Synechococcus in the Black Sea – an alternative explanation of the deep red fluorescence signal

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Introduction

The Black Sea is known for its peculiar vertical structure: a strong stratification prevents deep-water ventilation, leading to complete absence of oxygen and abundance of hydrogen sulphide and ammonia in its deep layers. The oxycline is quite shallow (~100 m) and coincides approximately with the halocline and winter convection depth. Consequently, most biological activity, light and oxygen dependent, is confined to the first 100 m from the surface, while the deeper layers constitute an unfavorable environment for photo-autotrophic microorganisms . Since 2013 several Bio Argo floats were deployed in the Black Sea, providing real-time profiles to a depth of 1000 m for several biogeochemical variables. The floats consistently measured a chlorophyll a (Chl a) concentration increase between 100 to 1000 m depth, not visible in the corresponding profiles for the Mediterranean Sea and the Atlantic Ocean (Fig. 1). The profiles showed consistently the same pattern over the whole basin.

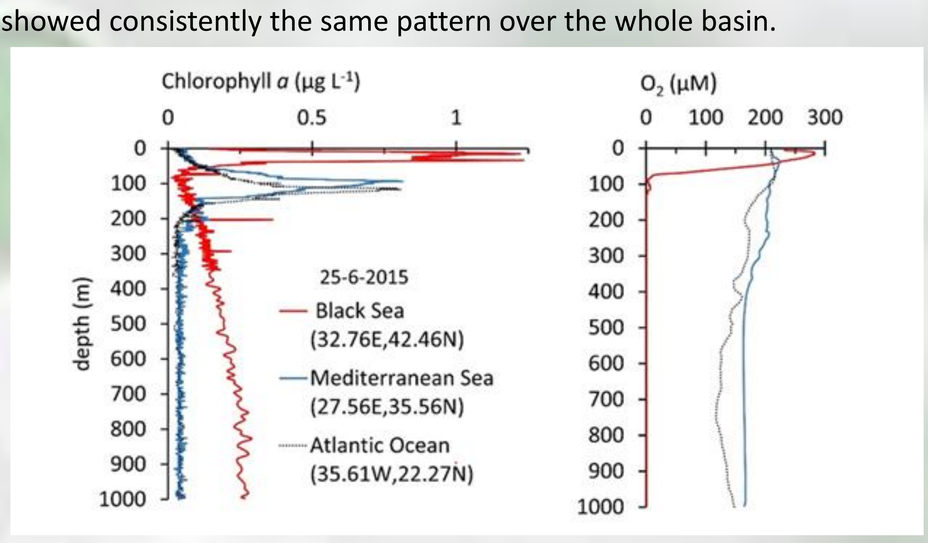


Fig. 1. Chlorophyll *a* (upper left) and dissolved oxygen (upper right) concentration profiles measured by three Argo floats (model PROVOR CTS-4 NUT). Dates and locations of the profiles are written in the legend.

These unusual values confirm previous observations (Broenkow et al., 1992; Anderson, 1982), and have been observed especially in subtropical areas of the Pacific and Atlantic Oceans, and in the Arabian and Black Seas (Broenkow et al., 1983; Röttgers and Koch, 2012). Studies of this absorption shoulder, termed "deep red fluorescence", have described it as a feature of the global ocean and associated it with the oxygen minimum zone. Since its detection, this signal challenged scientists: how to understand a consistent and widespread increase in Chl *a* in deep dark waters where photosynthesis is precluded? Answering this question through the study of fluorescent spectra, the hypothesis was formulated that "deep red fluorescence" could be attributed to the presence of a specific chromophore/fluorophore of pigment origin, possibly derived from autotrophic or heterotrophic microorganisms (Röttgers and Koch, 2012; Zhao *et al.,* 2017). Associating the signal with high CDOM and non-algal matter concentration, though, lead some authors to treat non-zero Chl *a* values at depth as an artefact caused by CDOM presence and to propose techniques for correcting fluorometric chlorophyll measures at depth (Organelli *et al.,* 2017; Xing *et al.,* 2017). This interpretation of "deep red fluorescence" is grounded in the widespread understanding of deep, dark, anoxic waters as a prohibitive environment for photoautotrophs. However, prior studies suggesting that this signal could derive from picocyanobacteria (Röttgers and Koch, 2012; Zhao *et al.,* 2017) lead us to consider and explore the presence of *Synechococcus* in these deep waters.

Data

✓ BGC Argo float data

The Chl *a* profiles were measured by Bio-Argo floats (model PROVORCTS-4 NUT, equipped with miniature and low power sensors: Aanderaa oxygen optodes 4330 and WETLabs ECO-Triplet fluorimeters) delivered to three marine sites: 7900591 in the Black Sea; 6901472 in the North Atlantic Ocean; 69001770 in the Mediterranean Sea.

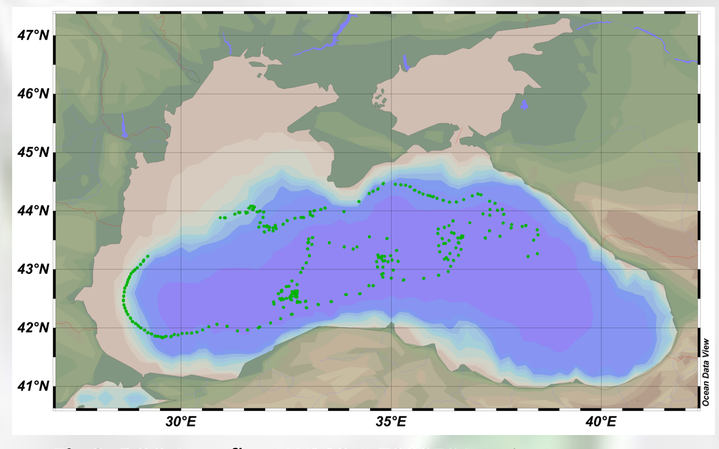
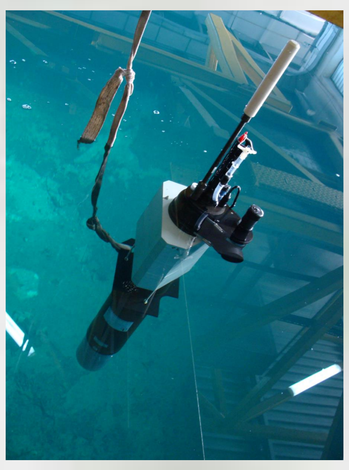


Fig 2. BGC Argo float WMO # 7900591 trajectory



✓ Shipboard sampling

Seawater samples for chemical analysis and *Synechococcus* counting were collected at st. 307 (43°10'N-29°00'E) of the Black Sea in 2015 and 2016 at selected depths from 0 to 1150m using a 12-Go Flo bottle CTD rosette sampler system. Temperature and salinity profiles were obtained from SBE-911 CTD system.

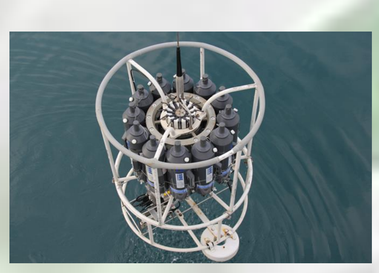
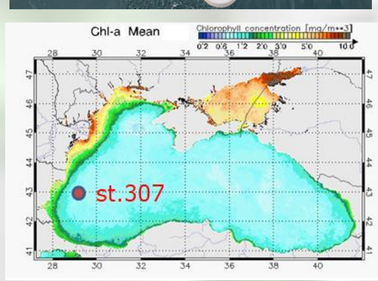


Table 1. Details of sampling and measurements collected at st.307 in the Black Sea during R/V Akademik cruises in 2015 and 2016

Period	Water Depths	Water sampling/ measurements
25.06.2015	Thermocline, DCM, 250, 500, 750, 1050 m	T(°C), S‰, NH_4, NO_2, NO_3, PO_4, NO_2, O_2 (µM), H_2S (µM)
28.07.2015	Thermocline, DCM, 250, 500, 750, 1050 m	T(°C), S‰, NH_4, NO_2, NO_3, PO_4, NO_2, O_2 (µM), H_2S (µM)
22.06.2016	Thermocline, DCM, 250, 500, 750, 1050 m	T(°C), S‰, NH_4, NO_2, NO_3, PO_4, NO_2, O_2 (µM), H_2S (µM)



Methods

- **Synechococcus sp. strain isolation:** *Synechococcus* sp. strains BS55D and BS56D were isolated on 25 June and 28 July 2015 from deep layers (750 and 1000 m). As a result, we successfully obtained monoclonal cultures from 750 m at both dates.
- **Sequencing, assembly and annotation of BS55D and 163 BS56D:** DNA of pelleted strains was isolated with a Qiagen MagAttract Kit, genomic 550-bp libraries were constructed with a KAPA Hyper Prep Kit and sequenced on an Illumina MiSeq instrument, using a 500-cycle MiSeq Reagent v2 Kit.
- **Phylogenomics, synteny plots and PBS comparison of BS55D and BD56D:** A maximum-likelihood phylogenetic tree with 259 universal markers and *Synechococcus* representatives from marine, brackish, euryhaline and freshwater habitats together with the novel strains was generated with PhyloPhlAn tool.
- **Laboratory experiment:** The two strains BS55D and BS56D were genetically very similar therefore only one (BS56D) of them was used to test survival and Chl *a* production under anoxic and dark conditions. In a 48-days of experiment, a total of 7 time points were selected for two treatments: the anoxic/dark and the oxic/light.
- ***Synechococcus* spp. enumeration -***Synechococcus* spp. from the field and during the laboratory experiment were counted with a Flow Cytometer. All samples were additionally checked at the epifluorescence microscope.
- **PhytoPAM Chl *a* and photosynthetic activity measurement :** During the laboratory experiment Chl *a* was measured by a Pulse-Amplitude-Modulation Phytoplankton Analyzer. The µg Chl *a* ml^{-1} was measured at each time point of the experiment, using *Synechococcus* absorbance spectra and specific Chl *a* calibration as reference.

Results and Discussion

Field measurements and *Synechococcus* spp. enumeration and isolation

The Bio-Argo data profiles show an increase of Chl a between 100 to 1000 m depth, reaching values of around 0.2- 0.3 µg L-1, which were not visible in similar profiles obtained in the Mediterranean Sea nor the Atlantic Ocean (Fig. 1). The profiles of Synechococcus cell abundance in the three dates indicated the presence of 103 cells ml-1 of phycoerythrin-rich picocyanobacteria of the genus Synechococcus from around 200 m to 1000 m, with the highest number (358 x 103 cells-1 ml-1) found at DCM. The observation at the microscope confirmed the presence of Synechococcus cells with high autofluorescence at depth (Fig.3) and with a mean cell size 291 of 0.60±0.03×0.38±0.02µm at 20m, 0.80±0.09×0.69 ±0.01µm at 750m.

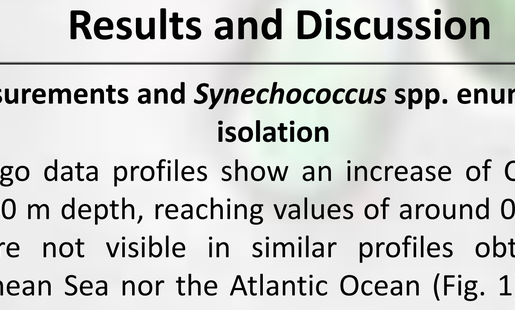


Fig. 3. *Synechococcus* spp. abundance profiles in the western gyre of the Black Sea (St. 307: 43°10'N-29°00'E) performed during two cruises in 2015 and compared with a new profile obtained in summer 2016. On the right: examples of epifluorescence microscopy visualisation of Synechococcus spp. at three depths.

Experiment and Chl a production in anoxic dark conditions

During the 48-day laboratory incubation, the strain BS56D had a positive growth rate (k=0.011 d^{-1}) in the oxic/light conditions and a negative growth rate (k=−0.014 d^{-1}) in anoxic/dark conditions (Fig. 4a). During the first week, the cells survived quite well also in the anoxic/dark conditions, even if not growing in number, and after 13 days they decreased by 23% from the initial number but they presented high cellular Chl a concentrations (pg Chl a $cell^{-1}$) reaching 15 pg Chl a $cell^{-1}$ and maintaining this concentration from day 13 to day 22 (Fig. 4b). Conversely, in oxic/light conditions, the cell number increased, and indicated photosynthetic activity of the PSII, lacking in the anoxic/dark treatment (Fv/Fm were 0.35 in oxic/light compared with 0.0 value for the anoxic/dark after 13 days of cultivation) (Fig. 4c).

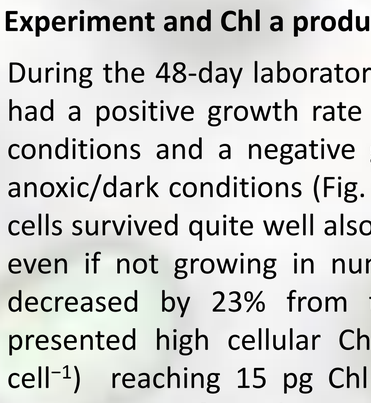


Fig 4. Experiment of *Synechococcus* BS56D growth in oxic/light and anoxic/dark conditions during 48 days. A) Number of *Synechococcus* (10^3 cells ml^{-1}), B) Chlorophyll *a* cell content (pg Chl *a* $cell^{-1}$), C) Photosynthetic activity of PSII (Fv/Fm). Statistical analysis (t-test) has shown significant differences between the two treatment (A: p=0.0103, B: p=0.0245, C: p=0.0018).

Genomic features of BS55D and BS56D

The genomes of BS55D and BS56D (novel strains isolated from 750m at 330 St. 307) have a total size of 2303823 and 2235215bp and a GC content of 61.23 and 61.61, for BS56D and BS55D, respectively.

In order to know to which clade to ascribe the novel strains, we performed a maximum-likelihood phylogenetic tree (Fig. 5) of 239 conserved genes comprising representatives from the marine sub-clusters 5.1 A/B, 5.2, 5.3 and freshwater *Synechococcus*. We used 9 genomes of *Prochlorococcus* to root the phylogeny. As could be expected and due to its marine origin, BS55D and BS56D strains fell inside the 5.1 sub-cluster close to the clades VIII/IX comprising Red Sea strains RS9916 and RS9917.

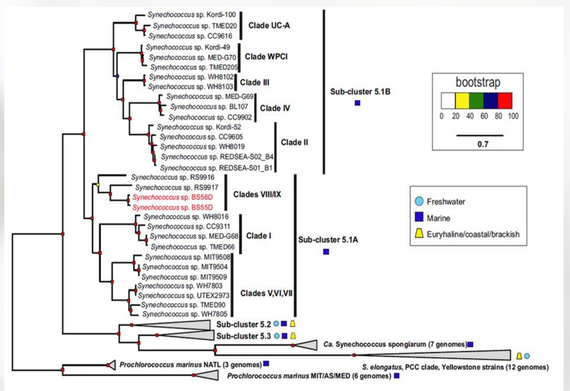


Fig. 5. Maximum-likelihood phylogenomic tree with representatives from marine, brackish, euryhaline and freshwater habitats together with the novel strains.

BS55D and BS56D strains contain the novel pigment type IIB reported in some freshwater strains . In addition, a total of six phycocyanin subunits were found, four of them inside the PBS operon and two more phycocyanins elsewhere in the genome. The presence of such amount of phycocyanin subunits has been reported in the green pigmented picocyanobacteria exposed to more light, but never in phycoerythrin-containing cells.

Conclusion

Results demonstrate the occurrence of "deep red fluorescence" in the meso- and bathypelagic waters of the Black Sea. They also confirm the presence of *Synechococcus* spp. in coincidence with this Chl *a* signal, and validate the ability of strain BS56D to survive in anoxic/dark deep waters. In addition, they show the accumulation of Chl *a* by strain BS56D under these environmental conditions. As such, they corroborate the hypothesized relation between the Chl *a* increase at depth and the presence of *Synechococcus* spp. in the mesopelagic and bathypelagic realm of the Black Sea, up to now considered unsuitable for the survival of photoautotrophic cells (Zaitsev, 2008), while also calling for more research on this process.

Acknowledgements

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