoms. Moreover, a current hypotheses explain TAG accumulation under N-limitation by protein degradation and catabolism of amino acids (especially branched amino acids), which generates tricarboxylic acid cycle intermediates, such as malate, that are used for fatty acid biosynthesis [39]. The N-limitation has an impact on lipids not only through down-regulation and degradation of photosynthetic complexes but also affects structural lipids of plastids. Lipids of the thylakoid membranes such as MGDG, DGDG and sulfoquinovosyldiacylglycerol are central to photosynthetic function of a chloroplast involvement in stabilization of photosynthetic complexes [40,41]. Recent experiments on Nitzschia closterium f. minutissima [42] and Phaeodactylum tricornutum [38] reported N-limitation-induced mobilization of MGDG and DGDG with a consequent degradation of thylakoid membranes and increase in TAG [38].

Diatoms such as B. biddulphiana and A. antediluviana, which form large cells, displayed the highest amount of lipids per cell. In contrast, T. weisflogii and T. pseudonana, diatoms with relatively small cell size that grow to very high densities, appear to have low lipid content per cell. One possible explanation for this is the previously observed exponential relationship between amount of lipid per cell and cell volume [43].

The most abundant group within TAG are lipids with 48 to 54 C in acyl chains (with different desaturation grades). They are most likely composed of glycerol bound with three of the main cellular fatty acids: palmitic (FA16:0) and stearic (FA18:0) acids and their desaturated derivatives. The abundance of TAG48:0 is in agreement with data obtained previously with approaches focusing on the fatty acids in diatom lipids [44]. These previous studies showed a high proportion of FA16:0 in cellular fatty acids of diatoms. Thus, one can assume that TAG48:0 is comprised of glycerol and three palmitic acid moieties. Some diatoms can accumulate more than 15% of their total fatty acids in form of myristic acid (FA14:0) [44–46]. Myristic acid has been shown to be incorporated into positions sn–1 and sn–3 in TAG from T. pseudonana [47]. Unfortunately, given the fact of high abundance of 14 C, 16 C, and 18 C fatty acids, the acyl chains of most of the lipid species cannot be unambiguously deduced without special analytical approaches. Recently, a fatty acid profiling revealed that in cells of T. pseudonana eicosapentaenoic acid (FA20:5) and docosahexaenoic acid (FA22:6) are the most abundant fatty acids [48]. On the other hand, the cells of B. biddulphiana accumulated much less of these fatty acids. The most intense peaks in fatty acid profiles of this diatom correspond to arachidonic acid (FA20:4), while content of the other poly-unsaturated FA (FA20:5 and FA22:6) was significantly smaller [48]. Moreover, the level of FA18:4 in B. biddulphiana is much higher when compared to T. pseudonana. Such differences in fatty acid profiles have been already reported by Dunstan et al. [49]. In his collection of studied algae, diatoms described as Centroles accumulated FA18:4, while Pennales, were relatively rich in FA20:4. Additionally, significantly higher proportion of FA22:6 was observed in fatty acid profiles of Centrales than of Pennales [49]. One should note that the evolutionary history of diatoms is more nuanced than the centric (Centrales)/pennate (Pennales) division. According to recent diatom taxonomy both Thalassiosira and Biddulphia are Mediophyceaen [50,51]. Thalassiosira and Biddulphia do differ in habitats—Thalassiosira is small-celled planktonic diatom while Biddulphia forms colonies in benthic/epiphytic habitats. These aspects should be taken into account while interpreting the observed molecular phenotype of those diatoms.
In plants galactolipids can be synthesised in two pathways: one “prokaryotic” and the other “eukaryotic”. The main difference between the pathways, other than their subcellular localization, is the enzyme preference for fatty acids at the sn–1 and sn–2 positions of glycerolipids. [52]. The “prokaryotic” pathway is localized to the chloroplast and the MGDG and DGDG synthesised there contain predominantly 18-C fatty acids in the sn–1 position and 16-C fatty acids in the sn–2 position. In the “eukaryotic” pathway, which is localized to the endoplasmic reticulum, FA18:1 (oleic acid) is incorporated into phospholipids in the sn–2 position. These phospholipids are the source of DAG for chloroplast-localized synthesis of galactolipids. Hence galactolipids synthesized according to the “eukaryotic” pathway contain predominantly 18-C fatty acid in both sn–1 and sn–2 positions [52]. Mono- and digalactosyldiacylglycerols from diatoms are mainly synthesised in the “prokaryotic” pathway, as these compounds contain 16-C fatty acids in the sn–2 position [53,54]. Thus, we can deduce the fatty acid chain length in galactolipids presented in this report. Five out of nine MGDG detected in analysed diatoms are probably MGDG 18-C/16-C. In the case of MGDG 36:6, we speculate that arachidonic acid (FA20:4) or eicosapentaenoic acid occurs in the sn–1 position. The plant model of galactosylglycerols synthesis is most likely true for diatoms as well, which is supported the study of Dodson et al. [53] who showed that galactolipids from T. pseudonana, Skeletonema marinoi and P. tricornutum contain primarily 20:5/16-C fatty acids. Similar conclusions about the fatty acid composition can be made for DGDG 36:6. Both galactolipids are the most abundant compounds in their classes in all 13 analysed cultures of diatoms. Radiolabelling experiments showed that in P. tricornutum FA20:5 is synthesized in microsomes [55]. The authors hypothesized that the FA20:5 can be transported to plastids as a free fatty acid or bound in DAG [55]. Regardless of whether the fatty acids are derived from lipid catabolism or de novo biosynthesis, diacylglycerol acyltransferases catalyze DAG conversion into TAG [56]. In our data, out of ten molecular species of the DAG class only DAG36:6 is found in all diatom extracts. The intensity and widespread detection of this compound suggest its high importance in the diatom lipid metabolism.

Primary metabolites profiling
In recent years there have been several publications presenting metabolic profiles of diatoms, including T. pseudonana [7], P. tricornutum [37], Skeletonema marinoi [58] and Cocconeis scutellum [59]. However, our knowledge of metabolism of these organisms is still limited. To increase the knowledge in this field this paper presents metabolic profiles for 13 different species and strains of marine diatoms cultivated in N-replete and N-limiting conditions.

The central metabolism of the studied diatoms is also affected by the applied growth conditions with reduced nitrogen level, although the type of response was very different between species (Fig 4). For example, H. sinensis and A. antediluvianus have reduced levels of most of measured compounds, whereas the three Thalassiosira species tend to accumulate various compounds as adaptation mechanisms to low-nitrogen conditions. This contradicts previously published metabolic profiles of T. pseudonana from cultures that were transferred from N-replete to nitrate-free medium [7]. In this report, primary amino acids levels, as main N-containing primary metabolites, were strongly reduced. An additional characteristic change in response to the N-limitation is the accumulation of citrate. This was observed in the short term medium shift experiments with T. pseudonana [7] and in nitrogen limitation experiments on Chlamydomonas reinhardtii [60]. Moreover, previous proteomic experiments on T. pseudonana have shown an increase of glycolytic and TCA enzymes in N-limited cells [36]. Increased levels of citrate were observed in H. sinensis, B. biddulphiana, and T. pseudonana CCMP1335 but only the profile of Ch. simplex showed a strong increase of citric acid in N-limited cells in
comparison to the N-replete control. In our experimental conditions Ch. simpex samples grew very rapidly, which may have resulted in very high demand for N and at the time of harvest strong accumulation of TCA intermediates, including citrate. This could explain the difference between Ch. simpex and other slower growing diatom cultures, which could better adapt to the limited availability of nitrate in the media (e.g. slowed down amino acid synthesis) and consequently the over-accumulation of photosynthetic carbon.

Conclusions

We have extracted and analysed lipids from 13 different strains and cultures of diatoms in order to explore the diversity of lipid metabolites in these organisms grown under two different conditions, i.e. nitrogen replete and nitrogen deplete conditions. The diatoms analysed represent different species and genera, shape and size, life habitats (benthic and planktonic) and colonization types (free living and chain forming). We identified 142 annotated lipid species mostly from the TAG class, many of them displaying species specificity. The growth in nitrogen-deplete or nitrogen-replete conditions affects lipid accumulation but has no major influence on the lipid profiles. We also observed an increase of TAG accumulation in low nitrogen samples, although this trend was not consistent across all 13 diatom strains. Overall, our results confirm that diatoms display a large diversity of lipid species. This observation is consistent with previous suggestions regarding the value of diatoms for producing lipids for either bioenergy or as feed stock.

Supporting Information

S1 Table. Normalized and log2-transformed intensity data of 142 annotated analytes measured in extract of 13 diatom strains. Following culture abbreviations were used: Ampant, Amphitetrans antediluvian; Bidbid, Biddulphia biddulphiana; Cerdea, Cerataulina daemon; Chasim, Chaetoceros simplex; Eunlae, Eunotogramma sp.; Hemsin, Hemiaulus sinensis; Lepdan, Leptocylindrus danicus; Rhiset, Rhizosolenia setigera; Thafra, Thalassionema frauenfeldii; Tp1007, Thalassiosira pseudonana CCMP1007; Tp1335, Thalassiosira pseudonana CCMP1335; Tw1587, Thalassiosira weissflogii CCMP 1587; Tweiss1336, Thalassiosira weissflogii. The “N” suffix marks samples from low nitrogen growth conditions.

S2 Table. Log2-transformed ratios of 96 primary metabolites and corresponding T-test p-values. Following culture abbreviations were used: Ampant, Amphitetrans antediluvian; Bidbid, Biddulphia biddulphiana; Cerdea, Cerataulina daemon; Chasim, Chaetoceros simplex; Eunlae, Eunotogramma sp.; Hemsin, Hemiaulus sinensis; Lepdan, Leptocylindrus danicus; Rhiset, Rhizosolenia setigera; Thafra, Thalassionema frauenfeldii; Tp1007, Thalassiosira pseudonana CCMP1007; Tp1335, Thalassiosira pseudonana CCMP1335; Tw1587, Thalassiosira weissflogii CCMP 1587; Tweiss1336, Thalassiosira weissflogii.

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Author Contributions

Conceived and designed the experiments: MAB JSS RKJ LW. Performed the experiments: FAA NHH SAK AAM MAB. Analyzed the data: MAB MM FAA MPA. Wrote the paper: MAB RKJ MPA LW.

References

1. Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281: 237–240. PMID: 9657713
2. Falkowski PG, Barber RT, Smetacek V (1998) Biogeochemical controls and feedbacks on ocean primary production. Science 281: 200–206. PMID: 9660741
3. Boyd PW, Jickells T, Law CS, Blain S, Boyle EA, Buesseler KO, et al. (2007) Mesoscale Iron Enrichment Experiments 1993–2005: Synthesis and Future Directions. Science 315: 612–617. PMID: 17272712
4. Maldonado M, Price N (1996) Influence of N substrate on Fe requirements of marine centric diatoms. Marine Ecology Progress Series 141: 161–172.
5. Cermeño P, Lee J, Wyman K, Schofield O, Falkowski P (2011) Competitive dynamics in two species of marine phytoplankton under non-equilibrium conditions. Marine Ecology Progress Series 429: 19–28.
6. Edwards KF, Thomas MK, Klausmeier CA, Litchman E (2012) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. Limnology and Oceanography 57: 554–566.
7. Bromke MA, Giavasis P, Willmitzer L, Hesse H (2013) Metabolic Analysis of Adaptation to Short-Term Changes in Culture Conditions of the Marine Diatom Thalassiosira pseudonana. PLoS ONE 8: e67340. doi:10.1371/journal.pone.0067340 PMID: 23799147
8. Hildebrand M, Davis AK, Smith SR, Traller JC, Abbriano R (2012) The place of diatoms in the biofuels industry. Biofuels 3: 221–240.
9. Goldman JC, Ryther JH, Williams LD (1975) Mass production of marine algae in outdoor cultures. Nature 254: 594–595.
10. Armano Y, Takahashi K, Machida M (2012) Competition between the cyanobacterium Microcystis aeruginosa and the diatom Cyclotella sp. under nitrogen-limited condition caused by dilution in eutrophic lake. Journal of Applied Phycology 24: 965–971.
11. Hamm C, Smetacek V (2007) Armor. Why, When, and How. Evolution of Primary Producers in the Sea. pp. 311–332.
12. Tomaru Y, Toyoda K, Kimura K, Hata N, Yoshida M, Nagasaki K (2012) First evidence for the existence of pennate diatom viruses. ISME J 6: 1445–1448. doi: 10.1038/ismej.2011.207 PMID: 2237541
13. Griffiths M, Harrison SL (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology 21: 493–507.
14. Dunahay T, Jarvis E, Dais S, Roessler P (1996) Manipulation of microalgal lipid production using genetic engineering. Applied Biochemistry and Biotechnology 57–58: 223–231.
15. Apt KE, Grossman AR, Kroth-Pancic PG (1996) Stable nuclear transformation of the diatom Phaeodactylum tricornutum. Molecular and General Genetics MGG 252: 572–579. PMID: 8914518
16. Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C (1999) Transformation of Nonselectable Reporter Genes in Marine Diatoms. Marine Biotechnology 1: 239–251. PMID: 10383998
17. Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE (2000) Transformation of the diatom Phaeodactylum tricornutum (Bacillariophyceae) with a variety of selectable marker and reporter genes. Journal of Phycology 36: 379–386.
18. Sakaue K, Harada H, Matsuda Y (2008) Development of gene expression system in a marine diatom using viral promoters of a wide variety of origin. Physiologia Plantarum 133: 59–67. doi: 10.1111/j.1399-3054.2008.01089.x PMID: 18346072
19. Poulsen N, Chesley PM, Kröger N (2006) Molecular genetic manipulation of the diatom Thalassiosira pseudonana (Bacillariophyceae). Journal of Phycology 42: 1089–1095.
20. Schneider M (2001) Phospholipids for functional food. European Journal of Lipid Science and Technology 103: 98–101.
21. Kooistra WHCF, Gersende R, Medlin L, Mann DG (2007) The origin and evolution of the diatoms: their adaptation to a planktonic existence In: Falkowski PG, Knoll AH, editors. Evolution of primary producers in the sea. Heidelberg: Academic Press. pp. 207–249.
22. d’Ippolito G, Sardo A, Paris D, Vella F, Adelfi M, Botte P, et al. (2015) Potential of lipid metabolism in marine diatoms for biofuel production. Biotechnology for Biofuels 8: 1–10. doi: 10.1186/s13068-015-0212-4 PMID: 25763104

23. Levitan O, Dinamarca J, Hochman G, Falkowski PG (2014) Diatoms: a fossil fuel of the future. Trends in Biotechnology 32: 117–124. doi: 10.1016/j.tibtech.2014.01.004 PMID: 24529448

24. Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. 1. *Cyclotella Nana* Hustedt, and *Dinoflagellates* (Cleve) Gran. Canadian Journal of Microbiology 8: 229–8. PMID: 13902807

25. Berges JA, Harrison PJ (1995) Relationships between nitrate reductase activity and rates of growth and nitrate incorporation under steady-state light or nitrate limitation in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). Journal of Phycology 31: 85–95.

26. Giavalisco P, Li Y, Matthes A, Eckhardt A, Hubberen H-M, Hesse H, et al. (2011) Elemental formula annotation of polar and lipophilic metabolites using 13C, 15N and 34S isotope labelling, in combination with high-resolution mass spectrometry. The Plant Journal 68: 364–376. doi: 10.1111/j.1365-313X.2011.04682.x PMID: 21699588

27. R-Core-Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing.

28. Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nature Protocols 1: 387–396. PMID: 17406261

29. Cuadros-Inostroza A, Caldana C, Resteghig H, Kusano M, Lisec J, Pena-Cortes H, et al. (2009) Target-Search—a Bioconductor package for the efficient preprocessing of GC-MS metabolite profiling data. BMC Bioinformatics 10: 428. doi: 10.1186/1471-2105-10-428 PMID: 20015393

30. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54: 621–639. doi: 10.1111/j.1365-313X.2008.03492.x PMID: 18476868

31. Yang Z-K, Liu Y-F, Ma Y-H, Xue J, Zhang M-H, Yang W-D, et al. (2013) Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation. Biotechnology for Biofuels 6: 67. doi: 10.1186/1754-6834-6-67 PMID: 23642220

32. Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology 128: 219–240.

33. Dunstan GA, Volkman JK, Barrett SM, Garland CD (1993) Changes in the lipid composition and maximization of the polyunsaturated fatty acid content of 3 microalgae grown in mass culture. Journal of Applied Phycology 5: 71–83.

34. Raven JA (1987) The Role of Vacuoles. New Phytologist 106: 357–422.

35. Obata T, Fernie A, Nunes-Nesi A (2013) The Central Carbon and Energy Metabolism of Marine Diatoms. Metabolites 3: 325. doi: 10.3390/metabo3020325 PMID: 24957995

36. Hockin NL, Maskin T, Mulholland F, Kopriva S, Malin G (2012) The Response of Diatom Central Carbon Metabolism to Nitrogen Starvation Is Different from That of Green Algae and Higher Plants. Plant Physiology 158: 299–312. doi:10.1104/pp.111.184333 PMID: 22065419

37. Mock T, Samanta MP, Iverson V, Berthiaume C, Robison M, Holtermann K, et al. (2008) Whole-genome expression profiling of the marine diatom *Thalassiosira pseudonana* identifies genes involved in silicon bioprocesses. Proceedings of the National Academy of Sciences of the United States of America 105: 1579–1584. doi:10.1073/pnas.0707946105 PMID: 18212125

38. Yang ZK, Ma YH, Zheng JW, Yang WD, Liu JS, Li HY (2014) Proteomics to reveal metabolic network shifts towards lipid accumulation following nitrogen deprivation in the diatom *Phaeodactylum tricornutum*. Journal of Applied Phycology 26: 73–82. PMID: 24600163

39. Ge F, Huang W, Chen Z, Zhang C, Xiong Q, Bowler C, et al. (2014) Methylcrotonyl-CoA carboxylase regulates triacylglycerol accumulation in the model diatom *Phaeodactylum tricornutum*. Plant Cell 26: 1681–1697. PMID: 24769481

40. Juergens MT, Deshpande RR, Luckner BF, Park JJ, Wang H, Gargouri M, et al. (2015) The regulation of photosynthetic structure and function during nitrogen deprivation in Chlamydomonas reinhardtii. Plant Physiology 167: 558–573. doi: 10.1104/pp.114.250530 PMID: 25489023

41. Mizusawa N, Wada H (2012) The role of lipids in photosystem II. Biochimica et Biophysica Acta (BBA) —Bioenergetics 1817: 194–208.

42. Su X, Xu J, Yan X, Zhao P, Chen J, Zhou C, et al. (2013) Lipidomic changes during different growth stages of *Nitzschia closterium* f. minutissima. Metabolomics 9: 300–310.

43. Hitchcock GL (1982) A comparative study of the size-dependent organic composition of marine diatoms and dinoflagellates. Journal of Plankton Research 4: 363–377.
44. Renaud SM, Thinh L-V, Parry DL (1999) The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture 170: 147–159.

45. Tonon T, Harvey D, Larson TR, Graham IA (2002) Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae. Phytochemistry 61: 15–24. PMID: 12165297

46. Orcutt DM, Patterson GW (1975) Sterol, fatty acid and elemental composition of diatoms grown in chemically defined media. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 50: 579–583. PMID: 1122739

47. Li S, Xu J, Chen J, Chen J, Zhou C, Yan X (2014) Structural elucidation of co-eluted triglycerides in the marine diatom model organism Thalassiosira pseudonana by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 28: 245–255. doi: 10.1002/rcm.6784 PMID: 24375875

48. Bromke MA, Hochmuth A, Tohge T, Fernie AR, Giavalisco P, Burgos A, et al. (2015) Liquid chromatography-high-resolution mass spectrometry for fatty acid profiling. The Plant Journal 81: 529–536. doi: 10.1111/tpj.12739 PMID: 25440443

49. Dunstan GA, Volkman JK, Barrett SM, Leroi J-M, Jeffrey SW (1993) Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35: 155–161.

50. Sorhannus U (2004) Diatom phylogenetics inferred based on direct optimization of nuclear-encoded SSU rRNA sequences. Cladistics 20: 487–497.

51. Theriot EC, Cannone JJ, Gutell RR, Alversen AJ (2009) The limits of nuclear-encoded SSU rDNA for resolving the diatom phylogeny. European Journal of Phycolology 44: 277–290. PMID: 20224747

52. Browse J, Somerville C (1991) Glycerolipid synthesis: biochemistry and regulation. Annual Review of Plant Biology 42: 467–506.

53. Domergue F, Spiekermann P, Lerchl J, Beckmann C, Kilian O, Kroth PG, et al. (2003) New Insight into Phaeodactylum tricornutum Fatty Acid Metabolism. Cloning and Functional Characterization of Plastidal and Microsomal Δ12-Fatty Acid Desaturases. Plant Physiology 131: 1648–1660. PMID: 12692324

54. Xu J, Chen D, Yan X, Chen J, Zhou C (2010) Global characterization of the photosynthetic glycerolipids from a marine diatom Stephanodiscus sp. by ultra-performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight mass spectrometry. Analytica Chimica Acta 663: 60–68. doi: 10.1016/j.aca.2009.11.026 PMID: 20172098

55. Allen AE, Laroche J, Maheswari U, Lommer M, Schauer N, Lopez PJ, et al. (2008) Whole-cell response of the pennate diatom Phaeodactylum tricornutum to iron starvation. Proceedings of the National Academy of Sciences of the United States of America 105: 10438–10443. doi: 10.1073/pnas.0711370105 PMID: 18653757

56. Allen AE, Laroche J, Maheswari U, Lommer M, Schauer N, Lopez PJ, et al. (2008) Whole-cell response of the pennate diatom Phaeodactylum tricornutum to iron starvation. Proceedings of the National Academy of Sciences of the United States of America 105: 10438–10443. doi: 10.1073/pnas.0711370105 PMID: 18653757

57. Vidoudez C, Pohnert G (2012) Comparative metabolomics of the diatom Skeletonema marinoi in different growth phases. Metabolomics 8: 654–669.

58. Vidoudez C, Pohnert G (2012) Comparative metabolomics of the diatom Skeletonema marinoi in different growth phases. Metabolomics 8: 654–669.

59. Nappo M, Berkov S, Codina C, Avila C, Messina P, Zupo V, et al. (2009) Metabolite profiling of the benthic diatom Cocconeis scutellum by GC-MS. Journal of Applied Phycology 21: 295–306.

60. Bölling C, Fiehn O (2005) Metabolite profiling of Chlamydomonas reinhardtii under nutrient deprivation. Plant Physiology 139: 1995–2005. PMID: 16306140