======= EXPERIMENTAL ARTICLES =======

Method for Isolation and Enumeration of Fungi Developing in Marine Sediments

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Abstract—A new design for a small growth container which was used for investigation of the microbiota of the White Sea littoral and sublittoral sediments is presented. These chambers made it possible to observe development of fungal mycelium under conditions of a natural ecotope (marine sediments) and to isolate this mycelium as pure cultures. The mycobiota isolated from the containers was compared to that obtained by standard plating method of the same sediments. The mycelium developed in 10% of the containers installed. Intensity of growth was found to be lower in the sublittoral than in the littoral. Most fungi isolated from the containers did not produce spores in pure cultures. Apart from non-sporulating cultures, colonies of marine species *Paradendryphiella salina* and *Acremonium fuci* were obtained. Standard plating of the sediments often resulted in isolation of *Penicillium* and *Tolypocladium* species, while the share of sterile isolates was considerably lower than in the case of isolation using growth containers.

Fungal diversity in marine sediments is usually determined by plating on solid media (Kohlmeyer, J. and Kohlmeyer, E., 1979; Pivkin et al., 2006; Pivkin, 2010; Bubnova and Nikitin, 2017), similar to research on terrestrial soils (Gams, 1992; Carlile et al., 2001; Jeewon and Hyde, 2007). As in the case of soils, plating of marine sediments does not reveal whether emerging fungal colonies originate from active mycelium or from dormant spores. It is therefore unclear whether the fungi revealed by this approach are a functional component of the microbiota involved in the degradation processes or occasional findings. The standard plating technique evidently does not reveal the complete fungal diversity in the substrates (Gams, 1992; Jeewon and Hyde, 2007). This method is, for example, unusable for the isolation of obligate symbiotrophs. The results of molecular genetic research on fungal diversity in soils and marine sediments differed from those obtained by plating. For instance, they may reveal different predominant taxonomic groups (Jeewon and Hyde, 2007; Andreakis et al., 2015; Zhang et al., 2015; Rämä et al., 2017). Moreover, molecular studies usually encounter the phylotypes which can not be assigned to know species, as well as with uncultured forms (Nagahama and Nagano, 2012; Andreakis et al., 2015; Zhang et al., 2015). Thus, both molecular and standard cultural methods have specific problems and limitations.

Various modifications of culture-based techniques have been developed for determination of the functional part of mycobiota in soils, such as direct plating of soil particles (Jeewon and Hyde, 2007) and soil particles washing, which was originally proposed by Parkinson and Williams in 1960 (Williams et al., 1965). Although soil washing requires special equipment (Williams et al., 1965), this method has been repeatedly used in further research on the soil mycobiota (Gams, 1992). Compared to the standard serial dilution method, this approach results in a reliably decreased share of abundantly sporulating fungi, such as *Penicillium* species, and in an increased share of sterile isolates. Special substrates may also be added to the soil in order to reveal specific trophic groups of fungi, e.g., hair for keratinophylic fungi or paper for cellulolytics (Gams, 1992; Jeewon and Hyde, 2007). Isolation of fungi from the mycelium developing in various traps or containers is an interesting approach. In this case, a glass chamber (such as a thin glass tube with holes in its walls) was filled with agar medium and inserted into soil. After a certain period of incubation, the chamber was removed and fungal cultures were isolated. This procedure was intended to isolate the fungi capable of development in soil. Such works have been carried out in the 1960s and were almost abandoned later, probably due to laboriousness of the process (Gams, 1992). Although the stimulatory effect of introduction of additional nutrient sources into soil on

fungal growth has been repeatedly noted, fungal development requires, apart from the nutrient substrate, other conditions of the ecotope. For instance, in marine ecotopes these conditions include low temperature, elevated salinity, and constant humidification, to which high pressure and low oxygen content are added in deep-water environments. Baits are often used in marine microbiology, e.g., for the isolation of cellulolytic and wood-decomposing fungi (Kohlmeyer, J. and Kohlmeyer, E., 1979; Pivkin et al., 2006). Modifications of the standard plating techniques for detection of fungal functional groups from soils and marine sediments have not been reported.

The goal of the present work was to develop traps for the isolation of fungi from marine sediments and investigation of their diversity. Design of the container should make it possible to carry out microscopic investigation of development of the fungal mycelium. Moreover, comparison of the fungal species diversity as determined using the containers and the standard plating technique.

MATERIALS AND METHODS

Sampling. The work was carried out at the Pertsov White Sea Biological Station (WSBS, Kandalaksha Bay, White Sea) in July–August 2016. The average water salinity was 26‰; July water temperature at the depth of 0–10 m was 12–14°C. The temperature of sublittoral sediments was the same as the water temperature at a given depth; the temperature of littoral sediments fluctuated significantly due to tidal currents and time of the day. Four sites were studied: two sublittoral (S1 and S2) and two at the mid-littoral level (L1 and L2). The site S1 was located at the 4-m depth near the WSBS pier; L1—in the WSBS bay; S2 at 8-m depth ~1 km west from the WSBS settlement, and L2 opposite site S2, respectively. The sediment at site L2 was silted sand, in all other cases siltation was pronounced. The works in the sublittoral were carried out using diving equipment. All works at sites S1, L1, and L2 were carried in parallel. Works at S2 were carried out 24 h later.

Isolation of fungi. The container design is described in the Results and Discussion section. The work at the littoral was carried out as follows: during the first week the containers were removed every 24 h after installation and at weekly intervals afterwards (2, 3, and 4 weeks); two batteries (20 containers) each time. At every site, 20 batteries were installed (a total of 200 littoral containers). At the sublittoral, four batteries were removed 1, 2, 3, and 4 weeks after installation. A total of 16 batteries were installed (160 sublittoral containers). After one week of incubation, sediment samples were collected at all sites in parallel with container removal. The sediments were collected into sterile 50-mL plastic tubes.

After removal, containers were transferred to the laboratory and washed with fresh water. The cable ties were removed, then the container was wiped dry and examined under a Leica DM 2500 light microscope (Microscopy Center, WSBS). In the case of mycelial growth, its properties were recorded (number of growth points at the edges of the containers, mycelial length, and degree of branching), it was photographed and washed with tap water, sterile water, and ethanol. The container was then disassembled and placed onto solid medium (Malt Extract agar with 0.3% total sugar content on White Sea water) supplemented with gentamicin (2 mL of 4% solution per 0.5 L of the medium, i.e., to the final concentration of 0.16 g/L). The plates were incubated at 6°C for up to 30 days (in some cases, up to two months). The colonies developing at the edge of the container (in the places where microscopy revealed fungal growth) were isolated in pure culture. Plating of the sediments according to the standard procedure was carried out in parallel on the same medium; the plates were incubated for 30 days at 6°C. Each sample (1 cm^3) was distributed over ten plates. Low growth temperature was used in order to suppress growth of abundantly sporulating, rapidly growing fungal species, so that slower growing colonies were able to develop.

RESULTS AND DISCUSSION

The final design of the growth containers was as follows: sterile agar medium (0.2 mL) prepared on White Sea water and containing various amounts of sucrose (0, 1, and 5 g/L, depending on the experimental variant) was applied to a sterile thin microscope slide (Minimed SP-2 Lux, 1 ± 0.1 mm thick) and covered with another slide. The medium was distributed uniformly between the slides. As a result, a chamber was formed, which consisted of two glass slides and the medium between them. Using thin slides and a thin medium layer made it possible to carry out microscopic observation at magnification of up to ×150. Thus, the presence or absence of a mycelium, as well as some of its features (length and branching) could be ascertained. For higher mechanical strength, the chambers were combined in batteries of five using sterile cable ties. Pieces of such ties were used to separate the containers in order to improve seawater circulation (Fig. 1). The remaining ends of the cable ties proved useful for retrieval of the containers, especially in the sublittoral. Each battery contained the chambers with different sucrose levels (two with 0 and 1 g/L and one with 5 g/L), which were marked accordingly with a at the glass edge. The batteries were transported to the installation site in sterile plastic bags and then were placed into separate holes and covered with the sediment. After removal, the containers were transferred to the laboratory in the same bags.

Microscopy of the containers provided the following results. First of all, the number of chambers exhib-
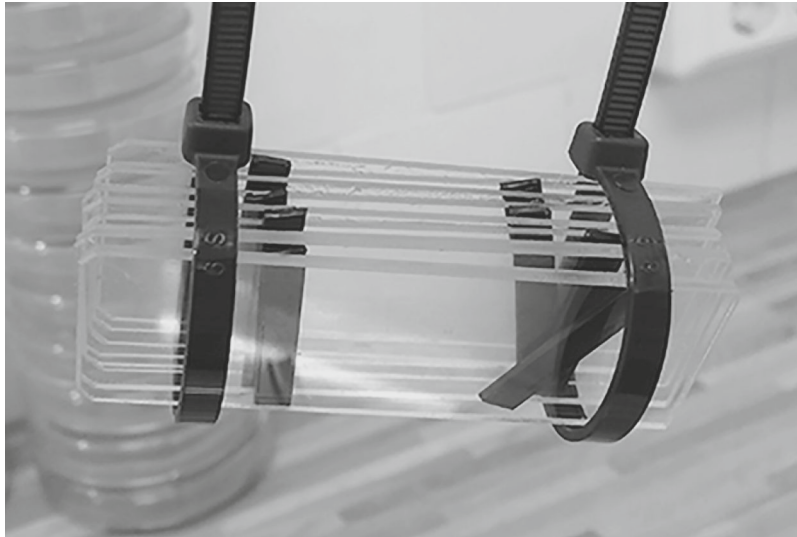
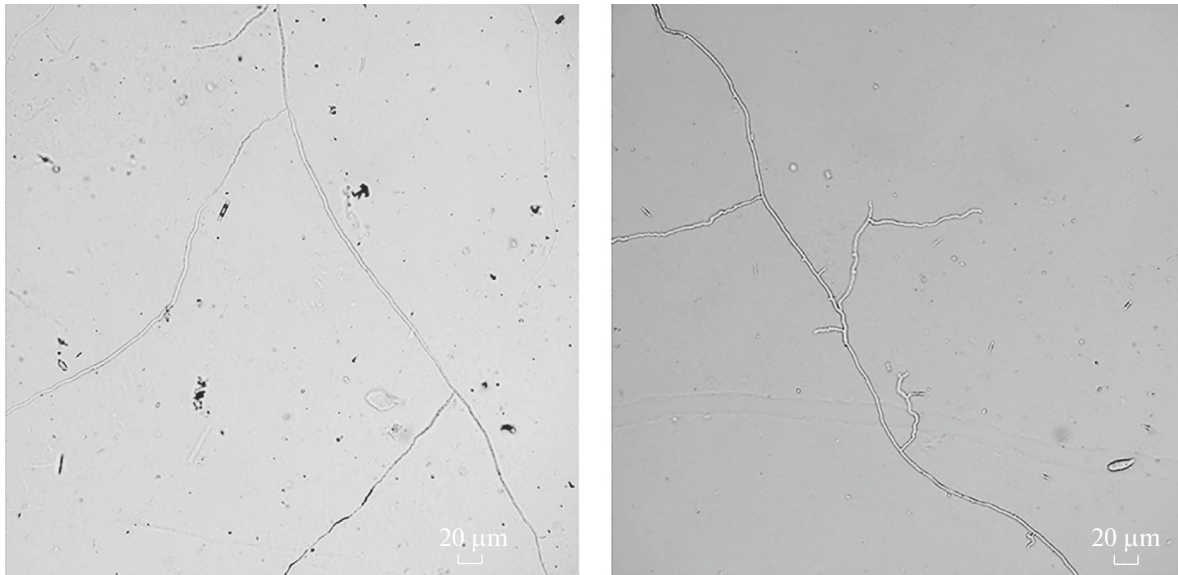**Fig. 1.** A battery of five trap chambers prepared for installation.

Fig. 2. Fungal growth in the trap containers incubated in a littoral sediment.

iting growth was quite low. Mycelial growth was revealed in 36 containers (Table 1), i.e., in 10% of their number. All sublittoral chambers contained only one center of development of the poorly branching mycelium 200−250 µm long. Five littoral chambers contained two to four centers of mycelial development, while each of the remaining ones contained only one. The average mycelium length was ~250 µm, it was poorly branching, with some branches of up to 500 µm long (Fig. 2). Thus, growth intensity was different in the containers incubated in the littoral and sublittoral, being higher in the littoral. Apart from fungal mycelium, diatoms and bacteria developed actively in many chambers; some of them colonized invertebrates.

Secondly, at the littoral no considerable difference was found in the number of growth-positive chambers incubated over one week. Only two chambers showed growth after the first week. Other cases of growth occurred more or less uniformly depending on time (Table 1). In the sublittoral all growth-positive chambers were collected after incubation for two or three weeks. Interestingly, the degree of mycelial development (length and branching) did not depend on duration of the incubation. Although the number of growth-positive chambers and intensity of mycelium growth were expected to increase with increasing time of incubation, this was not the case. The results obtained indicated that the concentration of fungal

Table 1. Number of chambers in which fungal growth was detected after different times of incubation

Time, days	Sublittoral		Littoral	
	site S1 (4 m)	site S2 (8 m)	site L1	site L2
1	—*	—	0	0
2	—	—	0	0
3	—	—	0	1
4	—	—	1	0
5	—	—	0	0
6	—	—	0	0
7	0	0	2	1
14	2	2	4	3
21	3	1	4	5
28	0	0	3	4
Total number of colonized chambers	5	3	14	14
	8		28	
	36			
Colonized chambers, % of the total chamber number	1.8	3.1	7	7
	5		14	
	10			

* At each time of incubation, 10 chambers from each littoral site and 20 chambers from each sublittoral one were examined. The sublittoral chambers were not examined at the incubation terms of one to six days.

propagules capable of development in the containers was very low. At the same time, both the littoral and sublittoral sediments are rather mobile and are constantly washed with seawater, which may carry propagules to and from the experimental sites. This was probably the reason for the absence of significant differences in container overgrowth at different terms of incubation. We therefore consider that installation of all chambers for 2–3 weeks and their simultaneous removal will be a rational approach to assessment of the intensity of mycelial growth. It should be noted that the total number of species increased with increasing exposure time. We think, however, that this is due to the greater number of containers retrieved, rather than to increased incubation time as such.

Lastly, no growth was observed in the medium not supplemented with sucrose, while in the medium with high sucrose content (5 g/L) bacterial growth commenced very early, which could hamper fungal development. Application of the medium with low sugar concentration (1 g/L) is therefore reasonable. Fungi are able to develop under such conditions, while bacteria are less abundant.

Isolation of fungi from the containers in which mycelia were detected by microscopy was not always successful. From the chambers incubated in the sublittoral, one colony of a marine anamorphic fungus *Paradendryphiella salina*, one colony of *Peni-cillium* sp., and four colonies of asporogenic fungi were isolated (Table 2). The latter formed poorly growing colonies, of which two were lost at the first transfer, as well as the *Penicillium* sp. colony. Poor survival of transferred cultures resulted probably from the differences in conditions (such as aeration) between the surface of agar medium and marine sediments. While cultivation of fungi in submerged culture could have probably helped to preserve most of them, such approach has not been previously used in marine mycology. The cultures obtained this way could have been used, for example, for DNA isolation and molecular genetic research. Higher fungal diversity was isolated from the containers incubated in the littoral sediments: a total of 20 colonies, including sterile mycelia, *P. salina*, *Acremonium fuci*, *Acremonium* sp., and *Cladosporium sphaerospermum* (Table 2). Thus, marine fungi and sterile morphotypes were found to develop in the chambers immersed into the marine ecotope. While the isolated marine fungi could be theoretically revealed by the standard plating technique, non-sporulating cultures were certainly a more interesting group. Their identification required molecular genetic investigation, which was beyond the scope of the present work.

As for the mycobiota diversity, application of growth chambers and of the standard plating technique produced different results. The fungal numbers

Table 2. Fungal diversity revealed in the White Sea littoral and sublittora sediments using trap chambers and by the standard plating technique

Fungi	Chambers		Plating	
	sublittoral	littoral	sublittoral	littoral
Mucor hiemalis Wehmer	0	0	2	0
Acremonium fuci Summerb., Zuccaro & W. Gams	0	2	3	3
Acremonium incoloratum (Sukapure & Thirum.) W. Gams	0	0	1	0
Acremonium sp. 1	1	0	0	0
Acremonium sp. 2	0	1	0	0
Cladosporium cladosporioides (Fresen.) G.A. de Vries	0	0	3	2
Cladosporium sphaerospermum Penz.	0	1	0	2
Fusarium oxysporum Schltdl.	0	0	0	2
Paradendryphiella salina (G.K. Sutherland) Woudenberg & Crous	1	4	1	4
Penicillium aurantiogriseum Dierckx	0	0	2	1
Penicillium chrysogenum Thom	0	0	6	8
Penicillium citrinum Thom	0	0	2	0
Penicillium glabrum (Wehmer) Westling	0	0	8	7
Penicillium griseolum G. Sm.	0	0	2	0
Penicillium thomii Maire	0	0	2	2
Penicillium sp. 1	0	0	0	0
Sarocladium strictum (W. Gams) Summerb	0	0	2	4
Tolypocladium cylindrosporum W. Gams	0	0	11	10
Tolypocladium inflatum W. Gams	0	0	6	12
Trichoderma harzianum Rifai	0	0	1	2
Trichoderma polysporum (Link) Rifai	0	0	3	0
Sterile	4	12	3	5
Total number of colonies	6	20	58	64

determined by plating was 26 and 32 propagules per 1 cm^3 in sublittoral sediments and 30 and 34 propagules per 1 cm^3 in the littoral sediments. The diversity determined by the standard method was higher than that revealed using the chambers, with predomination of the *Penicillium* and *Tolypocladium* species. The abundance of sterile mycelia was below 10%. Similar differences in species composition determined by the standard technique and using traps were previously reported for soil microbiota (Gams, 1992). Our studies confirm that abundant sporulation and high representation in standard platings do not always indicate the real role of a fungus in an ecotope.

Thus, the design of growth chambers described in the present work proved suitable both for microscopy and for the isolation of fungi. High numbers of sterile isolates certainly require the application of molecular genetic approaches for their identification, which will result in more precise information on diversity of the fungi capable of development in marine sediments. Two to three weeks of incubation was the optimal exposure in the White Sea conditions. High numbers of the chambers are required to enhance the probability of the isolation of fungi due to low rates of mycelia growth.

The chambers may be used to assess fungal development under various conditions, such as the root zone of littoral plants and shore areas with different geomorphology. Parallel application of growth chambers, standard plating techniques, and metagenomic analysis may provide interesting, mutually complementary results. Application of media with low concentrations of carbon sources is recommended for growth containers.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

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