Materials and methods for "Physiological adaptation of the diatom *Pseudo-nitzschia delicatissima* under copper starvation" dataset

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Material and methods

Culture conditions

Batch cultures of the marine pennate diatom *Pseudo-nitzschia delicatissima* (strain Pd08RB, solitary species isolated in 2008 by Beatriz Beker in the Bay of Brest, France) were grown at 16°C in polycarbonate bottles. Species was determined after sequencing of the ITS-1 fragment, using PnAllR and PnAllF primers (Hubbard et al., 2008) and obtained sequences were aligned using GenBank. Cultures were grown under cool-white light (OSRAM) over a dark:light cycle of 12:12 h with an irradiance of 130 µmol photons m^{-2} s^{-1}. Although the cultures were naturally xenic and grown without antibiotics, the culture media (see below) were sterilized using a microwave (Keller et al., 1988). Diatoms were pre-acclimated to each culture condition until their growth rate remained stable, and at least 20 generations were grown in the same conditions. Cultures were sampled in triplicates or quadriplicates (see below "Culture media") at mid-exponential phase of growth to maintain a pH lower than 8.5 and avoid CO_2 limitation, and at the same time of the day to avoid diel cycle variations.

Culture media

The culture medium consisted of artificial AQUIL seawater enriched with 300 µM nitrate, 10 µM phosphate, 100 µM silicate, 0.55 µg l^{-1} vitamin B_{12}, 0.5 µg l^{-1} biotin, 100 µg l^{-1} thiamin,

10 nM selenite and 100 nM molybdate (Price et al., 1989). The synthetic ocean water component (including all salts) and the macronutrients were passed through a Chelex 100 ion exchange resin to remove metal contaminants present in the chemicals (Price et al., 1989). The medium also contained a trace metal ion buffer system consisting of 100 µM ethylene diamine tetra acetic acid (EDTA), 50.3 nM Co, 79.7 nM Zn,121 nM Mn and 500 nM Fe. The buffer system generated free ion concentrations of Co, Zn, Mn and Fe of $10^{-10.88}$, $10^{-10.88}$, $10^{-8.27}$ and $10^{-19.2}$ M, respectively, at pH 8.1 according to the chemical equilibrium program MINEQL+ (version 4.62.3) for non-illuminated medium. In the replete medium (”Control”), 19.6 nM Cu were added, generating a free Cu concentration of $10^{-13.9}$ M. In the Cu starvation treatment (”Cu-starved”), no Cu was added to the medium.

All bottles and apparatus were acid cleaned, and all manipulations were conducted within a sterile laminar flow hood equipped with a Teflon® bench using sterile and trace-metal clean techniques (Bucciarelli et al., 2010). Cultures were run in triplicates for the Control and in quadriplicates for the Cu-starved condition.

Flow cytometric measurements

A flow cytometer FACScalibur (BD Biosciences, San Jose, CA USA) with an argon blue laser (488 nm) was used, with the same settings for all the duration of the experiment to allow comparison between treatments. Cell concentrations, morphological and physiological measurements of *P. delicatissima*, quantification of free-living bacteria associated to *P. delicatissima* and percentage of dead bacteria in the culture were assessed using flow cytometry (Lelong et al., 2011). As *Pseudo-nitzschia* spp. can form chains, cell concentrations were counted under microscope: each culture bottle was sampled in triplicate and the mean cell concentration is provided as an average.

Physiological measurements

Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log (cell concentration) versus time. Mortality of *P. delicatissima* was assessed by staining cultures with 0.1 µM of SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes. More than 95 % of living cells could be observed in all cultures, which ensures that the physiological measurements were performed on living cells.

Bio-dilution

When cells divide, the cytoplasm and its content are divided between the two daughter cells. To assess the bio-dilution effect at steady state, the production rate (in arbitrary units per cell per day (AU $cell^{-1}$ d^{-1}) of lipids can be calculated by multiplying their content (in AU $cell^{-1}$) by the acclimated specific growth rate (in d^{-1}) (Lelong et al., 2013).

Bacteria

To estimate free-living bacteria concentration and viability, bacteria were analyzed after 15 min incubation with a final concentration of 1/10000 of the commercial solution of SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg ml^{-1} (Lelong et al., 2011).

Cell volume

For each replicate, a minimum of 50 cells were photographed using a Leica microscope and the Axiovision imaging software. For each cell, cell length and cell width were measured. Cell volume was estimated using the following formulae of an ellipsoid (Hillebrand et al., 1999):

$$\text{Cell volume} = 1/6\pi * \text{length} * \text{width} * \text{width}.$$

Pulse-amplitude modulated (PAM) fluorimetry

Maximum quantum yield ($\Phi_{PSII} = (F_m-F_0)/F_m=F_v/F_m$), which is a measurement of the efficiency of the photosynthesis at the photosystem II (PSII) level, was measured using the AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic), where F_0 and F_m are respectively the minimum and maximum fluorescence of cells at 455 nm. The

measurement of Φ_{PSII} was performed after 20 min of dark adaptation of the cells at 16°C. Chl a fluorescence induction transient (OJIP) curves were also performed on cultures to determine if Cu starvation modified the photosynthetic electron transport of *P. delicatissima*. Measurements were performed applying the internal protocol with blue light (455 nm). Complete dataset of fluorescent variables acquired during this study are available in the supplementary files.

The relative electron transport rate through PSII (rETRPSII) was calculated as:

$$rETR = \Phi_{PSII} * I$$

with I being the light intensity (comprised between 0 and 500 µmol photons m^{-2}).

Pigment composition and chlorophyll content

Duplicate samples from each culture bottle were filtered onto glass fiber filters (GF/F) for Chlorophyll *a* and pigments, and rinsed with artificial seawater previously sterilized by microwave. Filters for Chl *a* were immediately stored in glass tubes at -80 °C. Chl *a* was measured by fluorometry after extraction into 90% acetone (Lorenzen, 1967).

Qualitative pigment composition was analyzed on pigments extracted from frozen cells (-80°C) by methanol using high-performance liquid chromatography (HPLC) according to the method described in (Ras et al., 2008), adapted from (Van Heukelem and Thomas, 2001). All the pigment standards were purchased from DHI (HØRSHOLM, Denmark). Duplicate cultures were used for pigment composition.

Cellular nitrogen and carbon

To determine the cellular C and N, culture samples were filtered as duplicates on a pre-combusted Whatman GF/F filter (450 °C for 4 hours) and rinsed with sterile artificial seawater containing no nutrient. The filters were dried at 60 °C overnight in pre-combusted glassware and stored until analysis using an elemental analyzer Thermo Fisher NA 2100 CN.

Lipids

About 9.10^6 cells from algal cultures were filtered on a GF/F filter. Boiling water was immediately added on filter to prevent lipid degradation through lipase activity. Total lipid extraction was performed placing the filter with algae in glass vials containing 6mL of a chloroform-methanol mixture (2/1, v/v). Lipid extract vials were then sealed under N_2 and stored at -20°C before further analyses. Lipid extracts were stored at -20°C under nitrogen (N2(g)) until analysis.

Lipid class composition analyses were performed by high-performance thin layer chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). Neutral and polar lipid classes were analyzed according to (Moutel et al., 2016). For polar and neutral lipid fatty acid (FA) analysis, an aliquot of the chloroform: methanol (2:1, v:v) extract was dried under $N_{2(g)}$ and then resuspended in chloroform:methanol (98:2, v:v) prior to neutral and polar lipid separation. Separation of neutral and polar lipids was realized by solid phase extraction (Le Grand et al., 2014). Polar and neutral fractions were transesterified and the resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) according to the method from (Le Grand et al., 2014). Lipid class and fatty acid standards were the same standards used in (Le Grand et al., 2014). FA were expressed as percentage of total FA in polar and neutral lipids. The level of unsaturation was calculated from the % FA derived from the gas chromatographic data according to the equation:

$$Unsaturation\ level\ = \sum [\%\ of\ fatty\ acid\ \times number\ of\ double\ bond]$$

Statistics

Effects of Cu starvation on the physiological parameters of *P. delicatissima* were tested using T-tests with the software StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA). The test of rank used was the Tukey test (variance homogeneity was first tested and confirmed for all the parameters). For all statistical results, a probability of $p < 0.05$ was considered significant.

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