# ODRA ESTUARY AS A HABITAT OF UNICELLULAR FUNGI

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## Abstract

Yeasts, able to form microbial complexes, are essential part of water ecosystems microbiota. Variations in biotic and abiotic factors occurring in natural habitat of microorganisms are often related to instability of microbial quantitative and specific structure. Many species are obligatory symbionts or are not able to grow in pure cultures. One of the most fundamental problem in monitoring of changes in microeukaryotic microorganisms is the presence of VBNC stages or those omitted due to their auxotrophic metabolism. On the basis of Odra estuarium it was proved possible to distinguish zones characterized by different mycocenosis profile. Spatial distribution of unicellular fungi is mainly the result of human activity and to a lesser extent depends on natural mechanisms in this ecosystem. Qualitative structure of yeasts in Odra Estuary is dominated by representatives of Ascomycota in water rivers. In lagoon and bay rivers, however, mainly yeasts of Basidiomycota were isolated. It was determined that species variety in Szczecin Lagoon reflects the mycocenotic quality of Odra waters. Yeasts representatives constantly present in particular zones of the estuary were *Candida* sp, *Cryptococcus* sp., *Rhodotorula* sp. and *Saccharomyces* sp.

## Keywords

Microbiot, microorganism, metabolism, unicellular fungi, ecosystem

## 1. Fungi in water environment

Microbiotic analysis of water ecosystems shows that fungi existing in these environments come from various ecological groups. According to Shearer et al. (2007) they represent all systematic classes of fungi and among those the most commonly isolated belong to Chytridiomycetes, Oomycetes and Ascomycetes,

seldom to Basidiomycetes. They are isolated from lakes, ponds, marshes, backwaters, streams, estuaries, seas and oceans, where the presence of available matter allows basic living functions. Barghoon and Linder (1944) for the first time described biology of filamentous fungi isolated exclusively from sea or oceanic environment. It laid the foundations for division of ecological groups of water fungi into inland and sea fungi. In 1979 Kohlmeyer and Kohlmeyer placed 209 taxons on the list of sea fungi species. Growing interest in water fungi, as well as development in experimental techniques, lead to increased number of identified species (Kohlmeyer and Volkmann-Kohlmeyer 1991). Until 2003 as much as 465 water fungi representatives were described (Shearer et al. 2007). On the basis of their incidence and essential behavioral characteristics obligatory and facultative sea species were determined. In case of the latter the influence of inland environment is insignificant for metabolic processes and reproduction. However, to maintain the basic cellular processes in obligatory sea species sea water environment is necessary. In biogeographic diversity of sea fungi, apart from determining factor - the concentration of chlorides in water, also water temperature is very important. Hughes (1974) tried to introduce the division into five biogeographic regions describing temperature dependence of fungi: arctic, moderate, subtropical, tropical and Antarctic. The above diversification system was quickly questioned by mycologists due to too wide overlap of temperature ranges between determined regions.

Each of the mentioned geographical regions and water ecosystems are also a habitat for yeasts (Guffogg et al. 2004, Schoenlein-Crusius et al. 2006, Butinar et al. 2005). Their presence in the environment reflects the ability to adapt to the profile of abiotic factors and relations with other organisms, with whom they shape biotic component of living niche. The presence of yeasts in natural ecosystems is therefore discussed with relation to their participation in biological and chemical dynamics of changes, in which they play role as reducers, consumers, predators or symbionts (Shearer et al. 2007, Bogusławska-Wąs 2010).

The occurrence of yeasts in water environment is related to especially high diversity in number of their taxonomic units, and due to their adaptability to environmental conditions they are regarded as euryecological organisms.

Cosmopolitan are usually significantly diverse in terms of nutritional requirements and ability to utilize organic compounds. Adaptation of certain individuals to conditions in the environment, where nutrients availability is usually periodical (Fry 1990), is metabolic reflection of specific environment colonized by yeasts (Phaff and Starmer 1987). Estuary areas are influenced by particular conditions, characterized by high dynamics of changes, and the relations between biotic and abiotic elements of the ecosystem are subject to constant fluctuation (Marques et al. 1997). It can lead to formation of ecological barriers determining the borders for migration of freshwater and sea organisms. Salinity is usually the critical factor. It seems, however, that changes in salinity do not significantly influence the occurrence of yeasts (Larsson et al. 1990) as much as their survival strategy (Bogusławska-Wąs 2010).

Essentially none of the studies so far explain the mechanism of yeasts adaptation to life in water environment. Only a few features were determined, which could predispose unicellular fungi to live in such ecosystems. These microorganisms are usually aerobic, non-sporulating, weakly fermenting forms, which require at least one growing factor in the form of vitamins. Their presence in water environment is not also determined by chloride concentration. Salinity is only one of the many factors determining ecological mechanisms of yeasts. According to Kohlmeyer and Kohlmeyer (1991) it is justified to divide these forms into obligatory (*Metschnicowia zobellii, M.krissii*) and facultative sea fungi (*Candida* sp.*, Rhodotorula* sp.*, Cryptococcus* sp.*, Trichosporon* sp.). The first group includes only those requiring the presence of chlorides for reproduction and metabolic processes. For others, usually freshwater and inland yeasts, the presence of chlorides is not necessary, and sometimes even harmful. Kutty and Philip (2008) emphasize, that eatuaries and seas are not a negative environment for yeasts isolated from soil and ontosphere of animals or humans. Unicellular fungi present there are most probably spread with

river current, sewage or wind. In majority of cases the presence of yeasts of inland origin in high-salinity waters is only periodical, otherwise they might have been omitted due to standard methods of isolation (Ekendahl et al. 2003).

## 2. Unicellular fungi in Odra Estuary

Estuary is not a typical environment. Its specific features are formed by partial environments, which do not exist independently. Their diversity is determined by relations between organisms functioning there, thus reflecting specificity of a particular reservoir. In Polish coast a typical estuary region, in its final part forming a bay largely supplied with freshwater, is the estuary of Odra river (Tórz 2007). On the basis of salinity structure it was classified as a homogeneous estuary. In contrast to stratified and partly stratified estuaries, no vertical gradient of salinity is observed and water flow occurs only in horizontal direction. Water circulation in non-tidal estuaries depends mostly on the size of inland water supply, which leads to mixing of waters. Intensity and spatial scale of this process depend on wind strength and direction (Kubiak, 1980, Skriptunov and Gorelits 2001). On the basis of spatial changes in macro-ions concentration Tórz (2007) divided Odra estuary into three zones: lower – covering the area of Pomeranian Bay directly connected to the sea, middle – being the area of brackish water of Szczecin Lagoon, and upper – characterizing freshwater environment of Odra river.

Estuaries are noted for high diversity of number and species structure of isolated microorganisms, which depends only little on hydrochemical parameters. It is usually determined by limitation in potential sources of organic matter metabolized by fungi. It is the reason of decreased frequency in their isolation along with longer distance from inland environment (Fig. 1). Mycocenosis diversity is also influenced by the presence of halocline rich in nutrients and flow of inland waters, including sewage (Bogusławska-Wąs 2007). In Pomeranian Bay waters the number of determined fungi did not exceed 21.0 cells$\cdot$L$^{-1}$, what is typical for open and clean waters (Hagler and Ahear 1987). Yeasts dragged from this part of estuary did not significantly influence the number of fungi isolated from lagoon waters (24.0 cells$\cdot$L$^{-1}$), much less river waters (Fig. 1).
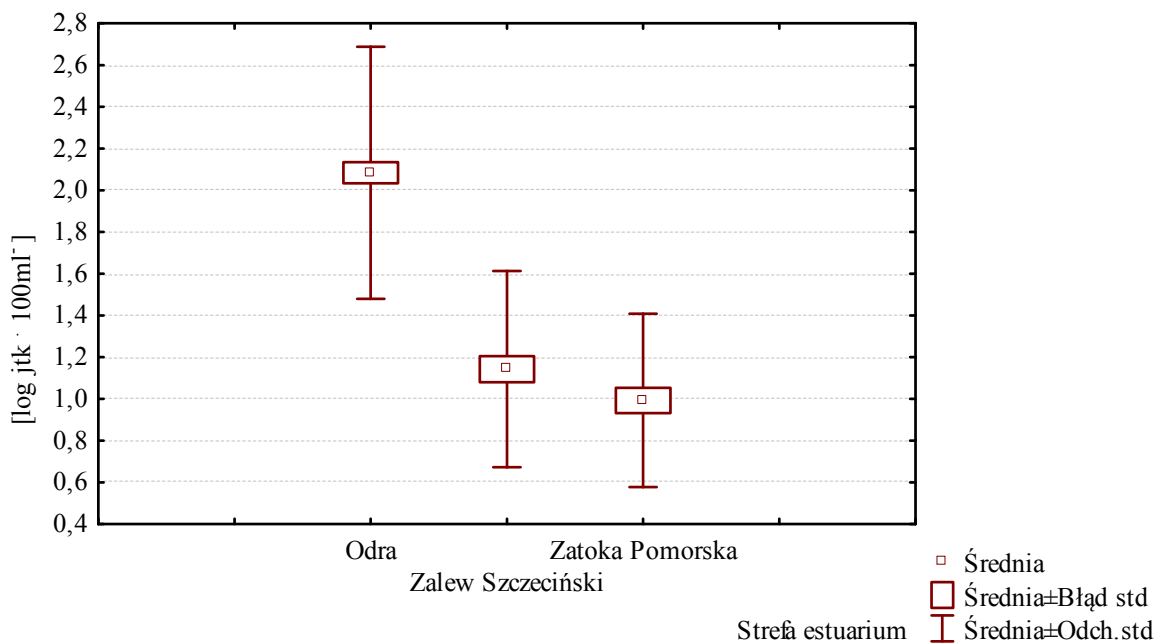
Fig. 1. Total count of yeasts isolated from particular zones of the estuary
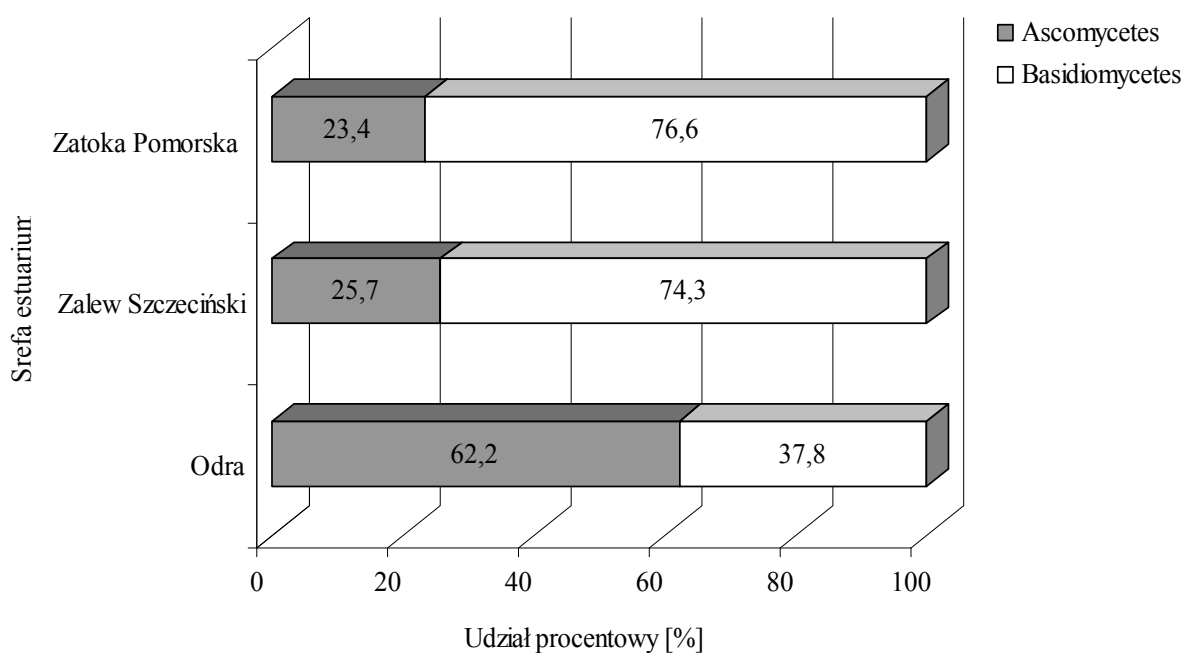(Bogusławska-Wąs 2010)



Fig. 2. Percentage distribution of yeasts of Ascomycetes and Basidiomycetes classes
isolated from particular Odra Estuary zones (Bogusławska-Wąs 2010)

In majority of cases the interpretation of the results does not refer to ecological group of marine fungi, but mostly to forms, which incidentally reached the sea (Kurtzman and Fell 2006, Nagahama 2006). In that case the analysis concerns the diversity in microbial species structure.

Its composition is influenced by biotic and abiotic factors characteristic for particular estuary zone. The analysis performed showed that Odra waters are dominated by representatives of Ascomycota, class Ascomycetes – 62.2% (Fig. 2).

It should be noted that isolation frequency of particular species of unicellular fungi changes along with the change of the area of examined estuary zones. Surface waters of Szczecin Lagoon and Pomeranian Bay are dominated by yeast species and yeast-like fungi of phylum Basidiomycota, class Basidiomycetes, which constituted, respectively, 74.3% and 76.6% isolated yeasts (Fig. 2). It is, undoubtedly, the result of considerably versatile adaptation skills and abilities to utilize nutrients by these fungi that are unavailable for Ascomycetes (Gadano et al. 2006).

According to Bogusławska-Wąs (2010) the determined proportions describing changes in number of yeasts Ascomycetes and Basidiomycetes in particular zones of Odra estuary do not correlate with their species similarity. It was showed by the values of species similarity index for particular zones (Jaccard index $JI$). Calculated values of Jaccard index unambiguously show species similarity only in zones of Szczecin Lagoon and Odra ($JI_{Odra, Lagoon} = 0,83$). In case of waters of upper estuary and the two other zones the values of calculated indexes were significantly lower - $JI_{Lagoon, Bay} = 0,56$ and $JI_{Bay, Odra} = 0,48$.

Changes in numbers of isolated fungi, their species affinity and area of isolation show, that Szczecin Lagoon is the zone, where species diversity is formed by river waters. However, the number of unicellular fungi in this zone of estuary is influenced by waters flowing from Pomeranian Bay.

Mycocenotic monitoring of this area shows that in river waters the increase in CFU of yeasts is related to the increased number of strains of the genus *Saccharomyces*, including *S. cerevisiae*, whose presence in examined environment is caused by the inflow of urban polluted waters (Bogusławska-Wąs et al. 2007). Mycocenoses of the second zone of the estuary are mainly formed by the species of *Rhodotorula* genus, including *Rh. glutinis* and *Rh. mucilaginosa* (Bogusławska-Wąs and Dąbrowski 2001, Bogusławska-Wąs 2010).

*Table 1.* **Occurrence of yeasts [%] in samples from Odra estuary**

| Species | Estuary zone | | |
|---|---|---|---|
| | Odra | Szczecin Lagoon | Pomeranian Bay |
| Aureobasidum sp | 0,07 | 0,29 | 3,75 |
| C. albicans | 1,51 | 0,15 | 3,10 |
| C. boidinii | 0,20 | 0,25 | 0,43 |
| C. colliculosa | 0,38 | 0,00 | 0,00 |
| C. famata | 4,73 | 0,98 | 1,44 |
| C. holmii | 0,36 | 0,59 | 0,00 |
| C. incospicua | 0,22 | 0,74 | 0,00 |
| C. kefyr | 0,27 | 0,15 | 1,22 |
| C. krusei | 0,42 | 0,15 | 0,00 |
| C. lambica | 1,40 | 1,82 | 0,36 |
| C. lipolytica | 1,47 | 0,74 | 0,94 |
| C. lusitaniae | 1,51 | 0,29 | 0,22 |
| C. membranaefaciens | 0,84 | 0,00 | 0,00 |
| C. norvegica | 0,16 | 0,15 | 0,00 |
| C. parapsilosis | 0,13 | 0,74 | 0,36 |
| C. pelliculosa | 0,62 | 0,00 | 0,72 |
| C. pulcherrima | 1,04 | 0,00 | 1,30 |
| C. quilliermondii | 0,31 | 0,83 | 4,90 |
| C. tropicalis | 3,42 | 0,29 | 3,82 |
| C. utilis | 0,07 | 0,15 | 0,00 |
| C. sake | 1,98 | 0,00 | 0,00 |
| C. zeylanoides | 0,40 | 0,00 | 0,22 |
| Cr. albidus | 2,69 | 4,76 | 8,72 |
| Cr. humicolus | 0,18 | 2,11 | 1,44 |
| Cr. laurenti | 6,00 | 9,58 | 17,72 |
| Cr. neoformans | 1,91 | 4,67 | 3,53 |
| Cr. uniguttulatus | 3,11 | 1,92 | 3,17 |
| Geotrichum spp | 0,91 | 0,20 | 9,80 |
| Kl. apiculata | 0,73 | 1,33 | 0,72 |
| NOZN | 1,11 | 1,67 | 5,40 |
| P. carsonii | 1,38 | 1,67 | 4,83 |
| Rh. glutinis | 6,68 | 6,24 | 17,46 |
| Rh. mucilaginosa | 12,90 | 46,27 | 2,71 |
| S. cerevisiae | 40,24 | 4,57 | 1,73 |
| S. kluyverii | 0,67 | 6,73 | 0,00 |

In Pomeranian Bay waters, apart from carotenoid producing fungi, the increase in total number of yeasts results from the presence of the species of the genus *Cryptococcus* (Bogusławska-Wąs 2010).

The representatives of these genera together with *Candida* sp can be described as constant residents of Odra estuary and their participation in total number of isolated yeasts are presented in Table 1. Analyses performed by Bogusławska-Wąs (2010) showed, that the presence of fungi of the genus *Rhodotorula* in Odra estuary is caused by metabolic regulations for which the limiting factor is organic nitrogen concentration. Moreover, the author claims, that the area containing river waters is only temporary habitat for the strains of *Rhodotorula* sp. Only when dragged into Szczecin Lagoon, in conditions of stabilized biogenic profile, the strains find the habitat that intensifies their functioning.

It should be also noted, that in waters of Odra and Szczecin Lagoon, within the genus *Rhodotorula*, the predominant species was *Rh. mucilaginosa*, whilst *Rh. glutinis* was only co-dominant. The proportions in this multi-species association changed in waters of Pomeranian Bay. Considerably lower in protein compounds, bay waters mainly support the survival of species able to utilize inorganic nitrogen compounds. It is no less important that the fluctuations in microorganisms habitat conditions affect the change of yeast ecological niche. *Rh. mucilaginosa* strains in Odra waters dominate in planktonic form, whilst in waters of Pomeranian Bay they change their ecological profile into adherent (Bogusławska-Wąs 2010).

Here it should be stressed, that for the majority of yeast species isolated from Odra estuary waters, Szczecin Lagoon forms an ecological barrier for their survival and/or metabolic activity.

## 3. Survival strategies of unicellular fungi in water environment

Many biocenoses are characterized by certain biological equilibrium and physiological tolerance of particular species determines the direction of changes within their structure (Marques et al. 1997). Yeasts interactions with other microfungi, bacteria and protozoa, as well as with higher hydrobionts, are based on

certain relationships, which fundamental aim is to keep alive in the environment. In natural water ecosystems the microorganisms, in order to survive, have to adhere to a certain survival strategy. It reflects the reaction of the population to fluctuation in nutrients availability. It mainly concerns allochtonic forms, whose metabolism is often not adjusted to new habitat conditions.

One of the basic factors determining the growth of microorganisms is the availability of nutrients. In case of their minimal concentration yeast strains exist in a state of minimal metabolic activity in maximally elongated generation time. Moreover, the diversity in yeasts populations in particular estuary zones, except their possible death, results from natural cell response to environmental stress. The mechanism of cell reaction results from regulation of genes described as CER – common environmental response (Causton et al. 2001) or ESR – environmental stress response (Gash et al. 2000).

According to McDougald et al. (1998) the natural cellular response to environmental stress is conversion to the VBNC state (*viable but non-culturable*). VBNC cells are defined as respiratory active but unable to grow on laboratory media (Colwell 2000). They are able to synthesize proteins *in vivo* and to keep low metabolic activity (Oliver 2005), which is higher in pathogenic microorganisms. Regulation of physiological properties results from the presence of signaling substances – i.e. autoinducers, and system of their transmission.

Unfortunately, the majority of works concerning water environment mycocenoses are based on determination of microorganisms able to grow in laboratory conditions. It is highly important because VBNC state do not influence the basic functions of the cells in the ecosystem. It is, however, reflected in the number of isolated strains and the data can be misinterpreted during determination of their presence. The fact that a species is not isolated in laboratory culture do not correlate with its absence in the environment. It only shows on particular survival strategy established by the microorganisms and its main aim is to maintain basic living functions in atypical environment. Such behavioral mechanism do not exclude

intracellular communication of yeasts and co-formation of complex populations (Fig. 3).



cell in VBNC state
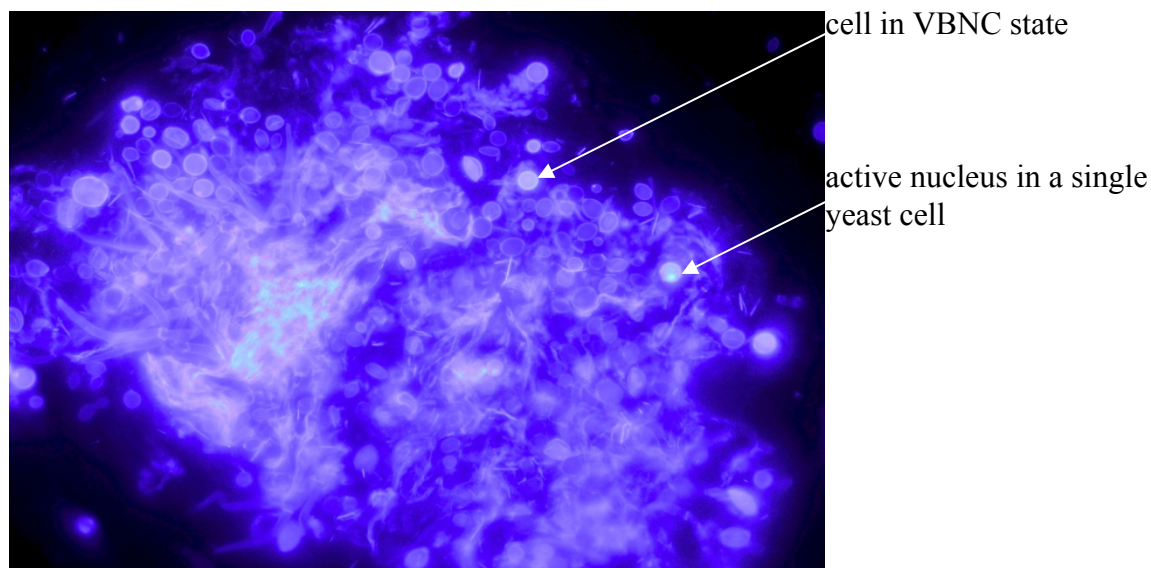
active nucleus in a single yeast cell

Fig. 3. Populations of yeasts with various forms of cells; visible in DAPI filter (Bogusławska-Wąs 2010)

Microorganisms described as VBNC are able to recreate their full metabolic properties but only in a certain time. Physicochemical profile of Odra estuary environment do not support development and maintenance of metabolic processes, but it does not have to lead to cell death. Supposing that yeasts enter water environment in stationary phase of growth, stored energetic reserves of glycogen and trehalose enable the cells, e.g. *S. cerevisiae*, to survive in starvation conditions for a long time (Granot and Snyder 1991, Granot and Snyder 1993). It is not entirely clear, whether cells conversion to VBNC state is a physiologically stable phase, after which regeneration occurs (Divol and Lonvaud-Funel 2005), or whether it is only a temporal state leading to death (Ohtomo and Saito 2001, Oliver 2005).

Interactions between yeasts and other groups of microorganisms living in the same ecological niche do not have to be based on competition, but they should be based on co-formation of organized population (Adams et al. 2008). The discovery of the ability of microorganisms to form heterogeneous consortium revolutionized ideas in microorganisms ecology and those concerning their functioning in water environment. It was somehow the consequence of the suggestion, that in natural

environment the potential occurrence of single cells is sporadic (Costertin and Lewandowski 1997, Vachova et al. 2009). In case of majority of microorganisms the natural behavioral consequence is formation of populations, and their fundamental role is to protect and maintain the living functions of "cooperators" (Theraud et al. 2004, Jin et al. 2005). Therefore, the presence of single cells is usually regarded as temporary state between the release of cells from biofilm and initiation of formation of a new organized microbial population.

In the structure of a formed complex the stratification of organization of multispecies population is determined by functionality and level of metabolic specialization (Carlsson 1997, Gilbert et al. 1997, Richard et al. 2003). Even though the studies on the formation of the populations by these microorganisms is essential for understanding ecophysiological processes occurring in natural environment, biofilm development by mycocenotic forms in water environment is practically unknown. According to Gomez-Suares et al. (2001) biofilm formation is the only successful way to persist in favorable environment. Experiments performed by Bogusławska-Wąs (2010) on *Cryptococcus* sp isolated from Odra estuary do not entirely support this statement. Strains able to form biofilm in the first stage of settlement colonize the surface regardless of nutritional composition of suspension medium. However, the critical condition is the activation of supporting mechanisms. Usually a modification of cell surface occurs, which affects the process of the first and critical stage of biofilm initiation. It is crucial, because non-constitutive genetically dependent cellular adherence ability is regulated by macromolecular structures of cell membrane (Mercie-Bonin and Fonade 2002). The stages of formed biofilm are very similar (Chandra et al. 2001), but dependent on the type of the ecosystem. The differences in time of development of particular stages are determined, as well as differences in biofilm structure (Fig. 4, 5). This structure significantly affect its functionality.

The mechanism of biofilm development, despite the potential determining its formation, is not identical for all the yeasts strains in Odra estuary. The necessity of a cell conversion to VBNC state does not allow the cell to initiate development of a

population, but only allows it to join already existing populations or persist in planktonic form. Some *Rhodotorula* strains, depending on the zone of Odra estuary, minimize the activity of adhered form in favor of planktonic form (upper estuary), what can be the cause of variable frequency of their isolation.
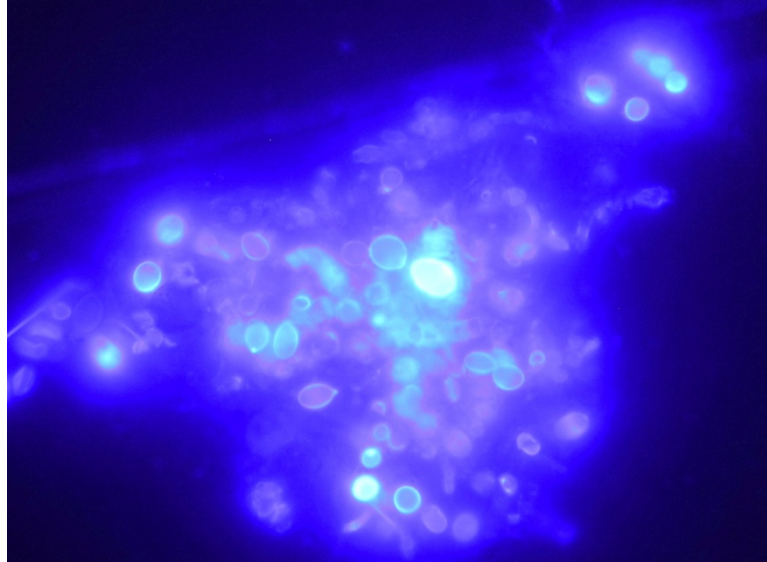


Fig. 4. Biofilm of *Rh.mucilaginosa* without filamentous structures
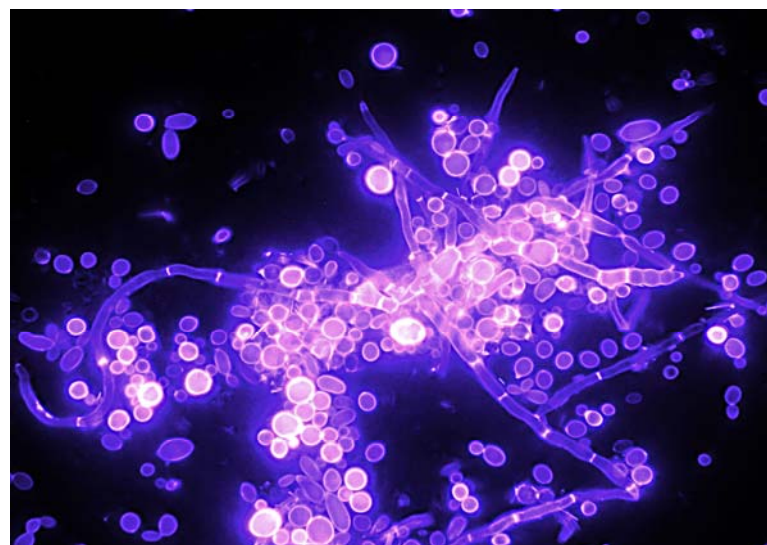


Fig. 5. Biofilm of *Rh.mucilaginosa* with filamentous structures

According to Costerton et al. (1987), the course of metabolic processes occurring in formed populations is significantly affected by the structure of microbial network in the ecosystem. Own observations show, that filter feeders (e.g. *Daphnia magna*), present in the environment, feed not only on bacteria but also on unicellular

fungi. For predators I the yeasts forming complex populations become a food source rich in nutrients. It is, however, interesting, that consumers mainly utilize extracellular polymer compounds released by microorganisms. Their release to external environment and chemical variety depend on genetic profile of a microorganism, as well as on adaptation to particular environmental conditions (Sutherland 2001, Yin et al. 2005).

Considerations concerning possible mechanisms of yeasts behavior suggest the need to interpret the results of complex analyses. The fact that the microorganisms is not isolated from a particular environment does not correlate with its absence. The interpretation of partial analyses without considering the mechanisms determining the presence of microorganisms in an ecosystem seems to be burdened with significant error. The result of that is underestimated number of total count of microorganisms present in ecosystems, lowered down to $1.0 - 10\%$. Incorrect estimation of qualitative and quantitative structure of yeasts results in the omission of their actual role in the environment. It particularly concerns the relations in microbial populations and the role of individual members. Unfortunately, performing a research in a limited context of such relations results in duplication of incorrect deductions. It is, therefore, not proper to accept *a priori* the presence of certain yeasts species depending on specificity of the environment.

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