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The First Marine Filamentous Fungi Discovered in the Bottom Sediments of the Oxic/Anoxic Interface and in the Bathyal Zone of the Black Sea

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Abstract

This is the first information about the filamentous fungi of the deep soft sediments at the transition zone from oxic to anoxic conditions in the Bosporus outlet area of the Black Sea (117.4, 149.9, 252.4, 262.7 and 295.7m) and maximal depth (2250m) of the Black Sea. At direct microscopy of the deep-water bottom sediments the filamentous mycobiota, belonging to Division. Ascomycota were found. The results indicate to the presence of so far unknown species of mycobiota in the bottom sediments of the oxic/anoxic interface and maximal depths of the Black Sea. The morphologic difference of mycelium indicates to species diversity of fungi in the extreme benthic conditions. Large accumulations of mycelium in the bottom sediments in Black sea deep-water zone is the evidence of fungi habitation in the hydrogen sulfide zone. By the present time this information is based on the individual studies and brings more questions than answers. However, the mycelium was found at several stations near Bosporus region of the Black Sea, which proves the regularities of the phenomenon. Further studies of this region's bottom sediments are needed to yield more information about the fungi of the soft sediments in the transitional oxic/anoxic water masses and deep water of the Black Sea.

Keywords: Filamentous fungi, hydrogen sulfide zone, deep-sea sediments, Black Sea.

Introduction

Traditionally, marine fungi, living in the seas, are divided into two groups: obligate or facultative. Obligate fungi are able to grow and reproduce exclusively in marine and estuarine habitats (Kohlmeyer and Kohlmeyer, 1979; Buchalo, 2003). In 1999, at the VII International Symposium on marine and freshwater Mycology in Hong Kong it was proposed to use as the main criterion in determining of the marine fungi their ability to grow and generate mycelium in natural marine conditions (Buchalo, 2003).

In the Black Sea 444 species of fungi that were referred to 7 divisions, 16 classes, 34 orders, 62 families, 168 genera exist in the marine environment. Micromycetes are presented by two ecological groups: 121 species of *obligate water fungi* (51 species of lower fungi, and 70 species of filamentous fungi (higher), 323 species of *facultative water micromycetes* (terrestrial fungi, which are able to function in marine water) (Dudka and Kopytina, 2011).

In the coastal waters of Ukraine, 340 species of fungi were found; this number is 109 in Romanian waters, 61 in Russian, 12 in Turkish and 34 in Bulgarian waters. In the sea 72 species are isolated, 45 of them–in water and bottom sediments of the hydrogen sulfide area (Artemchuk, 1981; Dudka and Kopytina, 2011; Zaitsev *et al.*, 2010).

The marine sediments are the substratum with the greatest species diversity of fungi (Kohlmeyer and Kohlmeyer, 1979; Pivkin *et al.*, 2006).

Study of the filamentous fungi in the bottom sediments of the Black Sea, especially in the hydrogen sulfide zone is insufficient. By the present time this is the first information of significant accumulation of mycelium of fungi in the soft sediments of the deep sea, so it will certainly be interesting to specialists-mycologists, hydrobiologists, biochemists, physiologists, and others. We believe that it is important to pay close attention of scientists to the fact that bottom sediments of the oxic/anoxic interface and the maximal depth of the Black Sea are inhabited by filamentous fungi.

Previous Studies

The first studies on fungi of the Black Sea (at an example of yeast-like forms) at different horizons of the water masses from the surface to a maximum depth were conducted in 1946 - 1955 (Kriss *et al.*, 1951, 1952; Kriss and Novozhilova, 1954). In the pelagic of the hydrogen sulfide zone 10 species of yeasts were isolated. The author managed to find budding yeasts even at maximum depths, where the concentration of hydrogen sulfide is very high (Kriss *et al.*, 1951). Later were established the viability of the yeasts and the preservation of the physiological functions in water hydrogen sulfide zone of the Black sea (Kriss and Novozhilova, 1954).

On the basis of studies of the pelagic zone of the Black sea to a depth of 2000m grown of 8 species of fungi were identified in the permanent hydrogen sulfide zone (Meyers *et al.*, 1967). The authors suggested that micromycetes may come into the hydrogen sulfide zone from the upper layers of water and remain viable, but do not develop.

In general, in 40-60s of the last century 21 species of fungi (including 5 filamentous) were isolated from the hydrogen sulfide zone water and conclusion was made about the relatively low density of propagules of fungi in the sea waters, as well as about decrease of species diversity and density of micromycetes with increase of depth and distance from the coast (Kriss and Novozhilova, 1954; Meyers *et al.*, 1967).

The first information about mycobiota of soft bottom sediments in the hydrogen sulfide zone of Black Sea appeared in 1999-2000 (Sergeeva and Zaika, 1999). Two forms of the fungi “A” and “B”, which the authors have previously included to Class Krassilnikoviae were found after the study of the bottom sediments from a depth of 1800 and 2250m by direct microscopy. Later they were assigned to the genus *Aspergillus* (Zaitsev and Kopytina, 2008). Together with the form «B» at a depth of 2250m accumulations of mycelium of fungi of uncertain systematic position was found.

In 2005, 31 species of higher fungi were discovered, 22 of which were isolated in laboratory conditions in the bottom sediments of the hydrogen sulfide bathyal; other species spores were detected by direct microscopy (Zaitsev *et al.*, 2007, 2008; Zaitsev and Polikarpov, 2008). Overview of micological research of the Black Sea hydrogen sulfide zone is included in the work of Kopytina and Zaitsev (2011).

Material and Methods

Hypoxic meiobenthic communities of the Istanbul Strait’s (Bosporus) outlet area of the Black Sea were studied along the depths gradient (80-300m) at the cruise R/V ‘Maria S. Merian’ (Germany) during 13^{th} - 17^{th} April 2010. Eighteen meiobenthic stations were chosen along this transect, ranging from the oxic to the anoxic zone (Figure 1).

For benthos studies we obtained virtually undisturbed bottom sediments at the stations in the range of depths 80-300 m by TV-multi-corer Ø=9.5 cm or gravity corer Ø=7 cm. Benthic stations were chosen along this transect, ranging from the oxic to the anoxic zone. On the other hand, our data suggest that some benthic eukaryotes can tolerate anoxic and sulfidic conditions.

Sediment cores, obtained by the multiple-corer,

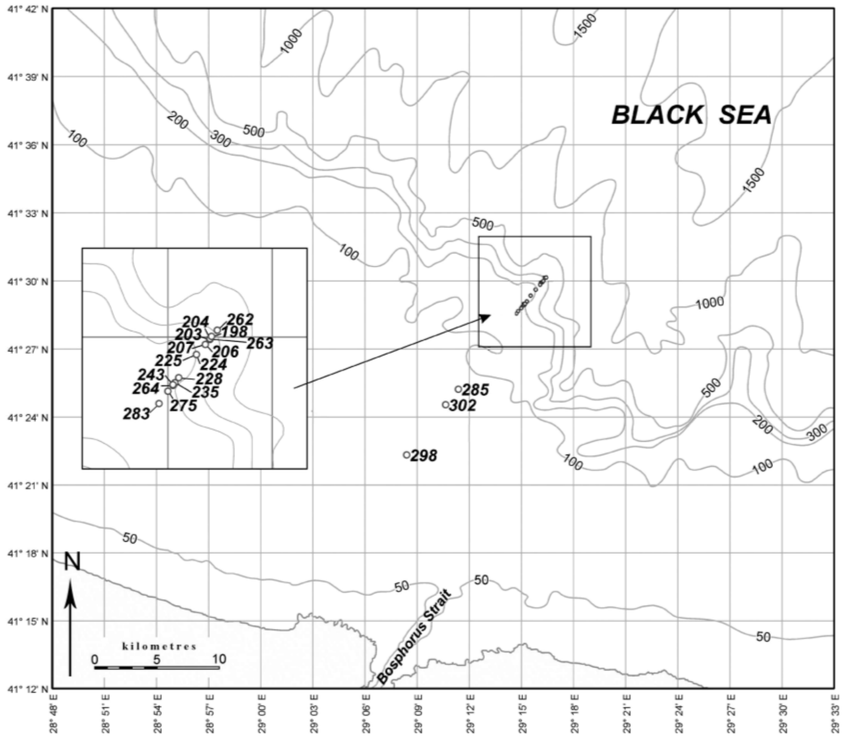

Figure 1. Meiobenthic stations in the Istanbul Strait’s (Bosporus) outlet area of the Black Sea

were sectioned at 0–1, 1–2, 2–3, 3–4 and 4–5cm intervals and all samples immediately preserved in 75% ethanol.

We preserved sediments samples in ethanol, which is known to preserve morphological structures of fauna without distortion. We avoided prior fixation in formalin in order not to damage calcareous taxa. The sediments were washed by distilled water through sieves with a mesh size of 1 mm and 63 μm. The fraction retained on the sieves was stained in Rose Bengal solution before being sorted in water under a binocular microscope for ‘live’ (stained) organisms which were identified to higher taxa. We extracted only those specimens that were stained intensely with Rose Bengal. Density of zoobenthos was calculated per m^2 of the bottom area, on the bases of number of benthic animals (macro- and meiobenthos) in column of the corers. We studied the fungi in detail with 100-1000x magnification, using Light microscope Olympus CX41 with digital camera and connected with PC.

In the Eastern part of the Black Sea at a depth of 2,250 m (St. 3314, 42°50' 0 N – 38°09' 7 E) collection of sediment was fulfilled with bottom sampler "Ocean-25" during the cruise no.23a R/V ‘Professor Vodyanitsky’ 01 July 1987. For the study of meiobenthos five samples were cut with the tube (18 cm^2 area) from the taken bottom sediment monolith and all of them were immediately fixed with 4% formalin separately. The bottom sediment station was represented by well-structured silt with a faint smell of hydrogen sulfide.

The method of the microscope analysis of samples obtained from a depth of 2250 m, was similar.

Results

During the study of meiobenthos near Bosporus region district, filamentous fungi were detected in five of eighteen stations (Table. 1). Fungi were registered in three areas: oxic (depth 117.4 m), suboxic (149.9 m) and hydrogen sulfide (252.4, 262.7 and 295.7 m) zones. At depth of 117.4 m, alevritic silt biotope of fungi was found with a large admixture of the dead shells of mollusks and foraminifera; at 149.9 m – in alevritic-pelitic silt–the surface layer is oxidized and deeper black recovered layer lies. The depths of 252.4, 262.7 and 295.7 m are characterized by biotopes of liquid black silt with a sharp smell of hydrogen sulfide. It is noteworthy that at a depth of 262.7 m at one station we revealed in the bottom sediment only numerous accumulations of mycelium, but benthic fauna was absent. At the other station hyphae of fungi were registered among enormous density of zoobenthic organisms (Table. 1).

In general, with increasing depth, a reduction of taxonomic diversity of zoobenthos takes place, but reduction of the abundance of benthos is not observed. Moreover, at the same depth (252.4 m), its population at two stations differed by three orders of magnitude (Table 1). We can assume that zoobenthos and filamentous fungi in the bottom sediments at this area of the Black Sea tend to have an accidental (aggregated) distribution. The environmental factors that cause inequality in distribution of fungi in general, and individual species or meiobenthos are not clearly known.

At the all stations pointed in horizons 0-1 and 1-2 cm septate mycelium, which formed dense aggregations of round shape has been revealed. In some aggregations substrates looking like remnants of the benthic fauna and flora (fragments of heavily transformed shells of bivalve molluscs) and empty shells of diatoms (*Coscinodiscus*) have been found.

As an example, the mycelium detected in the bottom sediments of oxygen zone (117.4 m), suboxic layer (to 149.9 m), below the upper limit (252.4 m) of the hydrogen sulfide zone and maximum depth (2250 m) of the sea (Figure 2, Figure 5) has been shown.

Fungi were found in two samples from five at the maximum sea depth (2250 m). They were clusters in the form of a bound septate floccus, which significantly differed from the considered above mycelium fungi.

In the future collection, material in the form of constant glycerin-gelatin preparations will permit to conduct a comparative analysis of mycelium from different areas and depths and hyphen (aggregates), detected in April 2010 in the samples of bottom sediments of the oxic/anoxic interface of the Istanbul Strait's (Bosporus) outlet area in the Black Sea during the cruise of the RV ‘Maria S. Merian’ (Germany).

Table 1. Stations and depths in the Bosporus Strait’s outlet area of the Black Sea, where the filamentous fungi were found (in the 0-5cm layers of corers of the bottom sediment). Fungi are not included in the abundance

Station	Depth, m	Description of bottom sediment	Taxonomic richness	Mean abundance of zoobenthos, ind. $*m^{-2}$
198	262.7	liquid black silt, H_2S	1(fungi)	0
203	252.4	liquid black silt, H_2S	4	1040
204	252.4	liquid black silt, H_2S	8	1.518660
262	295.7	liquid black silt, H_2S	7	18103.7
264	149.9	oxidized silt with recovered layers	16	584970.7
283	117.4	dense silt with the shells of dead mollusks and foraminifera	20	297145.1

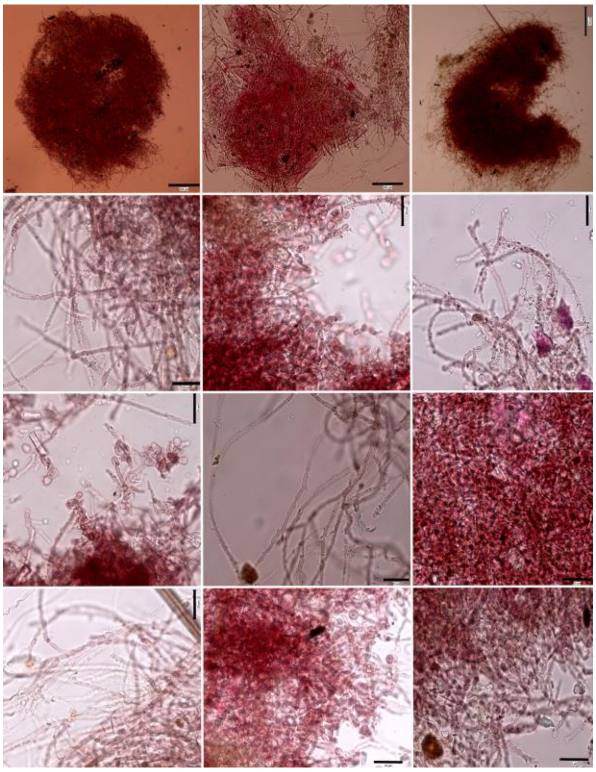

Figure 2. Fungi in the oxic conditions at the depth 117.4 m (Black Sea). In the top row accumulation (aggregates) mycelium, on the left and on the right scale bars = 200 µм; in centre =50 µм; in the rest of the ranks of the different aggregates of fungi fragments - scale bars = 20 µм.

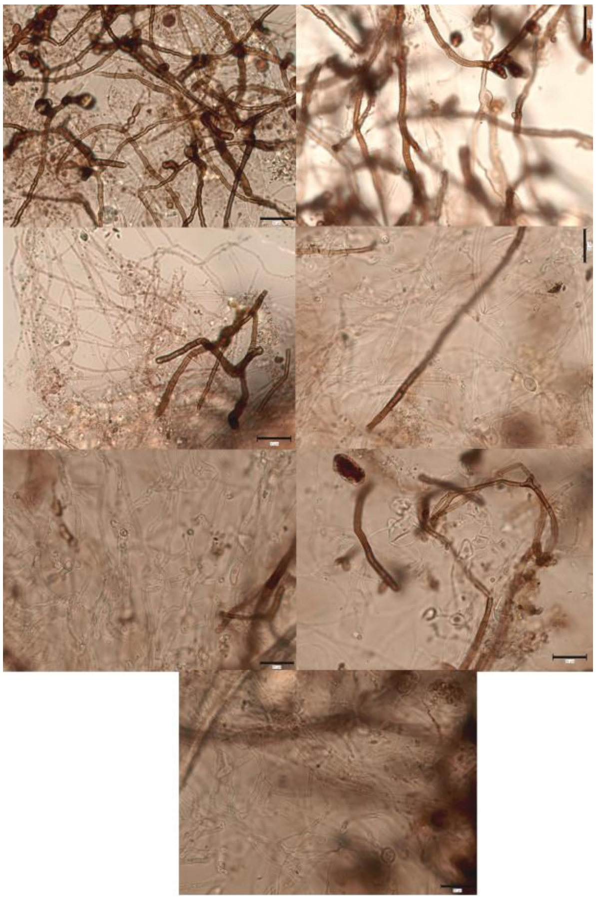

Figure 3. Fungi in the suboxic conditions at the depth 149.9 m (Black Sea). Scale bars = 20 µм.

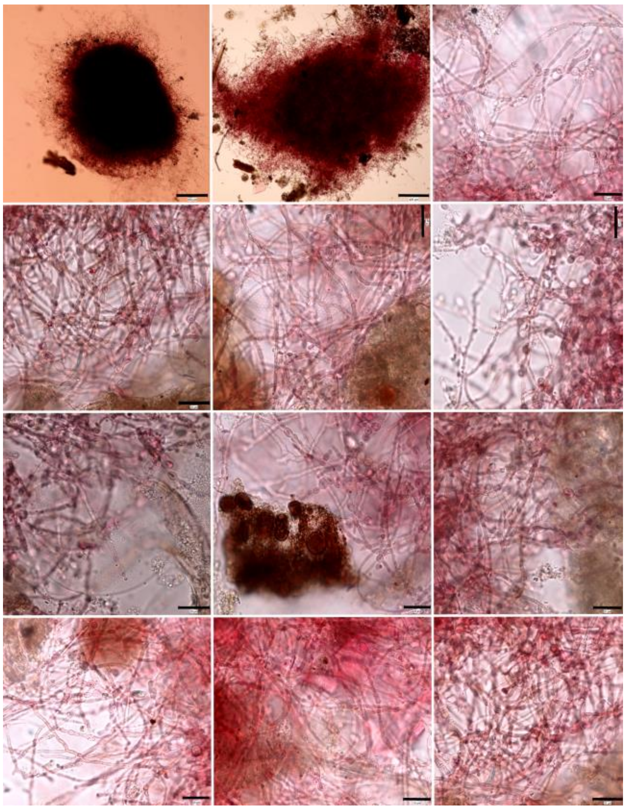

Figure 4. Fungi in the hydrogen sulfide conditions at the depth 252.4 m (Black Sea).
In the top row - two pictures on the left accumulation (aggregates) mycelium, scale bars = 200 μм (on the left) and 100 μм (on the centre); in the rest of the. ranks of the fragments of fungi from the different aggregates - scale bars = 20 μм.

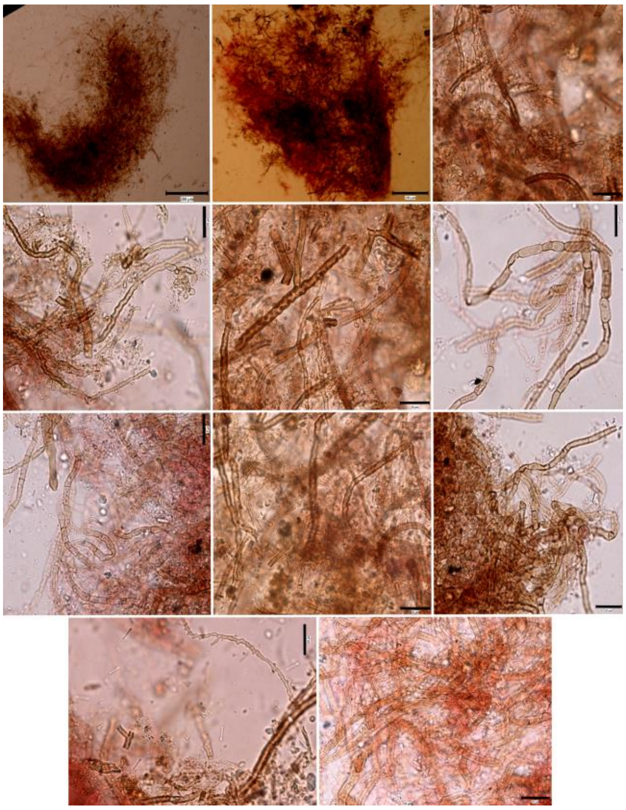

Figure 5. Fungi in the bathyal at the depth 2250 m (Black Sea). In the top row (left and centre) accumulation (aggregates) mycelium - scale bars = 200 μм; in the rest of the ranks of the fragments of fungi from the different aggregates, scale bars = 20 μм.

Discussion

Opinions of mycologists regarding the function of fungi in the hydrogen sulfide zone of the Black sea were different. Kriss with co-authors (Kriss *et al.*, 1952; Kriss, 1959) believes that constantly low water temperature (about 8°C), increased salinity, high pressure (up to 210 bar, or 210 bar), anaerobic conditions in the presence of hydrogen sulfide are not limiting factors of the environment for yeast fungi. They experimentally proved that most of yeast organisms are adapted to live in marine environments.

Others (Meyers *et al.*, 1967; Zaitsev *et al.*, 2007, 2008) believe that the micromycetes come from the upper layers of the water in the hydrogen sulfide zone and here in the anoxybiosis state (anaerobiosis) remain viable, but do not grow in it. Rationale: the main indicator of the viability of microorganisms in the deep waters of the sea is their growth under high hydrostatic pressure and low temperatures (Raghukumar and Raghukumar, 1998). For the Black Sea lack of oxygen and presence of hydrogen sulfide with high concentrations must be added to this. All the authors conducted experimental works other than the above conditions. Meyers *et al.* (1967) cultivated fungi at a temperature of 6-9°C (1967). Spores, which were studied by the method of microscopy, were found in physiologically inactive phase of organisms (Zaitsev *et al.*, 2007).

Evidence of the functioning of fungi. Yeasts extracted from the hydrogen sulfide zone clearly expressed denitrifying ability (reduction of nitrate to nitrite) (Kriss and Novozhilova, 1954). The yeasts and filamentous fungi also were extracted from the deep-sea sediments of the Arabian Sea (5000 m) and were grown in a lack of oxygen and they participated in the process of denitrification. In these samples, hyphae of fungi were found with the help of direct microscopy which proved directly functioning of fungi (Cathrine and Raghukumar, 2009). Many species of fungi belong to the group of facultative anaerobic microorganisms, which are able to use oxygen from the damaged material under anoxic conditions. Fungus mycelium can remain in a viable state for 1 month in anoxic soils reserving 10 - 30 % of total stock. The number of microscopic fungi, obtained from soil under anoxic conditions on the anaerobic incubation of the cultures, makes from tens to hundreds of CFU (colony-forming units) in 1 g of soil, with a species richness about 30 species (Kurakov *et al.*, 2008).

It is known that hyphal cells of terrestrial fungi, trapped in a deep-water zone of the sea, in the first period may blow up or be violated in their construction under the influence of high hydrostatic pressure and low temperatures. It has been revealed that abnormalities in cell morphology were caused mostly by temperature but not pressure (Damare *et al.*, 2006).

When comparing photo of mycelium of the Black Sea sample, revealed by direct microscopy of sediment, and this provided by the work of Damare *et al.* (2006), some similarity in the structure of the hyphae can be seen (Figure 6A, B),

The hypothesis concerning deep-water fungi of the Black Sea must be considered with great caution. If we accept that mycelium cells of the fungi swelled when they got into the deep-sea area, then due to unknown reason deformation of the cells occurred almost simultaneously in all species of fungi found in oxygen and hydrogen sulfide conditions. How can it be explained?

It would be possible to assume, that the micromycetes got into the deep-water conditions quite recently and are in the process of adaptation to the increasing pressure and low temperature. But this phenomenon can hardly occur in reality.

Unquestionably for fungi, different periods of time would be needed during which they can come to the studied depths from the upper horizons of the sea. In addition, we have not found any mycelium at the other thirteen stations. Such swelling of mycelium at the maximum depth is not observed, so is it possible in this case to assume that this type of process of adaptation has been already completed?

Fungi have managed to adapt to deep-sea

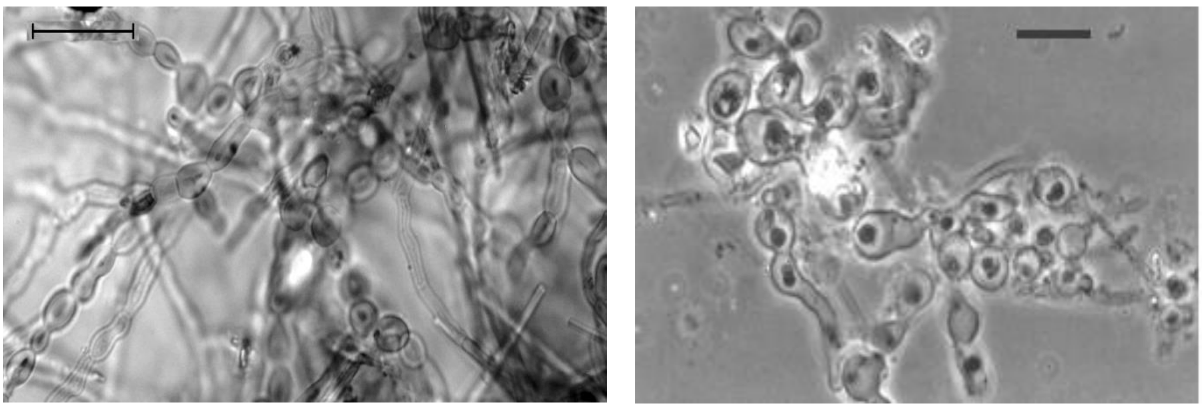

Figure 6. A - native accumulation of hyphae at 252.4 m (pressure about 25 bars). Scale bars = 20 µм. B- hyphae *Aspergillus terreus*, № A 4634, grown at 30 and 5°C and pressure 200 bars. Scale bars = 10 µм (by Damare *et al.*, 2006).

conditions, they grow and develop. It is known that representatives of the genus *Aspergillus* physiologically are very versatile and successfully colonize various substrates on land and in the sea (Damare *et al.*, 2006). But one can not exclude the possibility that these hyphae may belong to the obligatory sea micromycetes not yet known to science.

Anaerobes (from the Greek *an* - negative particle, *aer* - air and *bios* - life) are organisms that can live and develop under absence of free oxygen, for example, some types of bacteria, yeast, protozoa, worms. Obligate, or strict anaerobes, develop only in the absence of oxygen (for example, *Clostridium*) and the facultative, or conditional, anaerobes are able to develop in presence of oxygen (for example, *Escherichia coli*). Anaerobic organisms are common in soil, water and sediments (http://dic.academic.ru/dic.nsf/enc1p/5022). The Black Sea is no exception, there are eukaryotic organisms living under anoxia and hydrogen sulfide contaminated conditions (Sergeeva *et al.*, 2011a, 2011b).

Earlier, in the bottom sediments of the Black Sea at depths of 1990 and 2140 m specimens of Cladoceran crustaceans were found; and one of the caught specimens was alive. Caught adult and juvenile individuals, as well as developing juveniles in eggs, permitted to conduct a detailed study of these crustaceans and a new genus and species of Cladocera was described as *Pseudopenilia bathyalis* by Sergeeva (2003, 2004). Afterwards, family Pseudopenilidae, new for science, was established by Korovchinsky and Sergeeva (2008).

During the cruise 'M Merian', direct observations of deep-water Protozoa and Metazoa living under hypoxia/anoxia conditions in the Black Sea have been carried out *in situ* at the depths of 250 and 300m and it was found that they actively moved (Sergeeva *et al.*, 2011b).

In the North-Western sector of the Black Sea from the 730m, deep bottom sediments cultures of 7 species of terrestrial filamentous fungi from ground layers 0.0-1.5 cm and 1.5-5.0 cm, were received. The analysis of radionuclides distribution in the thickness of bottom sediments allowed determination of the rate of sedimentation, which made 1–2 mm year^{-1} in the studied region. This means that the age of the top-1.5 cm layer of the considered sediment is in the range from 7.5 to 15.0 years, the underlying horizon 1.5 - 5.0cm - maximum 50 years (Zaitsev and Polikarpov, 2008). This proves that life in the hydrogen sulfide zone can be either allochthonous or autochthonous, getting from the upper layers of the water or even from the land. Both versions of the mycobiota origin in the permanent hydrogen sulfide conditions have the right to exist.

Detection of organisms from different taxonomic groups in the horizons of water and bottom sediments, adjacent to the upper limit of hydrogen sulfide, and in the very hydrogen sulfide zone (Kriss and Novozhilova, 1954; Meyers *et al.*, 1967; Sergeeva, 2002, 2003, 2004, Sergeeva *et al.*, 2011a, 2013; Sergeeva and Zaika, 1999; Sergeeva and Zaika, 2013; Zaika and Gulin, 2011; Zaika and Sergeeva, 2012; Zaitsev *et al.*, 2007, 2008) attracted a lot of attention of scientists.

In the Black Sea the scale of reductive hydrogen sulfide zone is extremely large, covering a large part of its water column - pelagic (about 87% by volume) and benthic (region of board of the Black Sea depression - 40% and the bottom of the Black Sea depression - abyssal plain - 35%), that makes it unique among all the seas and oceans. In this case, the word «extreme» means «utterly going out of limits» (Polikarpov, 2012).

Conclusion

The results indicate to the presence of so far unknown species of mycobiota in the bottom sediments of the oxic/anoxic interface and maximal depths of the Black Sea. It is difficult to identify micromycetes according to hyphae to a certain systematic status, but the morphologic difference of mycelium indicates to species diversity of fungi in the extreme benthic conditions. Large accumulations of mycelium in the bottom sediments of the Black Sea deep-water zone are the evidence of fungi habitation in the hydrogen sulfide zone.

This information is based, by the present time, on the individual studies and brings more questions than answers. However, the mycelium was found at several stations near Bosporus region of the Black Sea with the range of depths 117.4–295.7 m, which proves the regularities of the phenomenon.

Method of processing of the samples used in the study of meiobenthos will permit to increase the amount of data about the resting stages (spores and conidium) and active functional state (accumulations of fungi mycelium). Expanding of methods of mycological research and efforts of specialists in various fields of science can bring, at first glance, the most extraordinary information about the taxonomic diversity of fungi, their spatial distribution in a wide range of water depths, ecology, and adaptation to extreme conditions, the role of the reservoir and the practical application of this knowledge.

We hope that the information about the clusters of fungi mycelium in the bottom sediments of the Black Sea hydrogen sulfide zone will attract interest of specialists in different areas of marine researches, and studies of mycobiota in the pelagic and benthic zone of the Black Sea deep-water areas will be continued.

Acknowledgements

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