

## ORIGINAL ARTICLE

# Global distribution patterns of distinct clades of the photosynthetic picoeukaryote *Ostreococcus*

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***Ostreococcus* is a marine picophytoeukaryote for which culture studies indicate there are ‘high-light’ and ‘low-light’ adapted ecotypes. Representatives of these ecotypes fall within two to three 18S ribosomal DNA (rDNA) clades for the former and one for the latter. However, clade distributions and relationships to this form of niche partitioning are unknown in nature. We developed two quantitative PCR primer-probe sets and enumerated the proposed ecotypes in the Pacific Ocean as well as the subtropical and tropical North Atlantic. Statistical differences in factors such as salinity, temperature and NO<sub>3</sub> indicated the ecophysiological parameters behind clade distributions are more complex than irradiance alone. Clade OII, containing the putatively low-light adapted strains, was detected at warm oligotrophic sites. In contrast, Clade OI, containing high-light adapted strains, was present in cooler mesotrophic and coastal waters. Maximal OI abundance (19555 ± 37 18S rDNA copies per ml) was detected in mesotrophic waters at 40 m depth, approaching the nutricline. OII was often more abundant at the deep chlorophyll maximum, when nutrient concentrations were significantly higher than at the surface (stratified euphotic zone waters). However, in mixed euphotic-zone water columns, relatively high numbers (for example, 891 ± 107 18S rDNA copies per ml, Sargasso Sea, springtime) were detected at the surface. Both Clades OI and OII were found at multiple euphotic zone depths, but co-occurrence at the same geographical location appeared rare and was detected only in continental slope waters. *In situ* growth rate estimates using these primer-probes and better comprehension of physiology will enhance ecological understanding of *Ostreococcus* Clades OII and OI which appear to be oceanic and coastal clades, respectively.**

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## Introduction

Over the past two decades, the diversity of small eukaryotic phytoplankton has been investigated with increasing intensity (for example, Rappé *et al.*, 1998; Diez *et al.*, 2001; Not *et al.*, 2007; Shi *et al.*, 2009). Picoeukaryotes have also been shown to contribute significantly to picophytoplankton biomass and primary production (for example, Li, 1994; Worden *et al.*, 2004; Grob *et al.*, 2007; Cuvelier *et al.*, 2010; Jardillier *et al.*, 2010). Several ‘new’ picoeukaryotic (≤2–3 μm diameter) groups have been reported (Vaulot *et al.*, 2008; Worden and Not, 2008), some having been successfully cultured and characterized, like *Ostreococcus* (Courties *et al.*,

1994). This photosynthetic picoprasinophyte falls in the order Mamiellales with *Micromonas* and *Bathycoccus* (Worden and Not, 2008).

*Ostreococcus* is the smallest known free-living eukaryote (~0.8–1.2 μm) and several studies have explored its diversity using environmental clone libraries (Guillou *et al.*, 2004; Worden, 2006; Viprey *et al.*, 2008; Worden and Not, 2008). Thus, far *Ostreococcus* diversity has been best resolved using the internal transcribed spacer (ITS), which is more diverged than the 18S ribosomal RNA (rRNA) gene (Rodriguez *et al.*, 2005). Laboratory photophysiology studies indicate that two differently photoadapted ecotypes exist and are represented by four distinct clades defined using ITS phylogeny, ITS Clades A, C and D being high-light adapted and ITS Clade B being low-light adapted (Rodriguez *et al.*, 2005). The same clade designations have been used for 18S ribosomal DNA (rDNA) trees (Guillou *et al.*, 2004; Viprey *et al.*, 2008), although not necessarily retaining bootstrap support in analyses of either marker

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(see also Rational section of Results and Discussion for details). These clades have also been termed Clades OI and OII based on 18S rDNA bootstrap support, with OI representing ITS/18S rDNA Clades A and C, OII representing ITS/18S rDNA Clade B and ITS/18S rDNA Clade D excluded because only one published sequence from a cultured strain was available at the time of analysis (Worden and Not, 2008; Worden *et al.*, 2009). Among isolates, *Ostreococcus tauri* as well as strains RCC356, RCC344 and RCC501 are regarded as high-light ecotypes (falling within Clade OI) whereas *Ostreococcus* RCC393, RCC143 and RCC809 (formerly RCC141) are considered low-light ecotypes (falling within Clade OII) (Rodriguez *et al.*, 2005; Six *et al.*, 2009). This photophysiology-based ecotypic differentiation has been likened to niche partitioning reported for strains of the cyanobacterium *Prochlorococcus* (Moore *et al.*, 1998; Moore and Chisholm, 1999; Six *et al.*, 2008).

*Ostreococcus* abundance has been explored in several studies using fluorescence *in situ* hybridization (FISH) (Not *et al.*, 2004, 2005, 2008) or quantitative PCR (qPCR) (Zhu *et al.*, 2005; Countway and Caron, 2006), in both cases with genus-level probes. However, ecotype distributions have not been explored systematically or quantitatively in nature. Moreover, comparison of clone library and metagenomic data have led to the proposal that the 'deep-adapted' clade is not low-light adapted *per se* but rather better adapted to life in open-ocean conditions, which include periods of time deep in the euphotic zone (Worden, 2006).

We explored *Ostreococcus* clade niche-partitioning in nature in relation to the laboratory-based hypotheses on irradiance and photophysiology. To this end, qPCR primer-probe sets were developed that target the proposed ecotypes independently. We quantified Clades OI and OII in marine samples and discuss how the data reshapes concepts of niche partitioning.

## Materials and methods

### Sampling

Twelve cruises were performed between 2001 and 2007 (Table 1). Samples were also utilized from the Scripps Pier (Worden *et al.*, 2004; Worden, 2006), the Monterey Bay Time Series (MBTS, [http://www.mbari.org/bog/Projects/CentralCal/summary/ts\\_summary.htm](http://www.mbari.org/bog/Projects/CentralCal/summary/ts_summary.htm)) and the Bermuda Atlantic Time-Series Study (BATS) station program. Water was typically collected in Niskin bottles mounted on a rosette along with a Conductivity, Temperature Depth sensor.

Euphotic zone DNA samples were collected by filtering 500–2000 ml seawater through a 0.2- or 0.45- $\mu$ m pore size Supor filter (Pall Gelman, East Hills, NY, USA). In all, 24 of 301 samples were first gravity filtered through a 2- $\mu$ m pore size polycarbonate filter (Osmonics, Trevose, PA, USA) and

subsequently onto a 0.2  $\mu$ m Supor filter. *Ostreococcus* is between  $\sim$ 0.8 and 1.2  $\mu$ m in diameter (Worden and Not, 2008), hence pre-filtration is not expected to significantly reduce counts. Filters were placed into sterile cryovials and frozen in liquid nitrogen before storage at  $-80^{\circ}\text{C}$ . BATS euphotic zone samples were filtered ( $\sim$ 75–100 l) as described in Treusch *et al.* (2009). For cruises with nutrient and Chl *a* data many of the data have been reported previously (see below).

### DNA extraction, clone library and plasmid preparation

All samples, except those from BATS in 2000 and 2003, were extracted using the DNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with filters being subjected to additional freeze fracture and bead-beating steps according to (Moisander *et al.*, 2008) or crushed using sterile pestles (63 CN cruise samples) at the start of extraction procedure. DNA was eluted using either one or two sequential elutions, resulting in a final volume of 50 or 100  $\mu$ l. Extracts were aliquoted and stored at  $-80^{\circ}\text{C}$ . For 2000 and 2003 BATS cruises a sucrose-based extraction was used, as in Treusch *et al.* (2009). 18S rRNA gene clone libraries were constructed from some samples (Table 1) as described previously (Cuvelier *et al.*, 2008) and sequences deposited under accession numbers HQ847732–HQ847735.

To generate 18S rDNA insert-bearing plasmid standards for qPCR, the 18S rRNA gene was amplified from culture DNA (*O. tauri* OTH95 and RCC809, extracted as above) using the eukaryotic primers 5'-ACCTGGTTGATCCTGCCAG-3' and 5'-TGATCCTTCYGCAGGTTACAC-3' (Moon-van der Staay *et al.*, 2000). PCR was performed using HotStar Taq polymerase (Qiagen) and cycling conditions were  $95^{\circ}\text{C}$  (15 min), proceeded by 32 cycles at  $94^{\circ}\text{C}$  (30 s),  $55^{\circ}\text{C}$  (30 s) and  $72^{\circ}\text{C}$  (1 min), and final extension at  $72^{\circ}\text{C}$  (10–15 min). PCR products were cloned (TOPO-TA, Invitrogen, Carlsbad, CA, USA) and plasmids purified (QIAprep kit, Qiagen) according to the manufacturer's procedures. Sequencing used Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA, USA). Clone UEPACAAp1, taken from a Pacific Ocean environmental clone archive (Worden, 2006), was also used. OTH95 and UEPACAAp1 have 98 and 99% identity to *O. lucimarinus* CCE9901 over 1722 positions, respectively, and represent Clade OI while RCC809 represents Clade OII. DNA from RCC789, a clonal ITS Clade D strain derived from RCC501, as well as DNA or plasmids from *Bathycoccus* and several *Micromonas* were also used (see below, Table 3, Supplementary Tables 2 and 3).

### qPCR, FISH, primer-probe design and qPCR analysis

Initially, published *Ostreococcus* genus-level SYBR-green qPCR primers (Zhu *et al.*, 2005) and a

**Table 1** Cruises on which the environmental sample set was collected

Location	Cruise	Station/number	~ Start or station		~ Site farthest from start		Dates (Date Month Year)
			Lat	Lon	Lat	Lon	
South Pacific	KM0703	Aust—S. Pac./26	18° 10'12"S	147° 25'12"E	15° 0'0"S	170° 0'0"W	Mar–Apr 2007
North Pacific	KM0715	ALOHA	22° 45'0"N	158° 0'0"W	NA	NA	Aug 2007
	CN107	CalCOFI line 67/8	36° 27'36"N	122° 25'8"W	33° 17'9.6"N	129° 25'41"W	Jul 2007
	CN207 <sup>a</sup>	CalCOFI line 67/7	36° 17'35"N	123° 8'2"W	33° 10'16"N	129° 15'25"W	Oct 2007
	MBTS	Sts C1, M1, M2	36° 47'60"N	121° 50'60"W	36° 42'0"N	122° 23'24"W	Jan–Dec 2007
	NA	Scripps Pier	32° 31'48"N	117° 9'0"W	NA	NA	Jan–Dec 2001
Trop. Atlantic	SJ0609	FL–E. Atl./19	18° 12'37"N	67° 28'27"W	12° 24'49"N	35° 16'14"W	Jun–Jul 2006
Florida Straits	WS0503 <sup>a</sup>	Sts 01, 04, 14	25° 30'07"N	80° 04'04"W	25° 29'59"N	79° 20'58"W	Mar 2005
	WS0518 <sup>a</sup>	Sts 01, 04, 14	25° 30'04"N	80° 03'59"W	25° 29'55"N	79° 20'54"W	Aug 2005
	WS0705	Sts 01, 04, 08	25° 30' 0"N	80° 3' 58"W	25° 30' 0"N	79° 42' 36"W	27 Feb 2007
North Atlantic	EN351	Shelf—BATS/5	31° 49'44"N	64° 10'30"W	40° 15'7"N	70° 25'23"W	Mar–Apr 2001
	EN360	Shelf—W. Sarg/5	39° 59'38"N	71° 48'1"W	34° 25'34"N	72° 3'28"W	Sep 2001
	OC374	Shelf—BATS/5	31° 38'55"N	64° 12'17"W	40° 12'57"	70° 6'19"W	Mar 2002
	OC413 <sup>a</sup>	N. BATS, BATS/2	35° 09'24"N	66° 33'46"W	31° 39'20"	64° 37'21"W	May–Dec 2005
Sargasso Sea	138 <sup>b</sup>	BATS	31° 34'59"	64° 8' 2.4"	NA	NA	14 Mar 2000
	173 <sup>b</sup>	BATS	31° 42'36"N	64° 13'16"W	NA	NA	14 Feb 2003
	174 <sup>b</sup>	BATS	31° 42'36"N	64° 13'16"W	NA	NA	04 Apr 2003
	179 <sup>b</sup>	BATS	31° 42'36"N	64° 13'16"W	NA	NA	13 Aug 2003

Abbreviations: Aust, Australia; BATS, Bermuda Atlantic Time-series Study; MBTS, Monterey Bay Time Series; NA, not applicable; Sts, stations.  
<sup>a</sup>Clone libraries sequenced.  
<sup>b</sup>Samples were provided by the BATS program (see acknowledgements).  
 Samples were collected on multiple dates from each MBTS stations (Supplementary Figure 2) and from the Scripps Pier (Worden, 2006).  
 Station name is provided for known time-series sites, and station number is provided for transect cruises, indicating the total number of stations from which samples were evaluated.

**Table 2** Nucleotide sequences of the clade-specific *Ostreococcus* primer-probe sets

Targeted clade	Full name	Sequence (5'–3')
OI	OI.08F	GGATTTTGGCTGAGAACGGTC
	OI.08R	CGATGAAGCACACCTCCTCAC
	OI.08P	6-FAM TGCACCTGACTGGTCTC MGBNFQ
OII	OII.08F	GGATTTTGGCTGAGAACGAA
	OII.08R	AAAGTAACCACGGTACTAAGTGGC
	OII.08P	6-FAM TGCACCTGTTGGTCTCA MGBNFQ

Abbreviation: MGBNFQ, minor-groove binding non fluorescent quencher.

Tyramide signal Amplification FISH (TSA–FISH) probe (Not *et al.*, 2004; with details in Cuvelier *et al.*, 2010) were used. To develop primer-probe sets that could discriminate between different clades, 18S rRNA gene sequences for cultured prasino-phytes, other organisms, and environmental sequences were retrieved from GenBank (last retrieval December 2008). Primer-probe sets for Clade OI and OII (Table 2), as well as *Bathycoccus* and *Micromonas* (Supplementary Table 1), were designed manually using Seqman (DNASTAR Inc., Madison, WI, USA). Melting temperature and secondary structures were checked using Primer Express (AB), Beacon Designer 7.0 (PREMIER Biosoft International, Palo Alto, CA, USA) and an online  $T_m$  calculator (IDT, San Diego, CA, USA). The reporter dye at the probe 5' terminus was 6-FAM (fluorescein) and a minor-groove binding non-fluorescent quencher or a Black-Hole quencher was

used at the 3' terminus. DNA extracts and plasmid standard curves from cultured relatives and more distant phytoplankton were used to test specificity of the *Ostreococcus* Clade OI and OII (Table 2), *Bathycoccus* (Supplementary Table 2) and *Micromonas* (Supplementary Table 3) primer-probe sets. The reaction efficiency, calculated as  $(10^{-(1/m)} - 1)$  where  $m$  is the slope of a linear regression against standard curve cycle threshold ( $C_T$ ) values, was 90–101% ( $n = 14$ , OI primer-probe) and 91–99% ( $n = 13$ , OII primer-probe). For *Micromonas* and *Bathycoccus* primer-probe sets it was 98 and 95–103%, respectively.

Before qPCR, plasmids were quantified using the NanoDrop system (Thermo Scientific, Waltham, MA, USA) and diluted to  $0.5 \times 10^9$  gene copies per  $\mu\text{l}$ , followed by 10-fold serial dilutions, creating a standard curve. Reaction volumes were 25  $\mu\text{l}$ , being composed of 12.5  $\mu\text{l}$  TaqMan Universal PCR Master Mix (AB), 2.5  $\mu\text{l}$  (each) of probe (250 nM final concentration), forward and reverse primers (900 nM final concentration), 3  $\mu\text{l}$  H<sub>2</sub>O and 2  $\mu\text{l}$  template (DNA extract or plasmid). For environmental samples, DNA was diluted according to inhibition test results (see below). qPCR cycling conditions were 10 min at 95 °C (initial denaturation) followed by 45 cycles at 95 °C (15 s) and 60 °C (1 min) using an AB7500. Data were collected during the annealing phase. Of 271 global environmental samples (not including MBTS), 81 were run in triplicate, and the rest in duplicate to conserve DNA. The 29 MBTS samples were run in triplicate (17 samples, which required dilution based on

inhibition results) or duplicate (12 samples). Each run (96 well-plate) included triplicated no-template controls and plasmid standard curves ranging from  $10^8$  to  $10^0$  copies per rxn, although dilutions from  $10^8$  to  $10^1$  were used for a small subset.  $10^0$  rDNA copies per rxn were detected  $39 \pm 19\%$  of the time among triplicates. The number of standard copies detected consistently, where the s.d. was smaller than the mean for triplicates, was  $10^1$  rDNA copies per rxn.

The possibility that environmental DNA extracts were inhibited was tested by spiking samples with  $2 \mu\text{l}$  of plasmid ( $10^4$  or  $10^5$  gene copies per rxn).  $C_T$  values from standard curve points with the same concentration were compared with plasmid spiked samples to ensure values were equivalent, or assess the extent of inhibition (Short and Zehr, 2005). Inhibition tests were performed on each sample using one of the four primer-probe sets developed herein. Although some samples were not diluted, typically dilutions ranged between 1:4 and 1:100 to avoid inhibition, or in some cases to conserve DNA. The resulting theoretical detection limits, based on the volume of sample filtered, the elution volume, the dilution used for the final run and consistent detection ( $10^1$  rDNA copies per rxn) was 0.1–12.5 rDNA copies per ml for 264 of 271 discrete samples (detection down to 0.1– $<3$  copies per ml, 151 samples; 3–13 copies per ml, 113 samples), between 16 and 20 rDNA copies per ml for six samples and for one sample the minimum detectable was 130 rDNA copies per ml. Owing to limited extract, 12 Florida Straits and 4 Sargasso Sea samples were not tested for inhibition; inhibition was not seen in other samples from these regions using the same extraction and dilution methods. Scripps Institution of Oceanography pier samples (five total), most of which contained high *Ostreococcus* counts, were not checked for inhibition. For each run, threshold and baseline values were calculated treating each measurement as a unique run (AB7500 software package, Applied Biosystems, Foster City, CA, USA). rDNA copies per ml in seawater samples were determined according to  $C_T$  values fitted on linear regression of  $C_T$  versus copy numbers (in log scale) of the standard curve, also taking into account sample volume filtered, elution volume, dilution and template volume. If the mean rDNA copies per ml in the environmental sample was at least twice the detection limit, the data was considered quantitative and in this case typically at least 10 copies per rxn were detected. The clade was considered detected but not quantifiable for ratios of sample copies to detection limit  $<2$  and  $\geq 1$ , and undetected for ratios  $<1$ .

#### Interrelating gene copies per ml and cells per ml

The genome sequence of *O. tauri* OTH95 (Derelle *et al.*, 2006) shows one rRNA gene cluster while *O. lucimarinus* CCE9901 (Palenik *et al.*, 2007)

and RCC809 (a representative of the putatively deep-adapted Clade OII), have two copies. Given the identical nature of this gene cluster, genome assemblies sometimes do not render the correct copy number. However, based on these genome sequences, qPCR rDNA copies per ml likely correspond to either an equivalent number of cells per ml, or two times the cell count, assuming G1 phase of the cell cycle and that the amplification efficiency of the plasmid standards matches that of cells.

#### Statistics and climatology

Environmental data for samples containing the respective *Ostreococcus* clades were tested for normality using SigmaStat (Systat software, Inc., Chicago, IL, USA), which typically failed. *T*-tests were performed using Mann–Whitney rank sum when environmental parameters did not have normal distributions and medians, rather than means and deviations, then reported. In addition, for the MBTS, which had a consistent set of environmental metadata, PRIMER-E v6 (PRIMER-E Ltd., Plymouth Marine Laboratory, Plymouth, UK) was used to investigate the relationship between gene copies per ml and physico-chemical data. An environmental matrix was assembled that contained the following variables: salinity, temperature, Chl *a*,  $\text{NO}_3$ ,  $\text{NO}_2$  and  $\text{PO}_4$  concentrations, and  $\text{NO}_3$  to  $\text{PO}_4$  ratios. The environmental matrix was normalized by subtracting the mean of each variable from every value and dividing by the s.d. Sea surface temperature climatology for 2009 (as in Figure 3) was determined using the daily 9 km blended microwave and infrared optimum interpolated sea surface temperature from remote sensing systems.

#### Ostreococcus growth curve and field growth estimate

OTH95 (axenic) and RCC809 (not axenic) were grown in K-media made with artificial (<http://www.mbari.org/phyto-genome/Resources.html>) or Sargasso seawater bases, respectively, at  $21.5^\circ\text{C}$  on a 14:10 light:dark cycle. Triplicate OTH95 cultures were grown in semi-continuous batch mode and monitored daily by fluorometry for each light-level investigated. Cells were acclimated to light levels, maintained for 10 generations of acclimated mid-exponential growth, and rates then calculated from four successive transfers (after the 10 generations). A previously described Sargasso Sea dilution experiment (Cuvelier *et al.*, 2010) was also further analyzed using flow cytometry (FCM) and qPCR results from herein. Average growth rate of all non-prymnesiophyte phytoplankton in the 70 m dilution experiment, including OII cells, was estimated as:

$$\mu_{\text{nonprymphyto}} = \ln[(\text{Aph}_{24} - \text{Apr}_{24})/(\text{Aph}_0 - \text{Apr}_0)]$$

abbreviations as follows: Aph, FCM abundance of photosynthetic eukaryotes; Apr, FISH abundance of pico-prymnesiophytes (from Cuvelier *et al.*, 2010);

subscripts indicate time point and values are for the 20% raw seawater treatment (80% filtered seawater), representing a conservative growth estimate (see Worden and Binder, 2003), or the Y-intercept of a regression (FCM-based only).

## Results and discussion

### Rationale, development and verification of qPCR primer-probe sets

*Ostreococcus* abundance in Sargasso Sea samples determined by TSA–FISH were not above the nonspecific negative control probe (~30 cells ml<sup>-1</sup>). Surprisingly, in one such sample, 32 of 36 18S rDNA sequences recovered from known photosynthetic taxa belonged to Clade OII and qPCR showed 2514 ± 401 genome copies per ml using genus-level *Ostreococcus* primers (Zhu et al., 2005; calculated using a standard curve based on genome copies and assuming 100% extraction efficiency for the genome copy-based standard curve). Anomalously low *Ostreococcus* counts by TSA–FISH have been seen in another open-ocean study (Jardillier personal communication). Small cell size or other factors may make TSA–FISH enumeration more challenging for *Ostreococcus* in certain samples than for other taxa. Inability to confidently enumerate *Ostreococcus* by TSA–FISH in our open-ocean samples and desire to enumerate the clades independently prompted us to design, test and implement Clade OI- and OII-specific qPCR primer-probe sets.

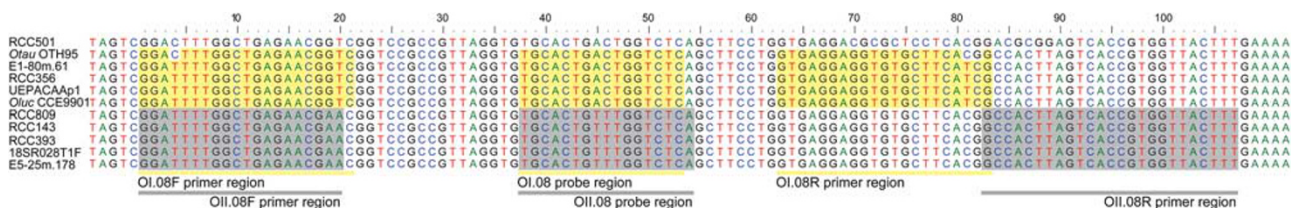
To verify specificity, primer-probe sets were tested against several species and strains (Figure 1, Table 2, Supplementary Table 1). No non-target amplification was detected using either *Ostreococcus* primer-probe set (Table 3). The *Micromonas* and *Bathycoccus* primer-probe sets were used for a subset of inhibition tests only. The former showed no cross-reactivity with non-target standards, while a low level of non-linear cross-reactivity by the latter was detected at C<sub>T</sub> values of 38–39 against an OI standard curve (Supplementary Tables 2 and 3). Given the high plasmid copy number (10<sup>6</sup>–10<sup>8</sup> per rxn) where *Ostreococcus* amplification was detected with the *Bathycoccus* primer-probe set, and the dramatically higher amplification efficiency

(several orders of magnitude) against its target taxon versus *Ostreococcus*, this primer-probe set should still be *Bathycoccus*-specific in environmental samples. Eukaryotic picophytoplankton concentrations (and hence gene copies per ml) at these plasmid levels (10<sup>6</sup>–10<sup>8</sup>) are rarely reported (see, for example, Li, 1994; Worden and Not, 2008; Cuvelier et al., 2010).

With regard to published phylogenetic studies the OII primer-probe set amplifies cultured strains formerly considered ‘deep-adapted’, all of which belong to ITS Clade B (Rodriguez et al., 2005), designated Clade OII based on 18S rDNA analyses (Worden and Not, 2008; Worden et al., 2009). The OI primer-probe set amplifies a combination of ITS/18S Clade A (for example, clone UEPACAAp1, and many other environmental clones) and Clade C (for example, *O. tauri*), both of which are considered to be more high-light adapted (Rodriguez et al., 2005). *Ostreococcus* RCC789 (the clonal version of RCC501), which belongs to ITS/18S Clade D was not amplified by either primer-probe set because of mismatches (Figure 1, Table 3). We scanned for similar sequences in GenBank and found that, apart from RCC501, isolated from Barcelona Harbor, Spain, only three other sequences representing this clade have been deposited (≥99–100% 18S rDNA identity to each other; 97–98% identity to *O. tauri* and *O. lucimarinus*). All are from estuarine or brackish settings, specifically the Baltic Sea (FN690726, Gulf of Finland and FN263267, Gulf of Gdansk, Poland) and Lake Pontchartrain (FJ350825, LA, USA). Five unpublished Roscoff Culture Collection (RCC) cultures from estuarine/lagoon or bay settings also appear to belong to Clade D (<http://www.sb-roscoff.fr/Phyto/RCC/>), suggesting this clade generally resides in brackish, not coastal or open-ocean, environments. Clade C also appears to be lagoonal based on isolation locations. Given the environmental samples investigated here, the primer-probe sets are likely amplifying cells from OI belonging to ITS/18S Clade A and OII (ITS/18S Clade B).

### Characteristics of the sample set

The environments investigated (Table 1) represent a range of euphotic zone conditions. Environmental



**Figure 1** Regions of the 18S rRNA gene targeted by the *Ostreococcus* qPCR primer-probe sets. Three sequences (highlighted in grey) are from *Ostreococcus* Clade OII that were characterized as deep-adapted in Rodriguez et al. (2005), as well as two environmental clones. Five sequences are shown for Clade OI (highlighted in yellow), composed of *O. tauri* and the four other sequences, two from cultures and two from environmental samples (including sequences from two of the clades identified in Rodriguez et al., 2005 as being more high-light adapted). RCC501 represents the third putatively high-light adapted clade and is not targeted by either primer-probe set. Where highlighted sequences have perfect identity to respective primers and probes.

**Table 3** Verification of *Ostreococcus* clade-specific primer-probe sets against target and non-target DNA samples

Organism source material					Primer-probe set			
Class	Genus	Strain	Clade	DNA	OI.08		OII.08	
					Detect.	Mismatch	Detect.	Mismatch
Prasinophytæ	<i>Ostreococcus</i>	OTH95	OI	C, P <sub>STD</sub>	Y	1, 0, 2	UD	3, 2, 0
Prasinophytæ	<i>Ostreococcus</i>	UEPACAAp1	OI	EP <sub>STD</sub>	Y	0, 0, 0	ND	2, 2, 0
Prasinophytæ	<i>Ostreococcus</i>	RCC809	OII	C, P <sub>STD</sub>	UD	2, 2, 2	Y	0, 0, 0
Prasinophytæ	<i>Ostreococcus</i>	RCC789	ITS D	C	UD	1, 0, 7	UD	3, 3, 5
Prasinophytæ	<i>Bathycoccus</i>	BBAN 7	NA	C, P <sub>STD</sub>	UD	7, 16, 5	UD	6, 6, 13
Prasinophytæ	<i>Micromonas</i>	RCC472	MII	C	UD	6, 4, 19	UD	5, 4, 6
Prasinophytæ	<i>Micromonas</i>	RCC451	MI	C	UD	6, 4, 19	UD	5, 4, 6
Prasinophytæ	<i>Micromonas</i>	UEPACOp3	MIV	EP	ND	6, 4, 22	ND	4, 4, 7
Prasinophytæ	<i>Micromonas</i>	CCMP1545	MV	C	UD	5, 2, 20	UD	5, 4, 7
Prasinophytæ	<i>Micromonas</i>	CCMP1646	MIII	C, P <sub>STD</sub>	UD	4, 2, 19	UD	4, 4, 6
Prasinophytæ	<i>Micromonas</i>	RCC299	MII	C	UD	6, 4, 19	UD	5, 4, 6
Cryptophyceae	<i>Rhodomonas</i>	CCMP1319	NA	C	UD	ND	UD	ND
Bolidophyceae	<i>Bolidomonas</i>	CCMP1866	NA	C	UD	ND	UD	ND
Prymnesiophyceae	<i>Isochrysis</i>	CCMP1323	NA	C	UD	ND	UD	ND
Dictyochophyceae	<i>Rhizochromulina</i>	CCMP2174	NA	C	UD	ND	UD	ND
Pelagophyceae	<i>Pelagomonas</i>	CCMP1756	NA	C	UD	ND	UD	ND

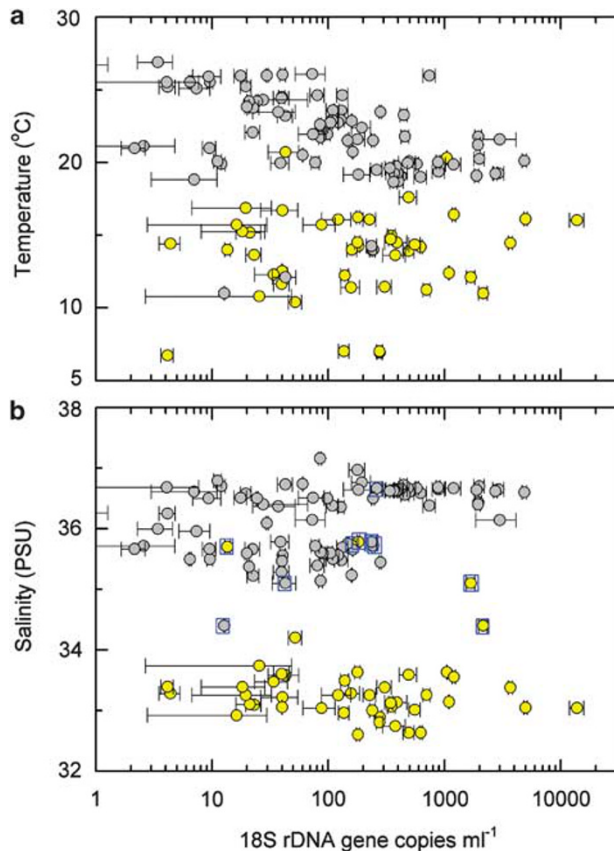
Abbreviations: C, culture DNA; Detect., indicates whether the primer-probe set rendered a detectable signal; EP, environmentally derived plasmid; EP<sub>STD</sub>, environmentally derived plasmid standard curve; Mismatch, provides the number of mismatches between the forward primer, probe and reverse primer to the source material indicated to the left; NA, not applicable; ND, not determined; P<sub>STD</sub>, plasmid standard curve (10<sup>1</sup> to 10<sup>6</sup>); UD, undetected (C<sub>T</sub> > 40, or no value detected); Y, yes detected; RCC472 is the same as CCMP492, RCC451 is the same as CCMP1764.

and other data have been published elsewhere for most cruises (Cuvelier *et al.*, 2008, 2010; Treusch *et al.*, 2009; Moisander *et al.*, 2010; Santoro *et al.*, 2010). Briefly, all Atlantic cruise samples were from relatively oligotrophic waters (see, for example, Steinberg *et al.*, 2001; Lomas *et al.*, 2010), with the exception of some stations on transit to the Sargasso Sea, which passed through continental shelf and slope waters, before crossing into the oligotrophic Gulf Stream and beyond. EN351, OC374 and EN360 traversed these settings (Table 1). The former two cruises also sampled stations north of BATS and BATS, while EN360 sampled more western Sargasso Sea sites. BATS and Sargasso Sea stations north of BATS undergo winter deep mixing (Steinberg *et al.*, 2001; Treusch *et al.*, 2009; Lomas *et al.*, 2010). The BATS euphotic zone was well mixed during OC374 (down to ~120 m with no apparent deep chlorophyll maximum (DCM); 5 March), with stratification developing during EN351 (DCM apparent at 60 m, 31 March) and OC413 (DCM developing at ~100 m, May–June). Sargasso sites were stratified during EN360 (DCM at 95 m; 22 September). The South Pacific transect was also oligotrophic (Moisander *et al.*, 2010) as were samples from the North Pacific Gyre (Station ALOHA). The eastern North Pacific transects (CN107 and CN207) originated in Monterey Bay and encompassed more varied conditions than other Pacific cruises, with eight stations spread over 800 km, ranging from coastal, to mesotrophic and relatively oligotrophic waters. Finally, 2007 MBTS samples were analyzed separately from the global set (see below).

#### Relationship of *Ostreococcus* Clades OI and OII to environmental parameters

Most environmental parameters were strongly delineated within the global sample set between those samples containing Clade OI and those containing Clade OII (Supplementary Table 4). Of the 271 global samples evaluated, 64 contained OI and 99 contained OII. OI was detected largely in colder, lower salinity coastal waters while OII was detected in gyre-like conditions with relatively warm waters and high salinities (Figures 2 and 3). The mean temperatures (Figure 2a) and s.d. for the two groups were significantly different, 14 ± 3 °C versus 22 ± 3 °C, for OI and OII, respectively. Salinity (Figure 2b) and depth were also significantly different ( $P < 0.001$ ), with median values of 33 and 36 PSU (Practical Salinity Unit), and 30 and 75 m for OI and OII, respectively. Fewer measurements were available for NO<sub>3</sub>, PO<sub>4</sub> and Chl *a* and data availability was uneven for oligotrophic versus coastal and mesotrophic sites. Still, NO<sub>3</sub> (Supplementary Figure 1) was significantly different ( $P < 0.02$ ) for samples containing OI (median 0.980 µM,  $n = 41$ ) versus OII (median 0.545 µM,  $n = 18$ ), as was PO<sub>4</sub> ( $P < 0.001$ ) for OI (median 0.686 µM,  $n = 41$ ) versus OII (median 0.002 µM,  $n = 23$ ) containing samples. Chl *a* concentrations at sites with OI were also higher than those with OII, with median values of 0.74 µg l<sup>-1</sup> ( $n = 35$ ) and 0.31 µg l<sup>-1</sup> ( $n = 30$ ), respectively.

In oligotrophic waters, that is, subtropical and tropical Atlantic, Gulf Stream, South Pacific and ALOHA, seawater temperatures were significantly higher where *Ostreococcus* was undetected



**Figure 2** Environmental parameters (a) temperature and (b) salinity as a function of *Ostreococcus* Clade OI (yellow) and Clade OII (grey) rDNA copies per ml. Only data considered quantifiable are shown. In (a), the three yellow symbols above 17 °C are from the Scripps Pier, which can warm significantly during day time hours at ~0.5 m where the samples were collected, while grey symbols below 15 °C are from OC374 and EN351 continental slope samples. In (b), data points from OC374 and EN351 slope samples are indicated by blue boxes, although, for some, only one clade was found at the particular depth; for data from all other samples shown only one clade was quantifiable. Error bars represent the s.d. of averaged technical replicates, where not visible they are within the symbol.

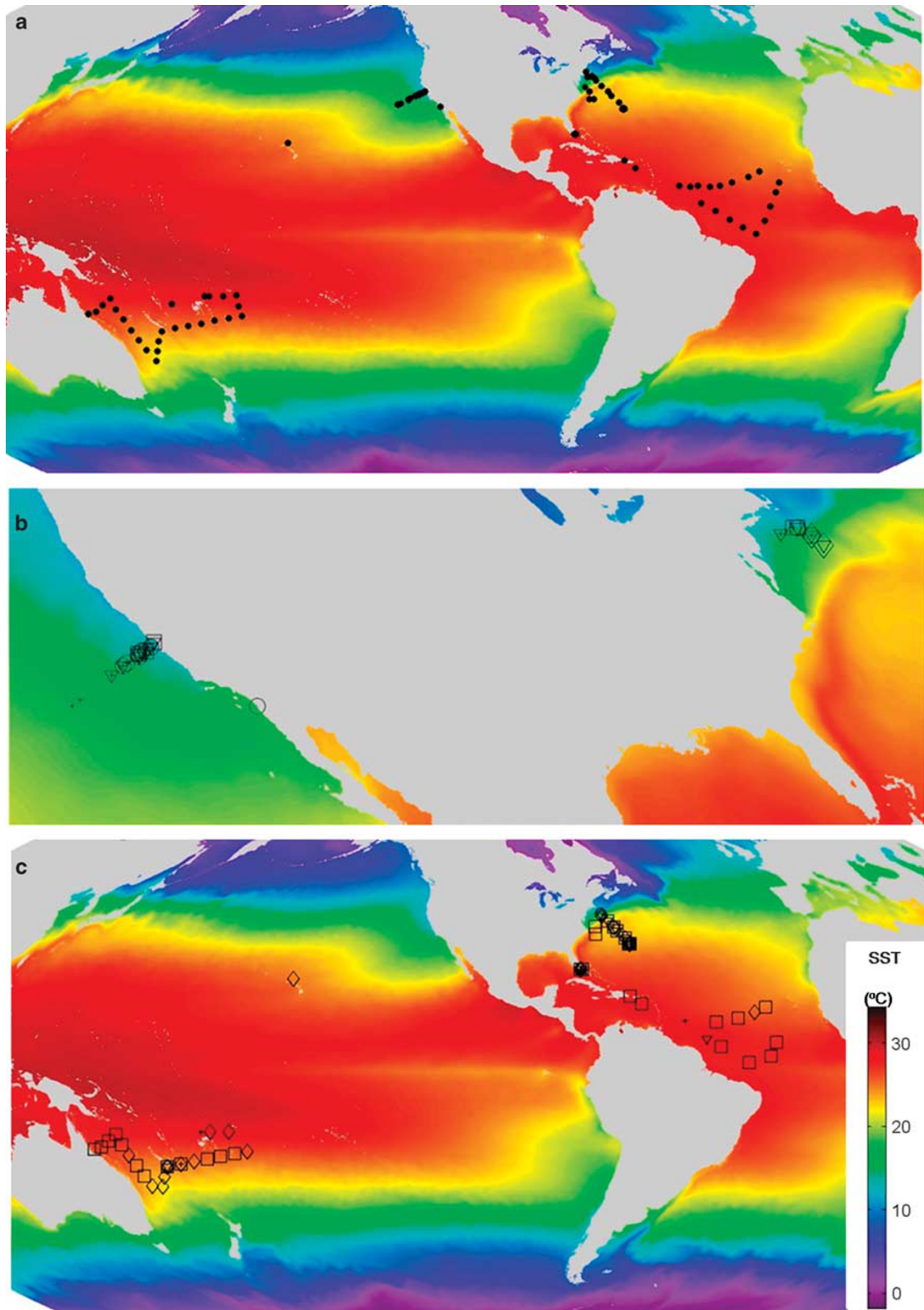
( $26 \pm 3$  °C) than where detected (only OII at these sites;  $22 \pm 3$  °C). The median sample depth for oligotrophic samples (from 200 m and up), was also significantly different ( $P < 0.001$ ) for samples with no *Ostreococcus* detected (6 m,  $n = 88$ ) versus OII containing samples (83 m,  $n = 85$ ). Nitrate was significantly lower ( $P < 0.001$ ), with median concentrations of 0.004 and 0.545  $\mu\text{M}$  and  $\text{PO}_4$  lower (0.000 versus 0.002  $\mu\text{M}$ , but not significantly different), where *Ostreococcus* was not detected versus samples containing OII, respectively. The latter two analyses compare measurements generated by different methodologies, however, in the case of  $\text{NO}_3$  only one sample was below detection using the less sensitive method and hence comparisons should be valid. Finally, Chl *a* concentrations were significantly ( $P < 0.02$ ) lower for samples with no *Ostreococcus* detected (median 0.17  $\mu\text{g l}^{-1}$ ,  $n = 44$ ) versus those containing OII (median 0.31  $\mu\text{g l}^{-1}$ ,  $n = 30$ ).

The MBTS covers a productive coastal/bay area (Monterey Bay) and these samples were analyzed separately to avoid weighting global results with data from a single region and year. OI, and only OI, was detected in 27 of 29 samples spanning February to December (Supplementary Figure 2). Average temperature for OI containing samples, or even those with  $\geq 1000$  rDNA copies per ml, was within the overall mean ( $12 \pm 1$  °C). MBTS environmental metadata was measured using a consistent set of protocols and a Relate test (Clarke, 1993) was performed to correlate qPCR data with the entire environmental matrix (depth, temperature,  $\text{NO}_3$ ,  $\text{NO}_2$ ,  $\text{PO}_4$ , Chl *a* and salinity) and each variable independently. A Euclidean distance matrix of environmental data was also compared with a Bray–Curtis dissimilarity matrix of gene copies per ml for each sample. However, there was no significant correlation for either the entire environmental matrix or the individual variables, nor did Euclidean distance provide significant results. For example, although the mean  $\text{NO}_3$  concentration was lower ( $9.3 \pm 5.7 \mu\text{M}$ ) for samples with  $\geq 1000$  rDNA copies per ml than for samples with fewer or none ( $13.6 \pm 7.6 \mu\text{M}$ ), the differences were insignificant. Nitrate ranged from 4.0 to 26.3  $\mu\text{M}$  and was 19.7  $\mu\text{M}$  and 24.0  $\mu\text{M}$  for the two samples in which OI was undetected. Furthermore, no statistical differences were detected between  $\text{NO}_3$ ,  $\text{PO}_4$ , Chl *a* data from samples containing  $\geq 100$ , 1000 or 5000 copies per ml versus those containing fewer than each of these abundance categories. Only differences presumably related to other aspects of seasonal variability (based on date of collection) seemed apparent—with generally lower OI rDNA copies per ml from April to August than other months (Supplementary Figure 2).

#### Co-occurrence and overall trends

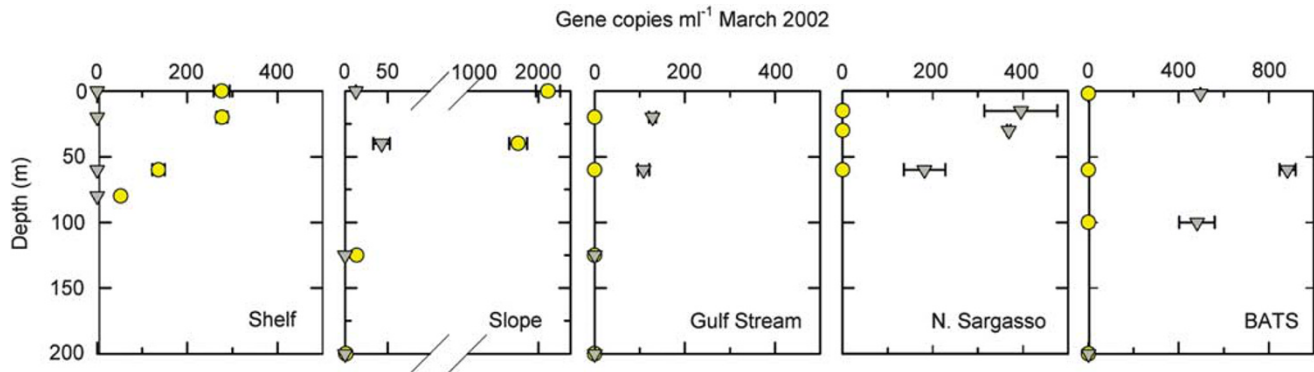
The two *Ostreococcus* clades were co-localized in continental slope samples from transects from coastal New England, USA to the Sargasso Sea. Specifically, OI and OII were quantifiable in the same slope water samples in early March (OC374, Figure 4) and late March/early April (EN351, Supplementary Figure 3a). Notably, a differential in abundance related to depth was not apparent and co-localization may have resulted from physical mixing of different water masses. In a September transect, OI was close to the detection limit at the Narragansett Pier and in low numbers in shelf waters, but not found elsewhere, while OII was detected in the Gulf Stream DCM and one of two Sargasso Sea Stations (EN360, Supplementary Figure 3b).

These data indicate that factors beyond light, such as temperature, salinity, nutrient availability or related variables, have a role in clade dynamics. With respect to depth, OI was detected as deep as 173 m ( $160 \pm 16$  copies per ml) at a slope station (Supplementary Figure 3a) with maximum abundance at 40 m at the base of the MBTS



**Figure 3** Distribution of *Ostreococcus* Clades OI and OII. (a) Global sample sites. (b) Sites at which Clade OI 18S rDNA copies per ml were quantitated (open symbols) or detected but not quantifiable (plus sign) and (c) the same for Clade OII. Depth ranges are 0–6 m (open circle), 10–59 m (open triangle), 60–99 m (open square) and  $\geq 100$  m (open diamond). Several measurements can be contained within a single depth range, but are represented by a single symbol for simplicity. As OI were not detected outside of the geographical range shown in (b) a high-resolution zoom to this area is shown. Note that Clade OI members have been detected in clone libraries in coastal European regions, for example, near Roscoff, FR.





**Figure 4** 18S rDNA copies per ml of *Ostreococcus* Clade OI (yellow) and Clade OII (grey) along a transect from the coastal Northwest Atlantic to BATS for all samples evaluated. Similar trends in Clade distribution were seen in EN351 in March–April 2001, although in EN360 in September 2001 few *Ostreococcus* were detected (Supplementary Figure 3). Error bars represent the s.d. of averaged technical replicates, where not visible they are within the symbol.

Station M2 mixed layer (Supplementary Figure 2). Across the global data set, the median depth for OI was 30 m, both for locations where it was detected and for samples with  $\geq 1000$  OI rDNA copies per ml. Notable OII counts (for example,  $2914 \pm 316$  copies per ml, March 2000, 40 m) were detected close to the surface at BATS. The influence of irradiance was not explored directly because sampling was performed throughout the day–night period and hence irradiance data were not comparable. However, even using depth as an irradiance proxy, establishing it as a driver for distributions was confounded by the fact that nutrient concentrations increased with depth (except under deep-mixing conditions) and could also serve as a driver. Parameters such as  $\text{NO}_3$  were significantly different among those samples containing one versus the other clade or open-ocean samples in which OII was present versus not present. Furthermore, OII was detected at relatively high abundances in BATS surface samples in March 2002 (Figure 4) and April 2003 ( $385 \pm 19$  rDNA copies per ml, 4 m). In Northern Sargasso Sea Stations, equivalent concentrations were found at 15 and 30 m in early March 2002 ( $33^\circ 13' 31''$  N,  $64^\circ 57' 8''$  W) with  $395 \pm 81$  and  $369 \pm 4$  rDNA copies per ml, respectively and at 15 and 150 m in March/April 2001 ( $33^\circ 14' 7''$  N,  $64^\circ 53' 19''$  W) with  $402 \pm 40$  and  $365 \pm 25$  rDNA copies per ml, respectively. Nutrient samples were not taken at these sites, however, the euphotic zone was well mixed during the sampling period. In addition to similar cell concentrations at the surface and DCM, temperature, salinity and *in vivo* fluorescence (and presumably nutrients by proxy) were more similar through the euphotic zone than in periods after stratification set in. Thus, nutrient availability appears to have a role in the vertical distributions of OII through the euphotic zone.

From a broader geographical perspective, the two clades appear to differentiate along lines of coastal versus oligotrophic water masses (Figure 3). Samples from mesotrophic locations close to the Pacific

coast, near-shore samples and the Scripps Institution of Oceanography Pier contained OI, as did Atlantic Narragansett Pier, Shelf and Slope samples. In contrast, apart from Atlantic slope sites, OII was not detected at these locations but present in the North Pacific Gyre, the South Pacific Ocean, the North Atlantic Gyre, subtropics and tropics. Similarly, only OII was detected in the Florida Straits, which are physically close to land but composed of oligotrophic Gulf Stream forming waters.

#### Correspondence between clone library and qPCR results

Evaluation of clone libraries and published sequences (for example, Worden, 2006; Viprey *et al.*, 2008; Worden and Not, 2008) support the qPCR findings (Figure 3). First, when the qPCR primer-probe sets developed here were applied to environmental samples from which *Ostreococcus* sequences were recovered, 18S rDNA clade sequence affiliations were consistent with clades detected by qPCR. In the Florida Straits and Sargasso, only OII 18S rDNA sequences were recovered (for example, clone FS\_St01Mar05\_5mB060T1F from 5 m station 01, 30 March 2005 has 100% identity to RCC143 over 707 positions). At the Scripps Pier (Worden, 2006) and in the eastern North Pacific (CN207), only OI 18S rDNA sequences were recovered (for example, clone CN207H3\_5m, 5 m station H3, 10 October 2007 has 99% identity to *O. lucimarinus* over 1765 positions) and only this clade was detected by qPCR. The high representation of deposited OI environmental sequences and strains likely indicates a sampling bias towards coastal settings, which are investigated more frequently. For example, the high-light adapted representative *O. tauri* was isolated from a shallow lagoon (Six *et al.*, 2008) and *O. lucimarinus* from  $\sim 0.5$  m in coastal California waters (Worden *et al.*, 2004). From a broader perspective, in addition to the proposal that the ‘deep-adapted’ clade (OII) are not

strictly low-light adapted but rather better adapted to life in open-ocean conditions, including periods of time in deep euphotic-zone waters (Worden and Not, 2008), a Mediterranean Sea clone library study (Viprey *et al.*, 2008) suggested that *Ostreococcus* Clade A (within OI herein) prefers surface waters influenced by low temperature, low salinity Atlantic waters, whereas Clade B (OII) was present throughout euphotic zone waters including surface waters, for example, 5 and 15 m. Clade OII (but not OI) sequences have also been recovered from the Indian Ocean (Not *et al.*, 2008).

#### Light intensity and clade distributions

In our study, Clade OII was relatively abundant in Sargasso Sea surface waters. Without further research, factors such as physical mixing, potentially displacing OII to the surface, even if not growing there, or the possibility of differently adapted ecotypes within the 18S OII rDNA grouping cannot be ruled out as contributing factors. Still, the discrepancy between environmental results and culture-based inferences are not surprising given the light intensities explored in the laboratory studies.

In oceanic systems light levels of  $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , where either the onset of photo-inhibition (OI representatives) or lack of growth (OII representatives) has been observed for cultures (Rodriguez *et al.*, 2005), are probably rarely encountered. Irradiance quickly decreases with depth. We recorded 21 and 6% (Photosynthetically active radiation) relative to levels in 'air' (detector out of the water) at 2 m ( $590 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) and 15 m ( $175 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ), respectively, on a sunny day in oligotrophic waters ( $33^\circ 10' 16'' \text{ N}$ ,  $129^\circ 15' 25'' \text{ W}$ ). At 76 and 93 m, PAR was 12 and  $5 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  or 0.4 and 0.2% of surface (air) irradiance, respectively. Although PAR detector calibration could have a role (air measurement  $2863 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , a relatively high measurement) the percentages should reflect light attenuation. Thus, the same percentages applied to what is typically considered full sun irradiance ( $2000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) render PAR of  $412 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  at 2 m,  $122 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  at 15 m,  $8 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  at 76 m,  $2 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  at 106 m (the 0.1% irradiance level). The published  $\mu_{\text{max}}$  values for RCC141 and RCC143, belonging to OII, appear to occur between 85 and  $180 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  and photoinhibition was only apparent at  $\geq 400 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  under the experimental conditions used. Here, we observed a higher  $\mu_{\text{max}}$  for *O. tauri* (1.6–1.8 per day), occurring between 150 and  $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , and growth rates as high as 0.3 per day at  $4 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  (Supplementary Figure 4). These values are higher than those published previously for *O. tauri* or even those for OII representatives at similarly low irradiances (Rodriguez *et al.*, 2005). However, the results

cannot be compared directly because of potential differences in culturing conditions, for example, a different K-media seawater base. Moreover, RCC809 (representing OII) growth rates were not attained because we could not maintain this strain in acclimated, mid-exponential growth at any light level (because of its 'crashing', despite daily attention), which we felt necessary for establishing the relationship between irradiance and growth rate (as done for the *O. tauri* measurements). Still, these comparisons, and reflection on natural irradiance levels, emphasize difficulties with relating culture-based experiments to field conditions. Only the highest irradiances encountered in the field, occurring above  $\sim 10$  m seem similar to photoinhibition levels seen for cultures. Furthermore, water column stability influences the extent and duration that cells are exposed to high surface irradiance. Given that such exposure may be rare in a well-stratified column, and of limited duration in less well-stratified waters, it seems unlikely that high culture irradiances relate directly to natural conditions.

These observations do not negate reported photo-physiology-based differences. For example, maximal growth rates were reported to be  $\sim 1.1$  per day at  $180 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  (OTH95, RCC356 and RCC420), or for RCC501, at  $400 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ . In contrast, OII isolates grew at  $0.2 \pm \sim 0.2$  per day to  $\sim 0.6$  per day at  $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for RCC143 and RCC141, respectively, with maximal growth rates at  $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  ( $\sim 0.65$  per day) and  $180 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  ( $\sim 0.9$  per day), respectively (Rodriguez *et al.*, 2005). Six *et al.* (2008) saw significant differences between the growth optima of a clonal version of RCC141 (that is, RCC809) compared with the original strain used by Rodriguez *et al.* (2005), and showed that below  $15 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  both RCC809 and OTH95 had growth rates of  $\sim 0.4$  per day. More recently, differences in photo-protection capacity (greater in *O. tauri*, representing OI) and light-harvesting capacity (greater in RCC809, representing OII), which correspond with conditions at the strain isolation site (Cardol *et al.*, 2008) have been reported. Responses to high-light exposure indicate these two strains differ in terms of recovery from photoinactivation (Six *et al.*, 2009). The extent to which these differences extend to other isolates, or natural populations, and how they respond under optimal growth conditions (for example, temperature) for each respective isolate, requires further investigation. Experiments show that *O. tauri* and RCC809 have the most disparate responses and photosystem characteristics, with *O. lucimarinus* (Clade A/OI) often appearing to form a more intermediate response (Six *et al.*, 2008, 2009). Differences in *O. tauri*, relative to *O. lucimarinus* and RCC809 may also reflect its unique isolation environment (a shallow lagoon).

Results from a single dilution experiment rendered a picoeukaryote growth rate of 0.5 per day at 70 m in the Sargasso Sea. This regression-based rate

represents the average for all small eukaryotic phytoplankton by FCM. The average minimum growth rate for all non-prymnesiophyte eukaryotic phytoplankton, with OII included, was 0.4 per day based on the 20% treatment (see also Cuvelier *et al.*, 2010). OII sequences were the most abundant in an associated clone library, having 100% identity to RCC143 (32 of 36 clones from photosynthetic taxa; also for 15 m clone library, e.g. OC413\_NSSJun05\_15mQ004T1F), although Clade MI/MII *Micromonas*, *Bathycoccus*, a likely photosynthetic stramenopile (96% identity to *Pinguiochrysis pyriformis*, AB058926) were also present as were sequences likely from non-photosynthetic taxa (for example, novel alveolates). qPCR showed high OII abundance at T<sub>0</sub> (4864 ± 224 rDNA copies per ml; 6441 FCM eukaryotic phytoplankton cells per ml, with FISH prymnesiophytes excluded). Depending on how OII copy numbers relate to cellular abundance and assuming 100% recovery during extraction, either 38% (2 copies per genome, the more likely number) or 76% (1 copy per genome) of the eukaryotic phytoplankton belonged to OII, a significant portion of the picophytoeukaryote community present during this experiment.

## Conclusions

Ecotypic differentiation based on photophysiology and other characteristics has been demonstrated for the cyanobacterium *Prochlorococcus* (Moore *et al.*, 1998, 2002; Moore and Chisholm, 1999). The offset between irradiances where cultured *Prochlorococcus* strains grow or are inhibited appears to be greater over a narrower range of irradiances (than for *Ostreococcus*). Furthermore, these irradiances (used in *Prochlorococcus* studies) are more similar to those at different depths *in situ* and *Prochlorococcus* niche partitioning occurs along depth gradients at the same geographic location. Clear shifts can be seen from dominance by high-light adapted ecotypes in surface waters to low-light adapted ecotypes at depth although some clades tolerate a greater irradiance range than others (Johnson *et al.*, 2006; Zinser *et al.*, 2007).

These observations for *Prochlorococcus* are quite different than those for *Ostreococcus* here. Although photophysiology may have a role in allowing OII to survive in low-irradiance waters, it seems unlikely that it is the primary environmental driver for *Ostreococcus* clade distributions. One caveat is that our sampling was not exhaustive. For example, we had no open-ocean 'winter' samples (for example, January, Sargasso Sea). If OI and OII thrive together at this time, or partition the euphotic zone vertically to strata where one dominates the other, we would not have detected this due to the composition of our global sample set.

In light of our current knowledge, it seems premature to classify the clades as either depth-specialists or generalists. Comparative genomic

analyses and physiology studies should facilitate identification of specific niche defining factors. Based on this study, we conclude that *Ostreococcus* Clade OI thrives in cooler, more nutrient rich waters found in more coastal environments, including the base of the mixed layer in such settings. In contrast, Clade OII thrives in warmer, higher salinity open-ocean settings and its success in such environments may involve the capacity to grow at deeper nutrient depths in gyre euphotic zones.

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## Author contributions

JPZ and AZW orchestrated cruises. ED, MLC, JPZ and AZW performed cruise sampling. ED, MLC, SS, JPZ and AZW performed extractions/provided DNA. MLC performed *O. tauri* physiology studies, FISH, initial qPCR and the dilution experiment. ED designed, tested and implemented *Ostreococcus* primer-probe sets, as well as *Micromonas* and *Bathycoccus* primer-probe sets. MLC and AZW constructed clone libraries. ED compiled qPCR data. ED, SS and AZW compiled environmental data. AZW performed statistical analyses and global data synthesis. ED synthesized MBTS data. SS further verified *Micromonas* and *Bathycoccus* primer-probe and performed a subset of inhibition analysis. CLG processed and integrated sea surface temperature data, created projections and mapped *Ostreococcus* data. AZW wrote the paper and ED provided written contributions. SS and JPZ provided edits. All authors read and commented on the paper.

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