Supporting Information for

***In situ* associations between marine photosynthetic picoeukaryotes and potential parasites – a role for fungi?**

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**This supporting information file includes:**

**Materials and Methods**

**References**

**Supplementary Table 1**

**Materials and methods**

**Sampling.**

Seawater samples were collected on board the RRS James Cook between October 13th –December 1st 2009 (AMT19), along a ~7,500 km latitudinal transect of the Atlantic Ocean (~49.57°N 37.86°W - ~39.19°S 43.39°W), within the framework of the Atlantic Meridional Transect program (<http://www.amt-uk.org>). This transect encompasses subtropical oligotrophic gyres of the Northern and Southern hemispheres and the equatorial convergence area. Ten stations were sampled for this study, 6 in the Southern subtropical gyre (SG) and 4 in the Southern temperate (ST) region (Figure 1). Seawater samples were collected from the surface to 88m depth with 20-L Niskin bottles mounted on a sampling rosette of a conductivity-temperature-depth profiler (Sea-Bird Electronics). All samples were pre-filtered through a 20µm pore-size mesh when being decanted from the Niskin bottles. Seawater samples were amended with 0.05% (v/v) pluronic solution (Sigma-Aldrich) final concentration to minimize clumping of concentrated cells. Samples (5 L) were then gently filtered through 0.2 µm pore-size Cell-Trap units (MEM-TEQ Ventures Ltd, Wigan, UK) using a peristaltic pump (Watson Marlow 323S with a 313D pumphead), 8.0 mm dia. silicone tubing, and a flow rate of ~0.1 L min-1. Concentrated cells were extracted from the filter in a volume of ~1.8 ml of filtrate using a syringe following the manufacturer’s recommendations, and fixed for 1 h at 4 °C with 1% (w/v) paraformaldehyde (PFA—Sigma-Aldrich, Poole, UK). Cells were then harvested onto 0.2 µm pore-size polycarbonate filters (Whatman), dehydrated in an ethanol series (50% (v/v), 80% (v/v) and 100% (v/v), 3 min each), dried at room temperature and stored at -80 °C until analysis.

**Flow cytometric cell sorting**.

Pluronic F-68 solution (0.01% (v/v) final concentration filter sterilized through a 0.2 µm pore size polycarbonate filter) was added to 3L seawater samples and then seawater concentrated to a volume of 1.8 ml using a Cell-Trap system (MEM-TEQ Ventures Ltd, UK). The concentrated sample was then fixed with 1% PFA (w/v, final concentration), flash frozen in liquid nitrogen and stored at -80 °C until further processing. Samples were gently defrosted overnight at 4°C, then stained with SYBR Green I and flow sorted using a FACSCalibur instrument (Becton Dickinson) according to light-scattering properties (90° or side light scatter, SSC), relative concentration of SYBR Green I stain per particle (green fluorescence; FL1, 530 ± 30 nm), and chlorophyll content (red fluorescence; FL3, >650 nm). Two different PPE populations were sorted (Plast-S ca. 2 µm, and Plast-L ca. 3 µm in size) (see Hartmann *et al*., 2012 for more details). Between 10,000 and 20,000 Plast-S and Plast-L cells were sorted onto 0.8 μm pore size 13 mm diameter polycarbonate filters (Nuclepore, Whatman, UK) to reduce the retention of potentially by-sorted bacterioplankton cells. The filters were then dehydrated in an ethanol series (50% (v/v), 80% (v/v) and 100% (v/v), 3 min each, dried at room temperature and stored at -80° C until analysis.

**Tyramide Signal Amplification (TSA) - Fluorescence *in situ* hybridization (FISH).**

The abundance and distribution of free-living parasites and PPEs were analysed by a single round of TSA-FISH on filtered samples. Only cells measured as <5µm were counted and analysed. In addition, potential associations between phytoplankton and parasites were detected by two successive rounds of TSA-FISH on two different PPE size fractions sorted by flow cytometry: Plast-S cells (2±0.1 µm in size) and Plast-L cells (3.1±0.3 µm in size).

Nuclear 18S rRNA oligonucleotide probes EUK1209 was used to target total eukaryotes (Giovannoni *et al*., 1988). Nuclear 18S rRNA oligonucleotide probes PRYM02, PELA01 and CRYPT13, specific for *Prymnesiophyceae*, *Pelagophyceae* and *Cryptophyceae* respectively (Simon *et al*., 2000; Lepère *et al*., 2008) as well as the CHRYSO1037 probe targeting the chloroplast 16S rRNA gene of *Chrysophyceae* (Jardillier *et al.,* 2010) were used to target PPEs. To identify potential parasites six different 18S rRNA oligonucleotide probes were used targeting Syndiniales group II (ALV01), Perkinsozoa clade 1 and 2 (PERKIN\_01, PERKIN\_02), and a wide range of Fungi (*Eumycota*), including Chytridiales and environmental sequences branching within Cryptomycota (MY1574, Chyt1061, LKM11\_01) (Chambouvet et al. 2008; Mangot *et al*., 2009; Baschien *et al*., 2008; Jobard *et al*., 2010; Jones *et al*., 2011).

Fungal probe MY1574probe is specific for a large number of members of the kingdom *Fungi (*Baschien *et al*., 2008) (No matching eukaryotic sequences other than fungal rRNA sequences were observed) while Chyt1061 probe targets at 90% the fungal species in the order Chytridiales, the largest order of the division Chytridiomycota (chytrids) (Gleason *et al*., 2008), mainly represented by phytoplanktonic parasites in aquatic environments (Canter, 1950; Sparrow, 1960). A strong specificity of the probe for Chytridiales 18S rDNA sequences from 6 different environments and seven different world regions was found (Jobard *et al*., 2010). The LKM11\_01 probe covers the diversity of a large clade of Cryptomycota, although 13 clades have been recently found (Lazarus and James, 2015) which may lead to an underestimation of Cryptomycota in the studied environment.

Oligonucleotide horseradish peroxidase (HRP) probes targeting PPEs or parasites were labeled at the 5′ end with the fluorescent dyes CY3 and fluorescein, respectively, (PerkinElmer, UK, TSATM Cyanine 3 System and TSATM Fluorescein System). *In situ* hybridizations for putative parasites were performed first, as described by Not *et al*., (2002) with some modifications: To reduce potential background, filters were pre-incubated for 30 min at the hybridization temperature with 10 ml hybridization buffer (HB) comprising 40% (v/v) formamide, 0.9 M NaCl, 20 mM Tris-HCl pH 7.5, 0.01% (w/v) SDS (sodium dodecyl sulfate, Sigma-Aldrich, UK) and 10% (w/v) blocking reagent. A mixture of 9 ml HB + 1 ml 10 pmol L-1 probe was then added to the filters. Samples were hybridized for 12 h at 35 °C (PERKIN\_01 and 02, LKM1101), 42 °C (Alv01) and 46 °C (MY1574, Chyt1061) before washing twice for 30 min at 37 °C, 46 °C and 48 °C, respectively, in pre-heated (30 min) washing buffer (56 mM NaCl, 5 mM EDTA, 0.01% (w/v) SDS, 20 mM Tris-HCl pH 7.5) in a shaking incubator. Samples were then equilibrated in TNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 (Sigma-Aldrich, UK)) for 15–20 min at room temperature before adding 10 ml TSA mix (TSATM Fluorescein System, Perkin Elmer, UK) following the manufacturer’s instructions, and left for 30–40 min at room temperature in the dark. After the TSA reaction, samples were incubated twice at 55 °C in TNT buffer for 20–40 min. The filters were then washed 2 x 10 min in sterile water and left to dry at room temperature. When filters were dry PPE cell hybridization was initiated as described previously (see Hartmann *et al*., 2013) except that the hybridization step involved a 3 h hybridization step at 35 °C and a washing temperature of 37 °C. To determine the presence and composition of a fungal cell wall, TSA-FISH filters were counter-stained with the cell wall markers wheat germ agglutinin (WGA) (Invitrogen 5 µg ml-1) for chitin (Jones *et al*., 2011), and calcofluor white (Sigma, 1 mg ml-1) for chitin and/or cellulose. Finally, filters were mounted on a glass slide with an anti-fading reagent (Citifluor, UK). These whole cell *in situ* preparations were kept at 4°C in the dark ready for epifluorescence microscopy over 1 week without significant loss of fluorescence. Control experiments without any probes were performed for each experiment and showed no signal coming from potential endogenous horseradish peroxidases (data not shown). In each case, a total of 100-200 cells was counted for each PPE population, in duplicate when possible, using a BX60F5 fluorescence microscope equipped with a mercury lamp (Olympus Optical, Japan).

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