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Research Article

Phenotypic diversity of diploid and haploid *Emiliania huxleyi* cells and of cells in different growth phases revealed by comparative metabolomics☆

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SUMMARY

In phytoplankton a high species diversity of microalgae co-exists at a given time. But diversity is not only reflected by the species composition. Within these species different life phases as well as different metabolic states can cause additional diversity. One important example is the coccolithophore *Emiliania huxleyi*. Diploid cells play an important role in marine ecosystems since they can form massively abundant algal blooms but in addition the less abundant haploid life phase of *E. huxleyi* occurs in lower quantities. Both life phases may fulfill different functions in the plankton. We hypothesize that in addition to the functional diversity caused by this life phase transition the growth stage of cells can also influence the metabolic composition and thus the ecological impact of *E. huxleyi*. Here we introduce a metabolomic survey in dependence of life phases as well as different growth phases to reveal such changes. The comparative metabolomic approach is based on the extraction of intracellular metabolites from intact microalgae, derivatization and analysis by gas chromatography coupled to mass spectrometry (GC–MS). Automated data processing and statistical analysis using canonical analysis of principal coordinates (CAP) revealed unique metabolic profiles for each life phase. Concerning the correlations of metabolites to growth phases, complex patterns were observed. As for example the saccharide mannitol showed its highest concentration in the exponential phase, whereas fatty acids were correlated to stationary and sterols to declining phase. These results are indicative for specific ecological roles of these stages of *E. huxleyi* and are discussed in the context of previous physiological and ecological studies.

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Introduction

Coccolithophores are unicellular marine algae that due to the formation of calcified coccoliths play a significant role in the world's oceans. *Emiliania huxleyi* (Lohmann) Hay & Mohler, 1967 is the species with the highest abundance among coccolithophores and the most important calcifier in the world's oceans (Westbroek et al., 1989). It forms intensive blooms (e.g. Holligan et al., 1983) which are even detectable from space (Brown and Yoder, 1994). Such blooms especially occur in eutrophic regions often in a succession following diatom spring blooms in temperate latitudes (Tyrell and Merico, 2004).

An interesting trait of this alga is its complex haplo-diploid life cycle displaying two basic levels of ploidy (Green et al., 1996; Houdan et al., 2004). *E. huxleyi* exists in a heterococcolith bearing, diploid stage alternating with a motile, flagellated cell form that is haploid and bearing organic scales (Houdan et al., 2004). The diploid, bloom-forming life phase mostly found in nature is intensively studied. In contrast, the role of the haploid phase is still poorly explored. Green et al. (1996) stated a potential function of the haploid form in acting as gametes. In general, among algae a haploid–diploid life cycle is regarded to be favorable in a seasonally changing environment or in a habitat comprising two to some extent different niches (Stebbins and Hill, 1980). So Rokitta et al. (2011) interpreted differences detected at the transcriptome level of *E. huxleyi* life phases as a specialization to varying ecological niches. The transcriptional patterns of life phases varied concerning genome expression, proteome maintenance and metabolic processing with the latter being up-regulated in haploid relative

Abbreviations: 1N, haploid; 2N, diploid; CAP, canonical analysis of principal coordinates; Chl *a*, chlorophyll a; compl., complex; Exp., exponential; Decl., declining; deriv., derivative; F_0, initial fluorescence; F_m, maximum fluorescence; GC–MS, gas chromatography–mass spectrometry; met., metabolite; ME, methyl ester; No., number; PSII, photosystem II; RT, retention time; sac., saccharide; Stat., stationary.

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to diploid cells (Rokitta et al., 2011). Another potential indication for differences in physiological traits regarding the phenotype of life phases can be found in their potential to be infected by viruses, because whereas diploid cells are susceptible to viral infection, haploid ones are resistant (Frada et al., 2008).

This study addresses the differences in diploid and haploid *E. huxleyi* to gain insight into the specific properties these life forms on the metabolome level. In addition we explore the general variability by including a comprehensive metabolic profiling during different growth phases of the poorly explored haploid cells. In other studies metabolic profiling revealed strain specific differences in *Chaetoceros socialis* (Huseby et al., 2012) and the influence of growth phases on the metabolome of microalgae (Barofsky et al., 2009; Kluender et al., 2009; Lee and Fiehn, 2008). Further studies gave insights into physiological adaptation of unicellular algae to variable environmental conditions (Paul et al., 2009, 2013; Vidoudez and Pohnert, 2012). Such considerations are crucial if ecological and physiological conclusions are concerned and are also of fundamental relevance for explanations of the enormous species diversity in the plankton. Several analyses targeting specific metabolic pathways in *E. huxleyi* especially aiming at lipid biosynthesis have been undertaken (e.g. Chuecas and Riley, 1969; Fernández et al., 1994; Maxwell et al., 1980; Viso and Marty, 1993), but mostly exponentially growing diploid cells were used. Exceptions are two studies distinguishing lipid composition in logarithmic and stationary phase (Bell and Pond, 1996; Pond and Harris, 1996). A recent metabolic profiling approach on diploid *E. huxleyi* cells targeted a limited number of substances in particular amino acids and sugars (Obata et al., 2013), but took neither life nor growth phase variations into account.

In recent years, metabolomics has developed as a powerful tool to explore the physiology and chemical ecology of algae. Studies include the mapping of the metabolism of microalgae by either applying metabolic fingerprinting (e.g. Barofsky et al., 2009; Huseby et al., 2012), which does not include compound identification, or metabolite profiling approaches (e.g. Lee and Fiehn, 2008; Nappo et al., 2009; Obata et al., 2013; Vidoudez and Pohnert, 2012). We thus considered the methodology advanced enough to address the multitude of different life and growth phase stages introduced in this study. Hence, we adapted an existing metabolomic approach developed for diatoms that is based on gas chromatography coupled to mass spectrometry (GC–MS) (Vidoudez and Pohnert, 2012). Automated data processing and manual compound identification then enabled a comprehensive comparative metabolic analysis of the coccolithophore *E. huxleyi*. We observed specific patterns hinting for different metabolic traits possibly related to environmental roles in the life phases of this species. Further, analysis of growth phases resulted in the identification of candidate pathways and specific compounds varying over the course of growth.

Materials and methods

Solvents

The following solvents were used for extraction and sample preparation: methanol, pyridine, water (all Chromasolv® Plus, Sigma–Aldrich, Munich, Germany), ethanol (LiChrosolv, Merck, Darmstadt, Germany), and chloroform (HiPerSolv, VWR, Dresden, Germany).

Experimental setup and culture preparation

Life phase experiment

Diploid (2N) and haploid (1N) strains of *Emiliania huxleyi* were obtained from the Roscoff Culture Collection (2N: RCC1216, 1N: RCC1217). The haploid strain RCC1217 was isolated from the diploid strain, thus these cultures represent the two genetically identical life phases of a single strain. They were grown in K/2 (lacking Tris and Si) medium (Keller et al., 1987) at a temperature of 20 °C with a light/dark regime of 14/10 h and 80 μmol photons m^{-2} s^{-1}. Previous to the experiment two cultures (12 mL) of each life phase were diluted 5× in fresh medium resulting in two 60 mL cultures per life phase. This was performed two more times on every other day producing two 1.5 L cultures in 2 L Erlenmeyer flasks per life phase. One week after the first dilution the two pre-cultures for each life phase were combined to a 3 L culture, from which we generated ten biological replicates per life phase by diluting 300 mL of the combined culture 5× with medium. This resulted in a total volume of 1.5 L for each biological replicate contained in a 2 L Erlenmeyer flask. Pre-cultures remained stable concerning their ploidy level. Inoculation resulted in cell abundances of approximately 9×10^3 cells mL^{-1} (2N) and 8×10^4 cells mL^{-1} (1N) as pre-cultures were performing differently. 4× 1.5 L of medium were used as blank (one per sampling, see below). Among the diploid cultures three out of ten replicates did not grow, resulting in three biological replicates for early and four for late exponential phase sampling (see below). Cultures were shaken daily and 2× 1 mL was sampled under sterile conditions for estimation of cell abundance, chlorophyll *a* (Chl *a*) fluorescence and photosystem II (PSII) efficiency (see below). Samples for these parameters were taken 3 h after onset of illumination.

Growth phase experiment

20 L cultures of RCC 1217 (1N) were grown from five cultures (16 mL) diluted 5× in fresh medium resulting in five 80 mL cultures. Dilution was repeated every other day in total 3× resulting in five 2 L cultures that were combined to a 10 L inoculation culture. Four 20 L culture vessels (polycarbonate, Nalgene) were filled with 18 L of sterile filtered medium and 2 L of the inoculation culture were added resulting in a cell abundance of approximately 2×10^4 cells mL^{-1}. Culture conditions were as described above. Culture vessels were agitated daily by rolling and 10 mL were sampled immediately afterwards to estimate cell and bacterial abundance, Chl *a* fluorescence, and PSII efficiency (see below). Samples for these parameters were taken 3 h after the lights turned on.

Monitoring of growth parameters and photosynthetic activity

To estimate cell abundance in the life phase experiment 1 mL of sample was fixed by addition of 10 μL 25% aqueous glutaraldehyde (electron microscopy grade, Sigma Aldrich, Munich, Germany) resulting in a final concentration of 0.25%, vortexed and incubated in the dark at 4 °C for 30 min. To 100 μL of these samples 100 μL of a calibration standard (see below) and 300 μL of sterile filtered ultra-pure water were added. Measurement was performed immediately afterwards 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 side scatter was used as discriminator and samples were run for 1 min at a flow rate of 30 μL min^{-1}. Polystyrene fluorospheres (3.6 μm in diameter; Beckman Coulter, Krefeld, Germany) measured at 620 nm were used for data calibration and samples were measured in triplicates. In the growth phase experiment at least 400 haploid cells per replicate were counted 3× using a Fuchs-Rosenthal hemocytometer with an upright microscope (Leica DM 2000, Heerbrugg, Switzerland), whereas bacterial abundance was determined by flow cytometry. Therefore, samples were fixed as described above, immediately frozen in liquid nitrogen and stored at −80 °C. For further analysis a protocol originally optimized for viruses (Brussaard, 2004; Marie et al., 1999) was adapted. In brief: samples were thawed for approximately 5 min in a water bath at 35 °C. Next, 10 μL of SYBR gold (Invitrogen, Carlsbad, CA, USA) (final concentration 10^{-4} of commercial stock) were added and

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