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MICROBIAL ECOLOGY OF MYXOGASTRIDS

WITH PARTICULAR FOCUS ON *PHYSARUM POLYCEPHALUM*

Master Thesis

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Author:	Katrin Schnitzer, BSc Glanblick 31 9560 Feldkirchen
Supervisor:	Ao.Univ.-Prof. Dr. Martin Grube
Co-Supervisor:	Dipl.-Biol. Dr. Christian Westendorf
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Abstract

Myxogastrids (or Myxomycetes) belong to the unikont supergroup Amoebozoa and are distributed all over the world with the highest diversity located in temperate forests so far. During one trophic stage of their life cycle they live as multinucleate plasmodium, which migrates whilst feeding on decayed plant material and bacteria. The behavior of the plasmodium of *Physarum polycephalum* is in focus of current research, but little is known about its microbial ecology. In general, experiments on plasmodia (e.g. nuclear cell cycle, cell motility and differentiation) are conducted with crude cultures. The aim of this study was to implement sterile cultures of plasmodia to allow reproducible experiments with microorganisms. Previous researchers have already worked with putative sterile cultures, but sterility proofs were always restricted to cultivable bacteria. In this study, the migration method was used to obtain sterile surface cultures of *P. polycephalum, Badhamia utricularis* and *Fuligo septica* to subsequently cultivate them in shaken cultures as microplasmodia. The latter two species were collected in nature and identified with molecular (ITS region and 18S rDNA) and morphological methods. The sterility was investigated with the single strand conformation polymorphism (SSCP) technique and therefore extended for non-cultivable bacteria the first time. Sterility was not verified completely since a putative alphaproteobacterium was found in shaken cultures of *P. polycephalum*. Furthermore, growth was quantified in shaken cultures with different heat-killed bacteria. The persistence of these bacteria (alive) in and on the sterile macroplasmodia was investigated as well. These experiments were restricted to *P. polycephalum* since only a temporary shaken culture was obtained with *B. utricularis* and the plasmodium of *F. septica* could not be freed from bacteria. *F. septica* showed moreover characteristics that were not described in current literature, for instance growth on thick strand-like traces, indicating no self-avoidance. Bacterial contaminations that appeared in non-sterile and sterile cultures of *P. polycephalum* were isolated and identified by sequencing their 16S rDNA genes. One of these contaminants (*Paenibacillus* sp.) was also used for further experiments resulting in a negative correlation between growth and persistence. In general, *Paenibacillus* sp. revealed to be a remarkably persistent bacterium. This also holds for *E. coli*, which was detectable over five passages on water agar. The freeing of plasmodia from bacteria always resulted in subsequent death. These results were not consistent with previous studies, which describe three passages on water agar to be sufficient to obtain bacteria-free plasmodia.

Zusammenfassung

Myxogastria (oder Myxomyceten) stellen eine Untergruppe der Amoebozoa dar und sind weltweit verbreitet, wobei in gemäßigten Wäldern eine besonders hohe Diversität herrscht. In einem trophischen Stadium ihres Lebenszyklus leben sie als mehrkerniges Plasmodium, welches sich kriechend fortbewegt und von totem Pflanzenmaterial und Bakterien ernährt. Das Verhalten des Plasmodiums von *Physarum polycephalum* wird derzeit eingehend erforscht, wohingegen wenig über die mikrobielle Ökologie bekannt ist. Die meisten Versuche mit Plasmodien (z.B. Erforschung des Zellzyklus) wurden und werden mit unsterilen Kulturen durchgeführt. Das Ziel dieser Studie war unter anderem die Sterilisation von Plasmodien, um reproduzierbare mikrobielle Versuche durchführen zu können. Einige Autoren haben bereits mit angeblich sterilen Kulturen gearbeitet, jedoch beschränkten sich Sterilitätsnachweise bis dato auf kultivierbare Bakterien. In dieser Studie wurde die Migrationsmethode benutzt, um Plasmodien von *P. polycephalum, Badhamia utricularis* und *Fuligo septica* zu sterilisieren und sie anschließend als sogenannte Mikroplasmodien in Schüttelkultur zu kultivieren. Die beiden letzteren Stämme wurden in der Natur gesammelt und anhand molekularer (ITS Region und 18S rDNA) sowie morphologischer Charakteristika identifiziert. Die Sterilität von Schüttelkulturen wurde mit Hilfe einer molekularen Fingerprint Methode (SSCP-single strand conformation polymorphism) zum ersten Mal auf nicht kultivierbare Bakterien ausgeweitet. Sie konnte nicht vollständig bestätigt werden, da ein alpha-Proteobakterium in den Kulturen von *P. polycephalum* nachgewiesen wurde. Das Wachstum von Mikroplasmodienkulturen wurde mit verschiedenen hitzegetöteten Bakterien beobachtet. Die lebenden Bakterien wurden ebenfalls für Persistenzversuche in und auf sterilen Makroplasmodien herangezogen. Die Wachstums- und Persistenzversuche beschränkten sich dabei auf Schüttelkulturen von *P. polycephalum*, da von *B. utricularis* nur eine temporäre Schüttelkultur und von *F. septica* keine Sterilkultur hergestellt werden konnte. Jedoch konnten von *F. septica* in der Literatur noch nicht beschriebene Eigenschaften beobachtet werden, beispielsweise das Wachstum auf und in verdickten Schleimspuren ohne Selbsthemmung. Auftretende bakterielle Kontaminationen von nicht sterilen, aber auch sterilen Kulturen von *P. polycephalum* wurden isoliert und anhand ihrer 16S rDNA Sequenzen identifiziert. Eine dieser Kontaminationen (*Paenibacillus* sp.) wurde ebenfalls für nachfolgende Versuche verwendet, wobei sich eine negative Korrelation zwischen Wachstum und Persistenz zeigte. Generell erwies sich *Paenibacillus* sp. am persistentesten neben *E. coli*, der fünf Passagen auf Wasseragar nachweisbar blieb. Plasmodien die von Bakterien befreit wurden, starben kurz darauf. Diese Ergebnisse stehen im Gegensatz zu vorangegangen Studien, die bakterienfreie Kulturen von *P. polycephalum* nach drei Passagen auf Wasseragar beschreiben.

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List of Abbreviations

APS	Ammonium persulfate
Bis	N,N'-Methylenbisacrylamid
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
CLSM	Confocal laser scanning microscopy
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FISH	Fluorescence in situ hybridization
LPS	Lipopolysaccharides
MDE	Mutation Detection Enhancement
NaOH	Sodium hydroxide
NB-2	Nutrient Broth No.2
NCBI	National Center for Biotechnology Information
OD_{600}	Optical density at a wavelength of 600 nm
ONC	Overnight culture
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Semi-defined
SDS	Sodium dodecyl sulfate
SOBEM	Synthetic obligate bacteria–eukaryote mutualism
SSCP	Single strand conformation polymorphism
SSU	Small subunit
TCA	Trichloroacetic acid
TBE	Tris/Borate/EDTA
TEMED	Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane

1 Introduction

1.1 Life Cycle and Classification of Myxogastrids

The myxogastrids, which are also known as myxomycetes, acellular, plasmodial or true slime molds, consist of about 850 species. They are divided into the five orders Physarales, Stemonitales, Trichiales, Liceales and Echinosteliales (Poulain *et al.* 2011). Although the term Myxomycetes is commonly used, Myxogastrids is suggested as the correct term according to the International Society of Protistologists (Adl *et al.* 2012). Myxogastrids are distributed all over the world with the highest diversity located in temperate forests so far (Spiegel *et al.* 2004). Their life cycle consists of a haploid unicellular stage as myxamoebae (or swarm cells) and a trophic diploid stage as plasmodium. After the fusion of two myxamoebae (or swarm cells) of compatible mating types, the diploid zygote forms a multinucleate plasmodium by synchronous mitosis without cell division. The plasmodium represents the assimilative stage and shows rhythmic protoplasmic streaming. Three forms of plasmodia exist: the phaneroplasmodium (large and veined), the aphanoplasmodium (transparent veins) and the protoplasmodium (small and unveined). The fan shaped phaneroplasmodium, which is easily visible to the naked eye, has an advancing feeding edge and a posterior trailing network of veins (Mueller *et al.* 2004; Everhart & Keller 2008; Adl *et al.* 2012). Colored phaneroplasmodia, such as the yellow plasmodium of *Physarum polycephalum*, are particularly striking. No carotenoids, but tetramic acid derivatives, Physarochrome A, Physarorubinic acid A/B and Chrysophysarin A are responsible for the pigmentation (Steglich 1989; Atta-ur-Rahman 2003).

Furthermore the life cycle of myxogastrids includes resting or dormant stages, such as spores, microcysts and sclerotia (Everhart & Keller 2008). Under starving conditions, the plasmodium can either form sclerotia or fruiting bodies (sporulation). The sporulation is moreover controlled by visible light. Under favorable conditions sclerotia can reform the plasmodium (Rätzel *et al.* 2013).

Species are mainly identified by morphological characteristics of the fruiting bodies, such as structure, color and lime deposition. Morphological species determination during the plasmodial stage is impossible (Neubert *et al.* 1993). The classification of myxogastrids changed over time. They were classified within the Kingdom Plantae (Class Myxomycota), the Kingdom Animalia (Class Mycetozoa), as well as within the Kingdom Fungi (Class Myxogastrids) (Martin & Alexopoulos 1969). The classification in the (paraphyletic) Kingdom Protista was also suggested and is still used (Spiegel *et al.* 2004). Recently, molecular methods have led to

the resolution of taxonomic relationships (Martín *et al.* 2003; Fiore-Donno *et al.* 2005; Hoppe & Kutschera 2009; Fiore-Donno *et al.* 2009). Finally it was confirmed that Myxogastrids are part of the phylum Mycetozoa, which belongs to the unikont supergroup Amoebozoa (Adl *et al.* 2012).

1.2 Ecology of Myxogastrids

In nature myxogastrids occur on decaying plant material (e.g. logs, stumps, leaves), but also live in microhabitats on living trees or herbivore dung. Even aquatic environments are suitable habitats for some species (Stephenson *et al.* 1994). Little is known about their ecological role, but it seems as if metatranscriptomic approaches could reveal the underestimated ecological importance of Mycetozoa (Stephenson *et al.* 1994; Urich *et al.* 2008; Stephenson & Feest 2012). It was shown that plasmodia from *P. polycephalum* make selective nutritional decisions to obtain a balanced diet of carbohydrates and proteins (Bonner 2010; Dussutour *et al.* 2010). Extracellular secretions serve as the "externalized memory" of the plasmodium. It avoids areas in which extracellular slime is present, because it signals an already visited area and therefore less food availability (Reid *et al.* 2012; 2013).

Along the few articles published on food preferences, the myxogastrids are regarded to be both fungivorous and bacteriovorous (Stephenson & Feest 2012). Howard and Curry (1932*a*, *b*) examined the food preferences of myxogastrids for the mycelia and sporophores of different fungi. They assumed the interactions to be saprophytic and also parasitic. Pinoy (1907), who assumed myxogastrids to be obligate parasites or symbionts, suggested that bacteria were essential to the growth and spore germination and concluded that bacteria were used directly as food by plasmodia. The most significant results were obtained in two-membered cultures with *Bacillus luteus*. Since then, bacteria are the most frequently used food organisms in two-membered cultures (Aldrich & Daniel 1982). Additionally, live yeast cells (Cohen 1939) and even green algae (Lazo 1961) can serve as a food source. The food uptake mechanisms have been studied in detail with microplasmodia, which are fragmented plasmodia grown in shaken cultures. Microplasmodia engulf food particles using a cell membrane invagination system that depends both on age and size of microplasmodia (Wolf & Stockem 1979; Brix *et al.* 1987). Scanning electron microscopy revealed the existence of pseudopodia (temporary cytoplasmic extensions) which attach to food particles (Anderson 1993).

1.3 Myxogastrids and their Relationship to Microorganisms

It is known that myxogastrids are bacterivorous, but little is known about which bacteria can serve as a food source and if interactions between bacteria and myxogastrids exist.
Chapman and Coote (1983) determined the growth of *P. polycephalum* microplasmodia on ten bacterial species and *Saccharomyces cerevisiae*, prepared as heat-killed suspensions. They pointed out the importance of the bacterial surface structure. For instance, *P. polycephalum* showed a slower growth on smooth strains of *Salmonella minnesota* than on rough strains. Compared to rough strains, smooth strains have full-length O-chains in the lipopolysaccharides (LPS) of the outer membrane. Moreover *P. polycephalum* microplasmodia poorly grew on slime-forming bacteria and bacteria with high lipid containing cell walls (*Mycobacterium*). Saddler *et al.* (1979) demonstrated the ability of *P. polycephalum* microplasmodia to degrade bacterial LPS due to enzymatic degradation of lipid A. The enzymes showed intracellular and extracellular activity. Different degradative enzymes are responsible for the breakdown of the surface structure (e.g. glycosidase, glucanases, chitinase, proteases, phospholipases) (Comes & Kleinig 1973; Farr *et al.* 1974; 1975; Kilpatrick & Stirling 1977; Kuehn *et al.* 1977; McClory & Coote 1985). Antibiotic substances with activity against gram-positive and gram-negative bacteria, as well as yeasts, were isolated from *Physarum gyrosum* cultivated together with *Escherichia coli* (Schroeder & Mallette 1973). These antibiotics were proven to be rather bacteriostatic than bactericidal (Taylor & Mallette 1978).

The relationship between myxogastrids and bacteria was regarded as a “mutualistic balance”, but there was no evidence for symbiosis, which still holds until today. (Cohen 1941; Daniel & Baldwin 1964). However, myxogastrids are associated with other organisms, for instance with insects (mainly beetles). They use the fruiting bodies for feeding, laying eggs, as homes and could probably play a role in spore dispersal. Moreover, different fungi appear on myxogastrid fruiting bodies, some of them being obligately myxomyceticolous (Wheeler & Blackwell 1984; Stephenson *et al.* 1994).

Furthermore, there are known associations, which were established in the laboratory. Gastrich and Anderson (2002) induced an association between *P. polycephalum* and the alga *Chorella pyrenoidosa*. The algae were engulfed by the plasmodium resulting in color change from yellow to green. Plasmodia associated with algae had an increased longevity compared to the ones without algae.

By now, stable associations between bacteria and myxogastrids were neither observed nor established. On the contrary, the cellular slime mold *Dictyostelium discoideum,* which

belongs to the distantly related mycetozoan group Dictyosteliida, is associated with different bacteria. It acts as a "primitive farmer" because not all bacteria serve as a food source. Some of them are not digested but left behind as a "seed" for new bacteria populations. This specialized form of symbiosis is referred to as agriculture (Brock *et al.* 2011).

1.4 Growing of Slime Mold Plasmodia in (Pure) Culture

Myxogastrids can either be grown in moist chambers, crude cultures or pure cultures. De Bary (1864) began with the cultivation of plasmodia in moist chambers on natural substrates (e.g. bark, rotten wood). The plasmodia were sampled in the field or grown from spores. Gilbert and Martin (1933) discovered the occurrence of myxogastrids in moist chamber cultures by accident. They intended to grow the green alga *Protococcus,* found on bark, in a moist chamber but observed fruiting bodies of myxogastrids. Since then, fruiting bodies derived from moist chambers complement field collections (Stephenson *et al.* 1994).

In a crude culture myxogastrids are grown on artificial medium that is sterile, but the plasmodial inoculum is not freed from bacteria. Oat agar that consists of autoclaved rolled oats and agar was commonly used. It is thought that the bacteria do not overgrow the plasmodium, but can serve as a food source besides the nutrients of the medium. Crude cultures were for example used to study the life cycle of myxogastrids and are still commonly used for other studies (Nakagaki *et al.* 2000; Tero *et al.* 2010; Reid *et al.* 2012; Alim *et al.* 2013).

The establishment of pure cultures requires more effort. Early researchers claimed to have grown plasmodia in pure cultures, but Cohen (1939) considered previous experiments as doubtful and wanted to establish adequate criteria for purity. Since then, three different methods are known to free the plasmodium from contaminants. The migration method, in which plasmodia migrate over several non-nutrient water agar plates, was fully described by Cohen (1939). After migrating over the first agar plate, a piece of plasmodium, which did not cross its own track, is transferred to the next agar plate. During this procedure, which is usually repeated three times, the migrating plasmodium leaves contaminants behind. The migration method works especially for myxogastrid species that have a highly motile plasmodium with a loose, abundant slime (*Physarum*, *Badhamia*). The purification of species that migrate more slowly (*Fuligo, Didmyium*) and have an adherent, tenacious slime is more difficult. Motile and rapidly proliferating bacteria disturb the purification procedure (Cohen 1939; Sobels 1950; Daniel & Baldwin 1964). Currently, the migration method is frequently used to free plasmodia from bacteria before starting a sterile shaken culture.

Cohen (1939) also described the enrichment method, in which plasmodia are fed with living yeasts (*Saccharomyces cerevisiae* or *Saccharomyces ellipsoideus*) that are provided as a heavy suspension. The plasmodium moves along a streak of this suspension and contaminants are left behind. The following migration over one or two water agar plates removes *Saccharomyces* from the plasmodium. The enrichment method ensures constant vigor of the plasmodium, whereas during the migration method the starving plasmodium can die or sclerotize. However, Cohen (1939) considered the migration method overall as more successful and easier to establish pure cultures.

Antibiotic decontamination is the third method for the obtainment of sterile cultures. When penicillin G and dihydrostreptomycin sulfate were added to agar, five out of twelve cultures could be freed from bacteria (Sobels & Cohen 1953). Hok (1954) reported that low concentrations of bacitracin, tyrothricin and subtilin were also tolerated from the plasmodium. However, Lazo (1960) found changes in growth characteristics of plasmodia treated with streptomycin.

Although plasmodia can be freed from bacteria with these methods, the maintenance in bacteria-free culture was not possible for a long time. Cohen (1939) was not able to attempt prolonged growth of sterile plasmodia trying different media (e.g. yeast extract agar, oatmeal agar and pea agar). Daniel and Rusch (1961) finally succeeded in growing plasmodia in a partially defined, soluble medium, that consisted of tryptone, glucose, yeast extract, $CaCO_3$, inorganic salts and chick embryo extract. Bacteria-free plasmodia were shaken in this medium resulting in the fragmentation into multinuclear microplasmodia that vary in size and shape. Daniel *et al.* (1962) demonstrated that chick embryo extract can be replaced by hematin. Moreover, yeast extract was replaced by thiamin and biotin, whereas a mixture of amino acids replaced tryptone (Daniel *et al.* 1963; Daniel & Baldwin 1964), which was questioned by Carlile (1971). In summary, Daniel and Baldwin (1964) established three different completely defined media (termed as synthetic media) as well as the most frequently cited semi-defined medium that is commonly used from different authors until now (Hosoda 1980; Starostzik & Marwan 1995; Rajan *et al.* 2014). Nevertheless, Carlile (1971) was not able to grow *P. polycephalum* in the semi-defined medium and completely defined media of Daniel and Baldwin (1964), but modified the formula of the semi-defined medium successfully. The cultivation of amoebae from *P. polycephalum* without bacteria was not successful until McCullough and Dee (1976) developed defined and semi-defined media for the growth of amoebae in sterile shaken cultures. Until then, amoebae were cultured on formalin-killed *Escherichia coli* on agar.

Unfortunately, pure cultures (in amoebal and plasmodial stage) turned out to be only suitable for nutritional, but not life cycle studies. Hu *et al.* (1985) observed a decreased longevity in axenic liquid cultures that were maintained for longer than 700 days. Furthermore, plasmodia lose their ability to sporulate in axenic shaken cultures. Re-culturing on oats (Hosoda 1980) and the contamination of axenic cultures with bacteria or green algae can restore the ability to sporulate (Lazo 1961).

In summary, growth of *P. polycephalum* in axenic cultures is established by now, but it should be emphasized that sterility proofs were always restricted to bacteria, which are cultivable. Moreover, microscopic observations were regarded as sterility proofs as well. The aim of this work was to extend sterility proofs to a molecular level to detect possible non-cultivable bacteria.

2 Material and Methods

2.1 Media for the Cultivation of Plasmodia and Microorganisms

Semi-defined (SD) medium for the growth of (micro)plasmodia

No growth was obtained by using the frequently cited semi-defined (SD) growth medium from Daniel and Baldwin (1964). This was also reported by Carlile (1971), who described a modified composition of the SD medium, which we slightly adjusted. Table 1 shows the composition of the adjusted SD medium. Per ml medium 5 µg Hematin are required. For the stock solution 0.05 g Hematin was dissolved in 100 ml NaOH (1%). The solution was autoclaved separately and stored at 4°C in the dark. The SD medium (solid or liquid) was stored at 4°C as well. It was added prior to inoculation of the shaking culture or pouring to cooled agar, respectively.

Table 1: Semi-defined growth medium. Quantities given are for 1000 ml of medium. Adjusted to pH 4.6 with 10% NaOH.

Component	Quantity
Glucose	10 g
Peptone	10 g
Hematin	10 ml
Agar (optionally)	20 g (2%)
Citric acid•H_2O	3.54 g
K_2HPO_4	2.0 g
$MgSO_4$•$7H_2O$	0.6 g
$CaCl_2$•$2H_2O$	0.6 g
$FeCl_2$•$4H_20$	0.06 g
$ZnSO_4$•$7H_20$	0.034 g
Na_2EDTA	0.224 g

Water agar

Nonsterile growth (with oatflakes added) and the first steps of the sterilization procedure required water agar ranging from 1.2% to 1.5%. Plates for the cultivation of *Fuligo septica* plasmodia were prepared twice as thick as usual agar plates (see 3.1.2).

Nutrient Broth No.2 (NB-2) medium

Nutrient Broth No.2 (NB-2) medium from Oxoid was prepared in accordance with the manufacturer's instructions (with agar for solid media) and used for the cultivation of different bacteria.

YPD-medium

The YPD-medium was used for the cultivation of *Saccharomyces cerevisiae*. 10 g yeast extract, 20 g peptone, 20 g glucose and 20 g agar (variable) were used per liter medium.

2.2 Cultivation and Identification of Different Myxogastrid Species

2.2.1 Three different strains of myxogastrids and their sources

Sclerotia from *P. polycephalum* were received from Prof. Andrew Adamatzky (Department of Computer Science, University of the West of England, Bristol, UK). Fresh plasmodia from *B. utricularis* and *F. septica* were received from Ao.Univ.-Prof. Dr. Martin Grube (Institute of Plant Sciences, University of Graz, Graz, Austria). In 2012, *B. utricularis* was sampled in a forest called Leechwald (Graz, Austria), whereas *F. septica* was collected in 2013 in a forest called Forêt domaniale de Liffré, which is located 20 km northeast of Rennes (Bretagne, France). The identity of the collected strains was unknown and required identification with molecular methods as well as fruiting body determination.

2.2.2 Cell culture under non-sterile conditions and production of sclerotia backups

The plasmodia were cultivated on water agar (1.2 %) and fed with non-sterile oat flakes. Once two-thirds of an agar plate was overgrown, 2x2 cm pieces of plasmodium were excised together with the agar and transferred to a new agar plate. Based on observations, culture conditions were individually adjusted even if no annotations could be found in current literature. *F. septica* seemed to require moister conditions for growth. The plates were kept in boxes (slightly opened) to increase humidity.

If fresh cultures were required, previously made sclerotia backups were put on water agar and moistened with water. Sclerotia were derived using a protocol of Strachauer and Guttmann (2012). First mass production of plasmodia had to be induced. For this, a plastic box was filled with 400 ml water agar (1.2 %). Pieces of agar with plasmodium (2x2 cm) were excised from agar plates that were two-thirds overgrown with plasmodium and trans-

ferred to one end of the box. A line of delicate oat flakes (Kölln) was spread 10 cm from the agar pieces. Closing the box or leaving it ajar modified the humidity and was adjusted individually. To avoid dehydrogenation of the plasmodium, due to the dry oat flakes, it was necessary to moisten the oat flakes with some deionized water. Wet filter paper was also placed on both sides of the box. Whenever the plasmodium crossed the line of oat flakes, a new line of oat flakes was placed to the front. Once the plasmodium reached the end of the box and covered the agar surface, it was ready to harvest. Figure 1 shows a plastic box covered with the plasmodium of *B. utricularis*.

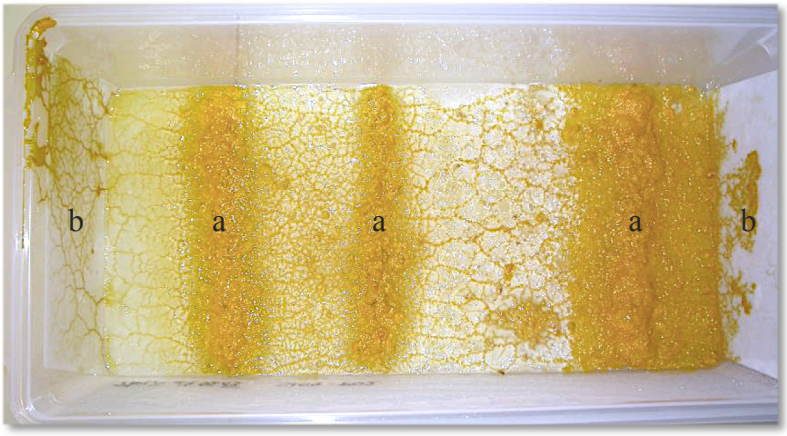

Figure 1: Mass production of plasmodia. Plastic box is filled with 400 ml water agar (1.2%) and covered with plasmodium of *B. utricularis*, which is ready to harvest. **a:** lines of oat flakes. **b:** wet filter paper.

The next step after mass production was to cover the bottom of a bucket with paper towels. Wet filter paper was put on the inner surfaces as well. Excised pieces of agar with plasmodium (2x2 cm) were transferred to the bottom of the bucket and fed for the last time with oat flakes. Incubation required 2-3 days and was stopped if two-thirds of the filter paper were covered with plasmodium. Finally, it was dried in a half open box. During the drying process of three days to one week, sclerotia formation took place. Subsequently the filter paper was cut into smaller pieces and stored in envelopes. Black parts, which indicate sporulation, were sorted out. If recent plasmodia were required, they were grown out of these backups. In regular intervals, cultures were renewed from growing these backups.

According to the protocol above sclerotia backups of *P. polycephalum* and *B. utricularis* were derived. Due to the slower growth, this method could not be applied to *F. septica* (see section 3.1.2).

2.2.3 Molecular and morphological identification of *B. utricularis* and *F. septica*

The identity of the collected samples (2.2.1) was determined by sequencing the ITS region as well as the 18S rDNA. Samples for DNA extraction were taken from the migrating front area of plasmodia from *P. polycephalum, B. utricularis* and *F. septica*, which were grown on agar plates (1.2 %). For this, some plasmodium was gathered carefully with an inoculation loop and transferred into lysis buffer. DNA extraction was performed with the DNeasy Plant Mini Kit (Qiagen). The DNA concentration was measured with a spectrophotometer (Implen NanoPhotometer) to determine the concentration of template DNA for the following PCR. The PCR was performed with the PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare). To amplify the ITS region of the myxogastrids the primers PHYS-5, PHYS-2, PHYS-3 and PHYS-4, designed from Martín *et al.* (2003) were used. Table 2 shows positions and sequences of these primers. To amplify the SSU, primers at both ends of the 18S rDNA sequence from *P. polycephalum* were designed with the program Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). They worked for *P. polycephalum* but not for *B. utricularis* and *F. septica* (multiple bands appeared in the gel). This is due to the numerous introns, which are included in the 18S rRNA gene of myxogastrids (Fiore-Donno *et al.* 2008).

The second approach was performed with primers designed from Fiore-Donno *et al.* (2008). Due to the large size and sequence divergence in the 18S rDNA they designed 35 specific primers. To find some ideal primers, a multiple alignment of 38 derived myxogastrid sequences from Fiore-Donno *et al.* (2008) was performed using MEGA software Version 5.2.2 (www.megasoftware.net). Introns were determined by the NCBI database (www.ncbi.nlm.nih.gov) and deleted with MEGA software. As a result the highly conserved regions within the SSU region were detected. Only six out of 35 primers, which were in the range of these highly conserved regions, were selected for the amplification (Table 3).

Table 2: Primers used for the amplification of the ITS region. Primers were designed from Martín *et al.* (2003) to amplify the ITS region of myxogastrids.

Primer	Positions	Sequence
PHYS-5	SSU-1932	5'…GGAAGCAGAAGTCGTAACAAGG…3'
PHYS-2	5.8S-30	5'…CTGCGCTCTTCATCGAAGC…3'
PHYS-3	5.8S-48	5'…GCATCGATGAAGAACGCAG…3'
PHYS-4	LSU-39	5'…TTCCTCCGCTGACTAATATGC…3'

Table 3: Primers used for the amplification of the 18S rDNA. Six out of 35 specific primers, designed from Fiore-Donno *et al.* (2008), were picked to amplify the 18S rDNA. Binding sites for these primers were found within the 18S rDNA exons of 38 myxogastrid species.

Primer	Sequence
S414F	5'…GAAGGCAGCAGGCGCGCAA…3'
SR12	5'…CGAACCCTTAACTTTCGC…3'
S12m	5'…GACGATCAGATACCGTCGTAGTC…3'
SR18	5'…GTCTCGCTCGTTATCGGA…3'
S13	5'…GAGTATGGTCGCAAGGCTG…3'
S44R	5'…GAACATCTAAGGGCATGACTG…3'

The PCRs were performed in a G-Storm thermocycler (AlphaMetrix Biotech) with the following conditions: 94°C for 1 min (initial denaturation), 35 cycles of denaturation (20 s at 98°C), annealing/extension (2 min at 52°C) and final extension (10 min at 72°C). For the amplification of the 18S rDNA from *F. septica* a Touchdown-PCR (from 59°C to 52°C) was performed. The PCR products were stored at 4°C. Subsequently, the PCR products were purified with the E.Z.N.A.Cycle Pure Kit (Omega Bio-Tek) and sequenced by Microsynth Austria GmbH (Vienna). The resulting sequences were adjusted with MEGA software by masking low quality segments on both edges of the sequences. Finally, they were joined and compared to the NCBI database with the Basic Local Alignment Search Tool (BLAST).

Species were also determined by their morphological characteristics. Plasmodia were exposed to visible light under room temperature (21-25°C) and starving conditions to induce fruiting body formation (Wormington & Weaver 1976). Subsequently, fruiting bodies were determined with the key from Neubert *et al.* (1993). Important features for the determination were for example size, structure, color, lime deposition, spores and the capillitium.

2.3 Growth of Myxogastrids in Pure Culture as Microplasmodia

Plasmodial surface cultures were purified with the migration method, referring to Cohen (1939) (see also 1.4). A plasmodial fragment (approximately 2x2 cm) from an agar plate with oat flakes is transferred to a new water agar plate. After migrating over the first plate a piece from the front of the plasmodium is transferred to the next agar plate. This pro-

cedure is usually repeated three times to remove contaminants. It is important that the plasmodium did not cross its own track (visible by its trail of slime), which would probably cause the uptake of already removed contaminants. After removing the contaminants another transfer to solid SD medium was conducted to build up more mass of the starving plasmodium. The plates were kept at room temperature (22-25°C) in complete darkness.

The following procedure was basically in accordance with the description of Daniel and Baldwin (1964) except the changed volumes referring to Carlile (1971). A 500-ml Erlenmeyer flask was filled with 50 ml semi-defined medium and 500 µl Hematin stock solution (described in 2.1). A piece of bacteria-free plasmodium from the surface culture was placed on the bottom of the Erlenmeyer flask. To avoid degeneration, it is important that the plasmodium does not submerge. After 48 hours of incubation without shaking at room temperature and in the dark, agitation was started on an orbital shaker (Infors HT) at 200 rpm and 25°C. The plasmodium fragments into multinuclear microplasmodia. Figure 2 shows the different sizes and shapes of microplasmodia of a 72-hour liquid culture.

To incubate a new shaken culture, 1 ml of a 72-hour-old shaken culture was transferred to 50 ml SD medium. To harvest microplasmodia, the liquid culture was centrifuged at 500 rpm for 3 minutes and resuspended in medium or water. If transferred to water agar or SD agar, microplasmodia fused and generated a sterile macroplasmodium within 6-12 hours for further experiments.

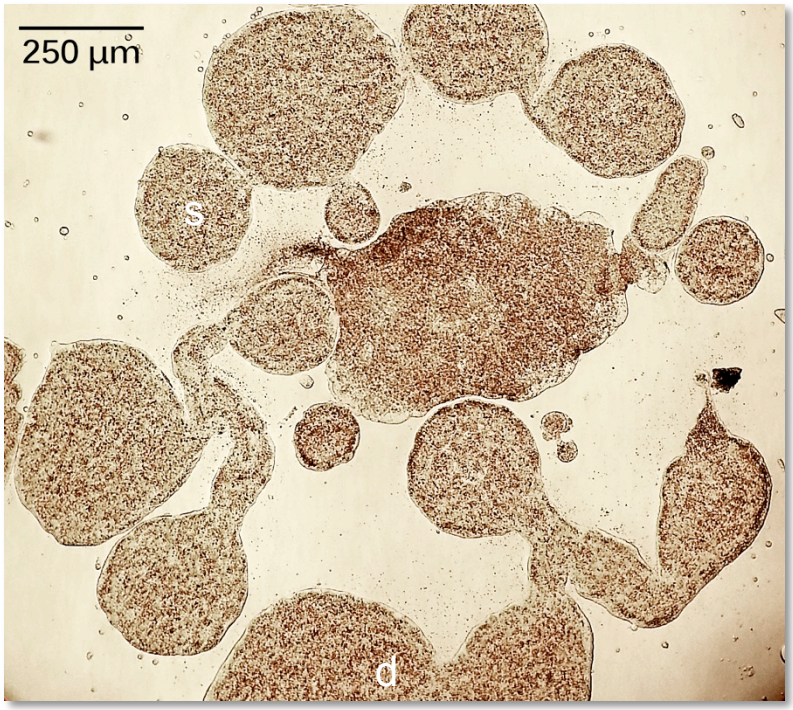

Figure 2: Microplasmodia of a 72-hour-old axenic shaken culture. Shaking macroplasmodia at 200 rpm and 25°C induced fragmentation into multinuclear microplasmodia. Different sizes and shapes of microplasmodia are visible: single microplasmodium (**s**) and dumbbell-shaped microplasmodium (**d**).

2.4 Identification of Bacterial Contaminants of (Micro)Plasmodia

During the experiments with *P. polycephalum,* different cultivable bacteria species were isolated under non-sterile as well as under putative sterile conditions. They were cultured on NB-2 agar plates at 30°C and identified by their 16S rDNA. The sources of these bacteria are described briefly in the following. Bacteria were derived from NB-2 plates, on which moistened backup sclerotia from *P. polycephalum* were placed and incubated. Moreover bacterial colonies, which appeared during the process of sterilization (second/third passage) in the plasmodial trace, were isolated and identified. Shaken cultures were plated on NB-2 plates from time to time to ensure their purity. Two bacterial isolates derived as contaminants of the shaken cultures (both fourth passage) were identified as well.

With a sterile wooden pick material from colonies of bacteria was taken, stirred in 10 µl deionized water and denatured for 5 minutes at 95°C. 1 µl of this suspension was used for the following PCR. To amplify the 16S rDNA the universal primers Com1 and Com2 (the latter not phosphorylated), designed from Schwieger and Tebbe (1998), were used (Table 4). Furthermore, the Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for the PCR reactions. Table 5 shows the PCR mixture for a 20 µl reaction volume. The PCR was performed in a G-Storm thermocycler (AlphaMetrix Biotech) under the following conditions: 98°C for 3 min (initial denaturation), 30 cycles of denaturation (20 s at 98°C), annealing/extension (40 s at 50°C) and final extension (7 min at 72°C). The PCR products were stored at 4°C. Subsequently the PCR products were purified with the E.Z.N.A.Cycle Pure Kit (Omega Bio-Tek). The half of the purified PCR products was required for sequencing by Microsynth. The other half was used for the SSCP procedure described in 2.5. The resulting sequences were adjusted with MEGA software by masking low quality segments on both edges of the sequences. Finally, they were compared to the NCBI database with BLAST. After the identification bacteria were stored in glycerol (40%) stocks.

Table 4: Universal primer pair Com1/Com2. Primers are used for the amplification of an approximately 400 bp long fragment of the bacterial 16S rRNA gene (Schwieger & Tebbe 1998). Phosphorylated (Ph) Com2 was only used for SSCP purposes.

Primer	Positions in *E. coli*	Sequence
Com1	519 to 536	5'CAGCAGCCGCGGTAATAC3'
Com2/Com2-(Ph)	907 to 926	5'CCGTCAATTCCTTTGAGTTT3'

Table 5: PCR mixture with primer pair Com1/Com2. 20 µl reaction volume. Phosphorylated (Ph) Com2 was only used for SSCP purposes**.**

Component	[µl]
H_2O	variable
5X Phusion HF Buffer	4
dNTPs (10 mM)	1
Primer Com1 (10 mM)	2
Primer Com2-(Ph) (10 mM)	2
Template DNA	variable
Phusion DNA Polymerase	0.1

2.5 Purity Determination of Microplasmodial Cultures with Single-Strand Conformation Polymorphism (SSCP)

2.5.1 Sampling, DNA extraction and amplification of 16S rDNA

Samples were taken from two 72-hour-old, putative sterile, shaken cultures of *P. polycephalum* (second passage) and one culture of *B. utricularis* (first passage). 300 µl microplasmodial suspension of each culture was disrupted with metal beads in a Tissue Lyser II (Qiagen) for three minutes at maximum speed. The DNA extraction was performed with the DNeasy Plant Mini Kit (Qiagen). The DNA concentration was measured with a spectrophotometer (Implen NanoPhotometer) to determine the concentration of the template DNA for the following PCR.

To amplify the bacterial 16S rDNA, the universal primers Com1 and Com2 (the latter phosphorylated) according to Schwieger and Tebbe (1998) were used (Table 4). The PCR procedure was the same as described in 2.4. The PCR products were purified with the E.Z.N.A.Cycle Pure Kit (Omega Bio-Tek) and used for the SSCP. For comparison and confirmation the 16S rDNA PCR products from different bacterial contaminants were also used for the SSCP (see 2.4).

2.5.2 Preparation of the single-stranded DNA samples for the SSCP and denaturation

First, the cleaned PCR products were digested by a λ-exonuclease (Fermentas). For each sample, 20 µl cleaned DNA was mixed with 0.75 µl λ-exonuclease and 2.3 µl 10x λ-exonuclease buffer (prepared as a mastermix). The samples were incubated for one hour at 37°C. The digestion of the phosphorylated strand (obtained with the phosphorylated reverse primer) leads to single-stranded DNA with sequence specific conformations.

Denaturation of the digested products required addition of 30 µl loading buffer to each sample. The loading buffer is composed of 950 µl formamide (95 %), 4 µl 2.5 M NaOH solution (10 mM), 5 µl bromphenol blue solution (5 %) and 41 µl deionized water. Subsequently, the preparation was heated for 5 minutes at 98°C to induce denaturation and was immediately put on ice for 5 minutes, which effects the different conformations of the single strands.

2.5.3 Preparation of the MDE gel electrophoresis and silver staining

For the sandwich assembly, two glass plates (one with wells) were cleaned with 70% EtOH. Afterwards spacers and a silicone sealing was placed on the glass plate with the wells and repellent solution (Acryl Glide) was applied. 1 ml of distilled water was spread on the glass plate without the wells for improved contact before placing a polybond film (Biometra). The two plates were assembled with proper alignment by fixing them with metal clamps.

Considering the length of the PCR products (~ 400 bp), a gel with a concentration of 8.75% and 60 ml total volume was prepared as follows: double distilled water (24.6 ml), 5x TBE buffer (12 ml) and 2x MDE solution (23 ml, Lonza) were mixed, whereas TEMED (28.3 µl, BioRad) and fresh APS 10% (283 µl, AppliChem) were added in the end (= start of the polymerization). Using a syringe, the assembled sandwich was filled with the gel. After the polymerization, the clamps and glass plates were removed and the gel on the polybond film was ready for further use.

The SSCP electrophoresis was performed in a TGGE Maxi system (Biometra). Half a liter TBE buffer was poured into each tank and buffer wicks were placed on the gel, as well as a cover film. The following step was the equilibration of the electrophoresis chamber (pre-run for 10 minutes, 400 V and 20°C) and the samples were loaded in the slots (9 µl each). To visualize the straightness of the lanes, the two outer slots on each side were loaded with 2 µl GeneRuler^{TM} 1kb DNA ladder. Electrophoresis ran for 22 hours (400 V, 26°C).

After the electrophoresis, the silver staining was carried out. For this, the gel was put in a box and the following steps were performed under shaking. First the gel was fixed with 10%

acetic acid (300 ml) for 30 minutes, followed by three times rinsing with distilled water (5 minutes each). After the rinsing, silver staining was conducted with 400 ml $AgNO_3$ (0.1%) and 1.5 ml formaldehyde (37%) for 30 minutes in darkness. Another washing step (10 seconds in deionized water) was followed by the development of the bands with 500 ml NaOH (3%) and 2 ml formaldehyde (37%) in darkness. As the bands became visible, the reaction was stopped with 300 ml acetic acid (10%).

2.5.4 Recovery of the SSCP bands and reamplification of the 16S rDNA

Visible bands were excised from the SSCP gel and soaked in 500 µl crash soak solution (extraction buffer respectively), which consisted of magnesium acetate (105.75 mg), ammonium acetate (1.93 g), EDTA (18.5 mg) and 20% SDS (250 µl) per 50 ml. The bands were frozen overnight at -20°C, then incubated at 60°C for one hour and finally stored at 4°C for ten days. Subsequently, the bands were centrifuged for 15 minutes at 13.000 rpm, thus the supernatant discarded and 400 µl isopropanol was added. The bands were incubated overnight at -20°C, centrifuged for 15 minutes at 13.000 rpm and washed with 400 µl 70% EtOH after discarding the supernatant. After another centrifugation step and discarding of the EtOH, the pellet was dried and resuspended in 30 µl TRIS (10 mM).

The reamplification of the cleaned DNA was conducted as described in 2.5.1 with the primers Com1 and Com2 (this time not phosphorylated) and 10 µl as a template. Cleaned PCR products that showed low concentrations were submitted to a nested PCR with the same primers. In the case of multiple bands, a preparative gel was run, of which the bands were excised under UV light. The DNA was recovered using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek) and sequenced by Microsynth. The resulting sequences were adjusted with MEGA software by masking low quality segments on both edges of the sequences. Finally, they were compared to the NCBI database with BLAST.

2.6 Observation of Microplasmodial Cultures under Sterile Conditions

The growth of microplasmodia of *P. polycephalum* in sterile shaken cultures was observed for 97 hours. The microscopic observations were sketched and the growth was quantified on the basis of pigment absorption, wet weight and dry weight.

For growth determination by measuring the pigment absorption, a modified extraction protocol of Chapman *et al.* (1983) was used. All steps were performed under dark conditions due to the sensitivity of plasmodial pigments against light. The observation started once a new

subculture was implemented by transferring 1 ml microplasmodial suspension from a previous 72-hour-old culture into 50 ml liquid SD medium. The cultures were shaken in complete darkness on an orbital shaker (Infors HT) at 200 rpm and 25°C.

Two samples (1 ml each) were collected after 0, 24, 31, 48, 52, 57, 74, 81 and 97 hours of growth and centrifuged at 14.000 rpm for 1 min. The supernatant was discarded and the pellet resuspended in 500 µl deionized water. The suspension was transferred into 15 ml Sarstedt tubes with 10 ml TCA/acetone (5 ml TCA plus 5 ml acetone) and incubated for 10 minutes to extract the pigment. The tubes were centrifuged at 500 rpm for 3 min and 1 ml of the supernatant was again centrifuged at 14.000 rmp for 8 minutes. The supernatant was measured with a spectrophotometer (Implen NanoPhotometer) at a wavelength of 415 nm. This wavelength was previously determined by a wavelength scan with the mentioned spectrophotometer.

To assay the growth on the basis of wet and dry weight, another shaken culture was used for this purpose. The procedure of subcultivating, shaking conditions and sampling, were the same as described before. Before taking the samples the reaction tubes were weighed. The collected samples (1 ml each) were centrifuged at 14.000 rpm for 1 min, the supernatant was discarded and the weight of the wet pellet was determined. Moreover, the dry weight was conducted by resuspending the wet pellet in 500 µl deionized water and letting it dry at 110°C overnight.

Statistical analyses were performed with the programming language R (www.r-project.org), especially using the package “grofit” for fitting of growth curves (Kahm *et al.* 2010). This package can fit growth curves obtained under different conditions and if required, can also generate dose-response curves. Parameters such as lag phase, maximal growth rate and stationary phase can be derived. The package fits data to different parametric models (Gompertz law, modified Gompertz law, logistic law and Richards law) and also provides a model free spline fit. Since no saturation (maximum growth A) was reached by the data, the model free fit, which applies a smoothed cubic spline, was used. The smoothness of the spline fit (smooth.gc) was set 0.5. The written R code can be found in Appendix A.

2.7 Microplasmodial Growth with Heat-killed Microorganisms

To investigate the ability of microplasmodia of *P. polycephalum* to utilize different strains of microorganisms as a food source the growth of microplasmodia with heat-killed (but undamaged) bacteria and yeast was observed. According to Chapman and Coote (1983), heat-killed preparations are necessary because living bacteria would overgrow the microplasmodia in the rich semi-defined (SD) medium. However, we were not able to reproduce the protocol of Chapman and Coote (1983) regarding the production of heat-killed bacteria. Considering other protocols (Campbell *et al.* 2001; Jazani *et al.* 2010), heat-killed bacteria were prepared. Overnight cultures of *Escherichia coli*, *Pseudomonas putida*, *Serratia plymuthica* and *Paenibacillus* sp. were derived in 5 ml liquid NB-2 medium (see 2.1) at 30°C in an orbital shaker (Infors HT). *S. cerevisiae* was grown in 5 ml liquid YPD medium (see 2.1) under the same conditions. *E. coli* and *P. putida* were derived from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Univ.-Prof. Dr. Gabriele Berg (Graz, University of Technology) provided *Serratia plymuthica* 3Re4-18. *Paenibacillus* sp. was isolated as a contaminant (see 3.2). ONCs were inoculated in 45 ml NB-2 medium and grown for another four hours at 30°C. Subsequently, the microorganisms were harvested by centrifuging at 5000 rpm for 8 minutes and resuspended in 2 ml PBS buffer (1x) each. After one washing step with PBS buffer, the pellet was resuspended in 2 ml PBS again. The suspension was heated for four hours at 85°C and stored at -20°C afterwards. The bacteria were plated on NB-2 agar plates (YPD agar plates for *S. cerevisiae*) to verify successful killing. The integrity of the cells was evaluated by light microscopy. The OD_{600} of the suspensions was determined with a spectrophotometer.

P. polycephalum was grown as microplasmodia in shaken cultures (see 2.6). 1 ml of a 72-hour-old microplasmodial culture (31st passage) was transferred in a 500-ml Erlenmeyer flask with 50 ml liquid SD medium (see 2.1) omitting peptone. Furthermore, 2 ml of the heat-killed preparation were added. *P. polycephalum* grown in SD Medium with peptone and without peptone served as positive and negative control, respectively. The cultures were shaken at 200 rpm and 25°C. 1-ml samples each were collected after 0, 24, 48, 66, 72 and 91 hours of growth and centrifuged at 14.000 rpm for 1 min. Wet weight and dry weight measurements were conducted as described in 2.6. Statistical analyses and the fitting of growth curves are also described in 2.6.

2.8 Persistence of Applied Bacteria in/on Slime Mold Plasmodia

It was investigated how long different bacteria species and a yeast persist in or on plasmodia of *P. polycephalum*. Axenic shaken cultures of *P. polycephalum* (31st passage) were obtained as described in 2.6. Microorganism suspensions of *Escherichia coli*, *Pseudomonas putida*, *Serratia plymuthica*, *Paenibacillus* sp. and *S. cerevisiae* were prepared as described in 2.7 with the difference that microorganisms were not killed. 1 ml of a 72-hour-old microplasmodial culture was transferred on a water agar plate. Moreover, 250 µl of a microorganism suspension (a yeast suspension respectively) was added on the same plate. Suspensions had an OD_{600} of 30 except the suspensions of *Paenibacillus* sp. (OD_{600} = 6) and *S. plymuthica* (OD_{600} = 13). The plates were kept at room temperature (22-25 °C) in complete darkness. Microplasmodia were allowed to fuse to a plasmodium and feed on the bacteria. After contact with the bacteria, the migration method was applied (for details see 2.3). During each transfer to a new plate, samples of the migrating front were taken to observe if microorganisms were still present. This procedure was performed until no microorganisms were present and/or the plasmodium died. The samples were dissolved in 100 µl PBS buffer, plated on NB-2 agar plates and incubated at 30°C. As a control, *P. polycephalum* without bacteria was transferred and sampled. The persistence was also controlled visually by searching for bacteria colonies on the slime tracks.

3 Results

3.1 Identification of Different Myxogastrid Species and their Cultivation

3.1.1 Molecular and Morphological Identification of *B. utricularis* and *F. septica*

The identity of *B. utricularis* and *F. septica* was unknown as they were collected in nature and not cultured from existing laboratory strains. Species were identified by sequencing the ITS region and 18S rDNA, as well as by determining their morphological characteristics. Table 6 shows the BLAST results, which were obtained using the amplified ITS region and SSU gene, respectively (see Table 2 and Table 3 for the used primers).

Table 6: BLAST results of the ITS region and SSU. Plasmodia of myxogastrids were identified by amplifying and sequencing the ITS region and comparing them to database sequences.

Source of Plasmodium	Sequence	Most similar sequence(s)	Accession number (GenBank)	Identical in %	Query coverage in %	E-value
P. polycephalum (control)	ITS region	*P. polycephalum* gene encoding the 5.8S rRNA and 26S rRNA	V01159.1	98	48	0.0
P. polycephalum (control)	18S rDNA	*P. polycephalum* rRNA small subunit (SSU rRNA) gene	X13160.1	100	99	0.0
Leechwald (Graz, Austria)	ITS region	*P. polycephalum* gene encoding the 5.8S rRNA and 26S rRNA	V01159.1	97	20	$8*10^{-28}$
Leechwald (Graz, Austria)	18S rDNA	*Badhamia utricularis* partial 18S rRNA gene	HE614597.1	96	99	0.0
		Badhamia foliicola strain 69058 small subunit ribosomal RNA gene	KC759100.1	96	99	0.0
Forêt domaniale de Liffré (Bretagne, France)	ITS region	*Fuligo septica* 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene	AJ584697.1	99	99	0.0
Forêt domaniale de Liffré (Bretagne, France)	18S rDNA	*Fuligo septica* 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene	AJ584697.1	98	100	0.0

According to the key from Neubert *et al.* (1993), the fruiting bodies were analyzed. The identification of *P. polycephalum* as a control was confirmed (Figure 3A). No full fruiting body formation could be conducted from *Fuligo septica,* but the beginning formation of an aethalium was observed. Aethalia are a morphological characteristic of the genus *Fuligo* (Physarales), but occur in other orders as well; for example in the order Liceales (genus *Lyco-*

gala) (Neubert *et al.* 1993; Everhart & Keller 2008). The different appearance of aethalia (e.g. color, structure) in other orders together with the sequencing results led to the identification of the plasmodium collected in the Bretagne as *Fuligo septica* (Figure 3B). Based on the sequencing results, the plasmodium collected in Graz could be determined as *Badhamia utricularis* or *Badhamia foliicola.* There was no match with *Badhamia* sp. using the sequences received with the ITS primers, due to the absence of the ITS sequences in the NCBI database. Morphological analyses revealed the identity of the species collected in Graz to be *B. utricularis* (Figure 3C). A sketch of the capillitium (sterile elements in fruiting bodies) from *B. utricularis*, which also had to be investigated for the identification, is shown in Figure 3D. The capillitium forms a coarse-meshed network, which consists of flat, ribbon-like tubes filled with colorless lime. Strands are 5-30 µm thick and bow-shaped or triangularly extended at junctions.

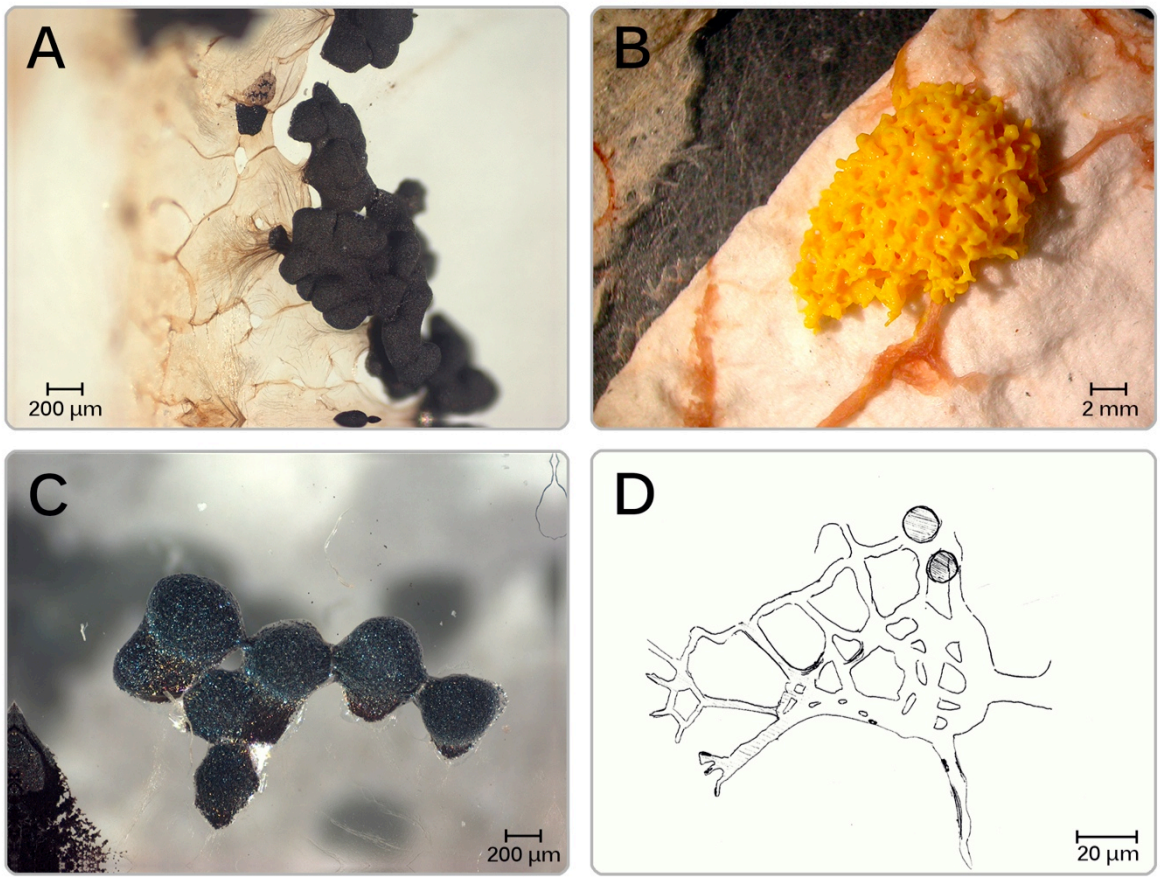

Figure 3: Morphological determination of fruiting bodies. Fruiting bodies of *P. polycephalum* (**A**) were determined as a control. The species that was identified as *F. septica* with molecular methods shows the beginning formation of an aethalium (**B**). Morphological analyses revealed the identity of the species collected in Graz to be *B. utricularis* (**C**). The capillitium from *B. utricularis* was investigated and sketched for the identification (**D**).

3.1.2 Non-sterile and sterile cultivation requirements and additional observations

During the cultivation of *P. polycephalum*, *F. septica* and *B. utricularis*, differences in growth and food preferences of these strains were observed. During the first three weeks of cultivation, *F. septica* was kept on agar plates with oat flakes, but did not use the oat flakes as a food source. This changed after several passages on water agar (supplied with oat flakes). In contrast, *B. utricularis* showed growth on oat flakes from the beginning of cultivation. Additionally, we observed furrows in the agar (mainly around consumed oat flakes) in cultures of *F. septica* that were older than approximately two weeks. Moreover, strand-like traces (about 2 mm thick) appeared, on/in which the plasmodium used to grow. Figure 4A shows the top view of a petri dish with the plasmodium of *F. septica* and left-behind thick traces. Figure 4B shows the plasmodium growing on the traces and in Figure 4C plasmodial strands that grow inside the traces can be seen as well. More vigorous growth was obtained by keeping *F. septica* on agar plates, which were twice as thick as usual. It seemed that *F. septica* had a preference for increased humidity. Furthermore, the longevity of plasmodia of *F. septica* was observed. When the food supply was stopped, plasmodia survived up to 8 weeks in the same petri dish without transferring.

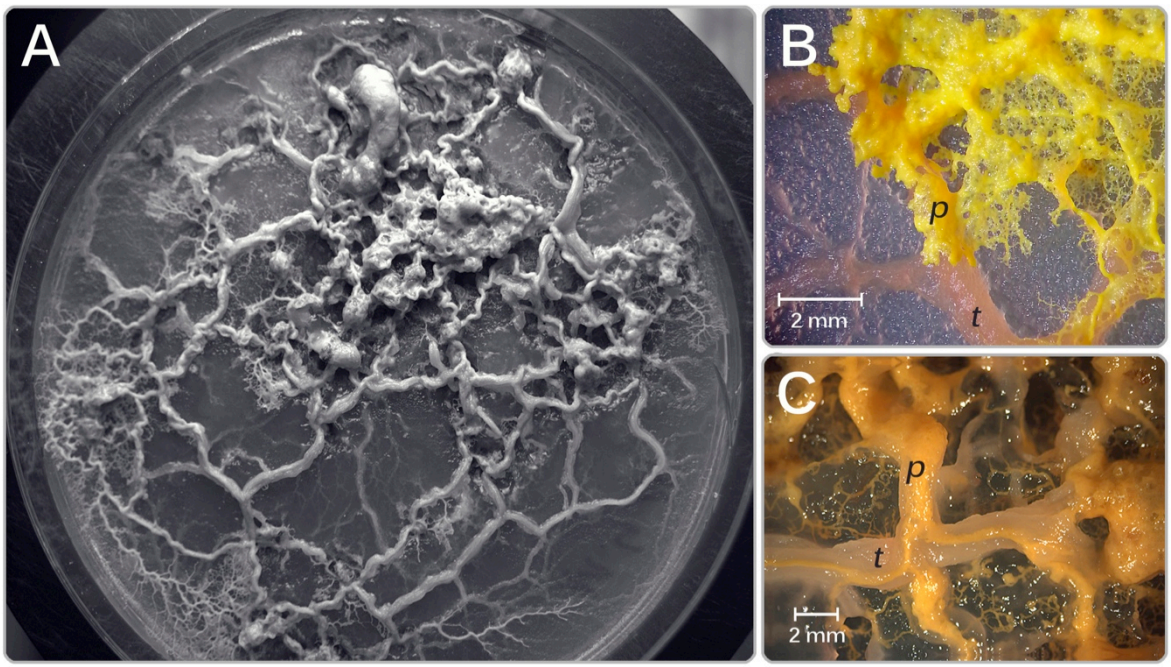

Figure 4: Plasmodium of *F. septica* growing on strand-like traces. Top view of a petri dish (Ø 90 mm) with the plasmodium of *F. septica* and left-behind thick traces (**A**). Plasmodium (p) growing on the traces (t) (**B**). Plasmodial strands (p) are also growing inside the traces (t) (**C**).

Backup sclerotia were derived from crude cultures of *P. polycephalum* as well as from *B. utricularis* and reactivation was possible. No sclerotia backup was derived from *F. septica*, due to its slower growth. Another approach was to place wet filter paper in several agar plates with plasmodia of *F. septica.* The plasmodium moved on the filter paper and was dried. The dried parts turned dark and seemed to be sclerotized, but could never be reactivated with water.

It was not possible to transfer *F. septica* in sterile shaken culture as the migration method remained unsuccessful. The migration method was successfully applied on *B. utricularis* and the transfer to a shaken culture was possible onetime. However, no vigorous growth could be attempted and growth stopped after three subcultivations.

Before the cultivation of *P. polycephalum* in shaken cultures led to success, several attempts regarding the medium were made. The growth in the SD medium of Daniel and Baldwin (1964) remained unsuccessful, but allowed us to observe the formation of so-called spherules. They have a noncrystalline cellulose-containing cell wall and are usually developed under unfavorable conditions (Ogawa *et al.* 2013). Using the adjusted SD medium of Carlile (1970) was finally successful. Furthermore one temporary culture of *B. utricularis* was obtained with this adjusted medium. The plasmodium died after three transfers with spherule formation, indicating again an inappropriate medium.

Further experiments were conducted with shaken cultures of *P. polycephalum*, since it was the only species, which could be both purified with the migration method and maintained in shaken cultures.

3.2 Identification of Bacterial Contaminants of (Micro)Plasmodia

Different cultivable bacteria species appeared under non-sterile as well as under putative sterile conditions during the experiments with *P. polycephalum*. Figure 5 shows bacteria colonies that appeared on the slime track of *P. polycephalum* under non-sterile conditions. Isolates were purified from sclerotia, slime traces and shaken cultures.

The species isolated from backup sclerotia of *P. polycephalum* was 91% identical to a partial sequence of the 16S rRNA gene from an unclassified bacterium called Bacterium LO393 (accession number: HM041150.1, query cover = 96%, E-value = 0.0). A similar result was received with the sequences obtained from the bacteria species, which appeared on the slime tracks (two isolates of second and third passage) of *P. polycephalum*. The sequences

were 92% identical to the mentioned unclassified bacterium (query cover = 95%, E-value = 0.0). Bacterium LO393 was found from Ramsden *et al.* (2010) whilst screening for antimicrobial-resistant bacteria in wastewater treatment plants.

Sequences derived from a bacterial contaminant of the shaken cultures (two isolates, each fourth passage) of *P. polycephalum*, which did not overgrow the cultures, but appeared when plated on NB-2 agar, was 100% identical to several *Bacillus* sp. and *Paenibacillus* sp. An example is the match with *Paenibacillus xylanilyticus* (accession number: FN555443.1, query cover = 99%, E-value = 0.0), an airborne bacterium (Rivas *et al.* 2005).

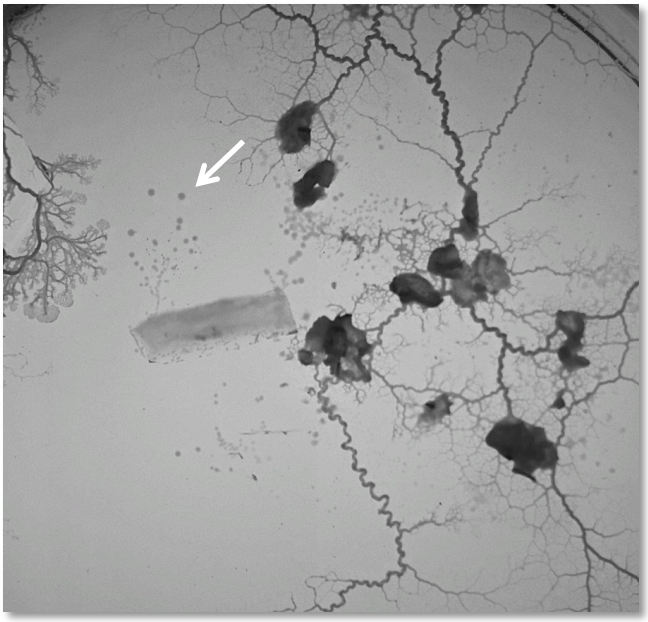

Figure 5: Bacterial colonies on slime track. Crude culture of *P. polycephalum* on water agar, fed with non-sterile oat flakes. Arrow shows bacterial colonies that appeared in the slime track after the retraction of the plasmodium.

3.3 Purity Determination of Microplasmodial Cultures with Single-Strand Conformation Polymorphism (SSCP)

Sterility proofs of putative sterile plasmodia that consider non-cultivable bacteria have never been conducted before. SSCP was used to prove the sterility of two microplasmodial cultures of *P. polycephalum* (second passage) and one temporary culture of *B. utricularis* (first passage). Bacterial DNA from the above-described contaminants (see 3.2) was used to confirm their identity, compare patterns and exclude mixed species.

Figure 6 shows the SSCP gel with the separated single-stranded DNA samples (obtained with primer pair Com1/Com2) from putative sterile shaken cultures of *P. polycephalum* and

B. utricularis as well as from the derived bacterial contaminants. Figure 7 shows the reamplificated PCR products, derived from the isolated DNA of the excised SSCP bands. Due to the low DNA concentrations a nested PCR was conducted with the samples 1c, 2d, 3a, 5a, 5b and 6a. After the nested PCR, all samples except 1c showed double bands. These samples were applied on a preparative gel and excised (see also 2.5.4). Figure 8 shows the preparative gel, in which the excised double bands are numbered with I and II. Table 7 shows the final BLAST results of the sequences that were derived from the SSCP gel as well as from the preparative gel.

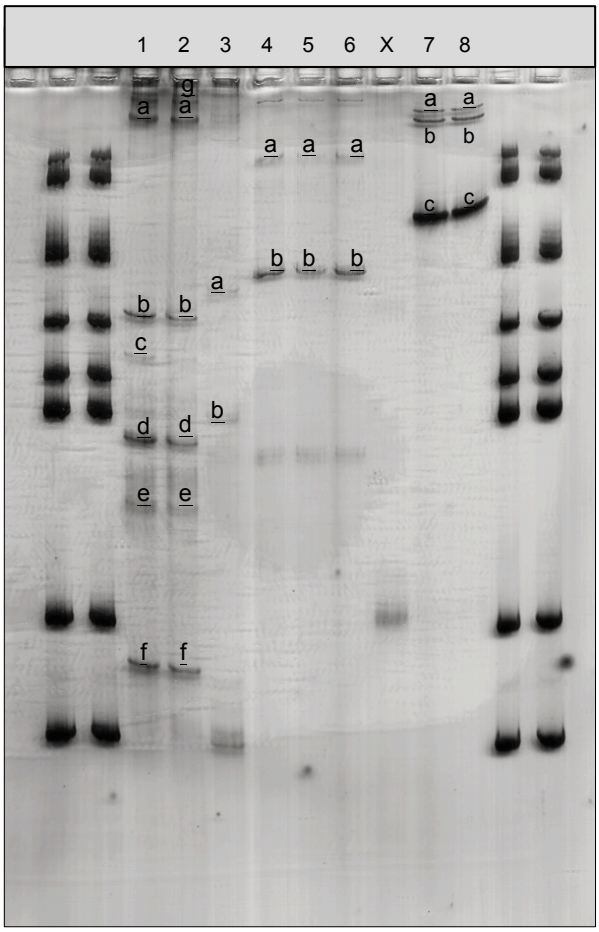

Figure 6: SSCP gel with separated single-stranded DNA samples. The separated samples that were derived from putative sterile shaken cultures of *P. polycephalum* **(lane 1 and 2)** and *B. utricularis* **(3)** are shown. Lanes 4-8 show the single-stranded DNA samples of different bacteria that were derived during experiments with *P. polycephalum* as contaminants from a sclerotia backup **(4)**, water agar plates during the migration method **(5 and 6)** and shaken microplasmodial cultures **(7 and 8)**. **X** was a non-usable sample. The PCRs were performed with the primer pair Com1 and Com2, the latter phosphorylated. Letters refer to the excised bands in each lane. To visualize the straightness of the lanes the two outer slots on each side were loaded with 2µl GeneRuler^{TM} 1kb DNA ladder.

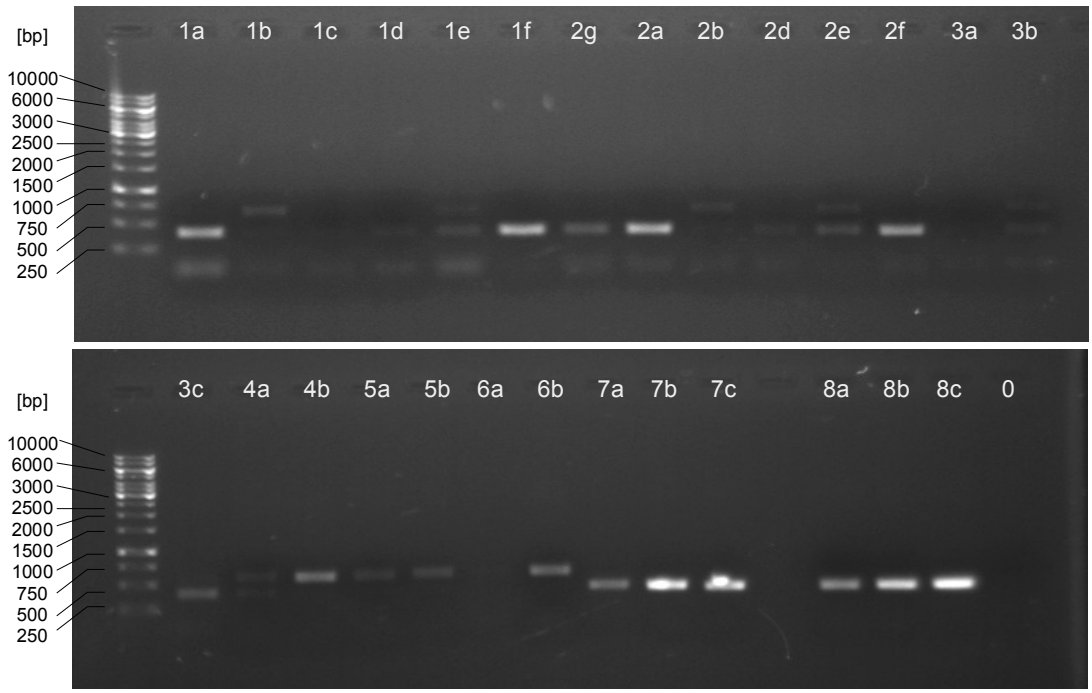

Figure 7: Reamplification of DNA from excised SSCP bands. Numbers refer to the lanes and letters to the excised bands of the SSCP gel (see Figure 6). Reamplification was performed with the primer pair Com1/Com2. 1% agarose gel with ethidium bromide, DNA ladder: GeneRuler^{TM}1kb, 0 = negative control.

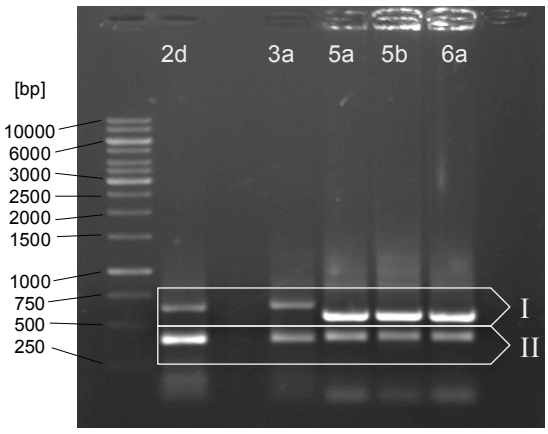

Figure 8: Preparative gel of samples with double bands. After the nested PCR double bands appeared that were applied on a preparative gel and excised. Numbers refer to the lanes and letters to the excised bands of the SSCP gel (see Figure 6). 1% agarose gel with ethidium bromide, DNA ladder: GeneRuler^{TM}1kb.

Table 7: Sequencing results of the SSCP. The BLAST results of the sequences obtained from the isolated DNA of the SSCP gel (Figure 6 and Figure 8) are shown. Two shaken cultures of *P. polycephalum* (both from the fourth passage) are numbered with I and II. The quality classification of the sequences refers to the Microsynth sequencing report.

Number of isolated band	Source of isolate	Quality of sequencing traces	Number of nucleotides of masked sequence	Most similar sequence	Accession number (GenBank)	Identical in %	Query coverage in %	E-value
1a	*P. polycephalum* shaken culture I	good	353	*P. polycephalum* mitochondrial DNA	AB027295.1	99	99	0.0
1b	*P. polycephalum* shaken culture I	good	577	*P. polycephalum* SSU gene	X13160.1	99	100	0.0
1c	*P. polycephalum* shaken culture I	superimposed signals	322	Uncultured alpha proteobacterium	AM989610.1	85	83	$1*10^{-76}$
1d	*P. polycephalum* shaken culture I	superimposed signals	353	*P. polycephalum* mitochondrial DNA	AB027295.1	96	99	$9*10^{-167}$
1e	*P. polycephalum* shaken culture I	superimposed signals	599	*P. polycephalum* SSU gene	X13160.1	94	41	$9*10^{-107}$
1f	*P. polycephalum* shaken culture I	good	361	*P. polycephalum* mitochondrial DNA	AB027295.1	99	99	0.0
2g	*P. polycephalum* shaken culture II	good	351	*P. polycephalum* mitochondrial DNA	AB027295.1	99	99	$5*10^{-179}$
2a	*P. polycephalum* shaken culture II	good	339	*P. polycephalum* mitochondrial DNA	AB027295.1	99	99	$5*10^{-174}$
2b	*P. polycephalum* shaken culture II	good	579	*P. polycephalum* SSU gene	X13160.1	97	99	0.0
2d_II	*P. polycephalum* shaken culture II	poor quality	38	*P. polycephalum* mitochondrial DNA	AB027295.1	100	100	$7*10^{-9}$
2e	*P. polycephalum* shaken culture II	partially superimposed signals	601	*P. polycephalum* SSU gene	X13160.1	99	41	$3*10^{-122}$
2f	*P. polycephalum* shaken culture II	good	356	*P. polycephalum* mitochondrial DNA	AB027295.1	99	99	0.0
3a_II	*B. utricularis* shaken culture	poor quality	28	No significant similarity	-	-	-	-
3b	*B. utricularis* shaken culture	partially superimposed signals	606	*B. utricularis* 18S rRNA gene	HE614597.1	99	44	$4*10^{-130}$
3c	*B. utricularis* shaken culture	poor quality	526	*P. polycephalum* mitochondrial DNA	AB027295.1	89	64	$3*10^{-122}$

Table 7 (continued): Sequencing results of the SSCP. The BLAST results of the sequences obtained from the isolated DNA of the SSCP gel (Figure 6 and Figure 8) are shown. For samples 4a to 8c search was refined for bacteria. The quality classification of the sequences refers to the Microsynth sequencing report.

Number of isolated band	Source of isolate	Quality of sequencing traces	Number of nucleotides of masked sequence	Most similar sequence	Accession number (GenBank)	Identical in %	Query coverage in %	E-value
4a	Bacterial contaminant from sclerotia backup	partially superimposed signals	507	Uncultured bacterium clone RKMbTu2868, 16S rDNA	KF845742.1	91	91	0.0
4b	Bacterial contaminant from sclerotia backup	good	534	Bacterium LO393 16S rDNA	HM041150.1	91	95	0.0
5a_I	Bacterial contaminant from water agar plate	good	186	Uncultured bacterium clone A16S_82, 16S rDNA	EU626480.1	86	29	$1*10^{-5}$
5a_II	Bacterial contaminant from water agar plate	poor quality	75	No significant similarity	-	-	-	-
5b_I	Bacterial contaminant from water agar plate	partially superimposed signals	156	No significant similarity	-	-	-	-
5b_II	Bacterial contaminant from water agar plate	poor quality	32	No significant similarity	-	-	-	-
6a_I	Bacterial contaminant from water agar plate	poor quality	131	No significant similarity	-	-	-	-
6a_II	Bacterial contaminant from water agar plate	poor quality	229	*Enterobacter asburiae* strain PW2a, 16S rDNA	KF673163.1	86	69	$2*10^{-43}$
6b	Bacterial contaminant from water agar plate	good	518	Bacterium LO393 16S rDNA	HM041150.1	91	98	0.0
7a	Bacterial contaminant from shaken culture	good	352	*Paenibacillus* sp. CH-f10 SSU RNA gene	KF768938.1	100	99	0.0
7b	Bacterial contaminant from shaken culture	good	351	*Paenibacillus taichungensis* strain p69_F01, 16S rDNA	JQ831276.1	99	99	0.0
7c	Bacterial contaminant from shaken culture	good	342	*Paenibacillus* sp. AS1(10) 16S rDNA	JX154347.1	99	100	$8*10^{-172}$
8a	Bacterial contaminant from shaken culture	good	364	*Paenibacillus xylanilyticus* 16 S rDNA	FN555443.1	99	100	0.0
8b	Bacterial contaminant from shaken culture	good	341	*Paenibacillus* sp. AS1(10) 16S rDNA	JX154347.1	100	100	$6*10^{-178}$
8c	Bacterial contaminant from shaken culture	good	339	*Paenibacillus* sp. AS1(10) 16S rDNA	JX154347.1	100	100	$8*10^{-177}$

As seen in Table 7 all excised bands, which were initially derived from microplasmodial cultures of *P. polycephalum* and *B. utricularis*, matched with *P. polycephalum* or *B. utricularis* except one band. The exception was the isolated band number 1c that matched with an uncultured alphaproteobacterium. Since the sequencing traces showed superimposed signals, this result requires further investigations. The received sequence and the mitochondrial DNA of *P. polycephalum* were subsequently aligned with the BLAST sequence alignment tool. No significant similarity between the mitochondrial and the alpha-proteobacterial sequence was found.

Since none of the contaminants were present in the shaken cultures it only served as a confirmation of the formerly received sequence results. The bacterium isolated from backup sclerotia and from slime track colonies was identified as Bacterium LO393, but the sequence also gave less significant matches with uncultured bacteria and *Enterobacter asburiae* (a plant growth promoting gammaproteobacterium isolated from soil). The contaminant of the putative sterile shaken cultures was confirmed to be *Paenibacillus* sp.

3.4 Observation of Microplasmodial Growth under Sterile Conditions

3.4.1 Microscopic and macroscopic observations during growth of microplasmodial cultures

Since no stable sterile microplasmodial cultures were established with *B. utricularis* and *F. septica,* the following experiments were restricted to *P. polycephalum*. To determine if the subcultivation of microplasmodial cultures after 74 hours is ideal, microscopic and macroscopic observations were made during 97 hours of growth. Figure 9 shows sketches of microplasmodia during growth in axenic shaken cultures at different time points. Sizes of microplasmodia were determined visually and varied among the whole time of observation between 125 µm and 750 µm. Shapes varied from circular to donut-shaped or dumbbell-shaped. Sizes and shapes shown in Figure 9 are not restricted to the given time point. However, the darker color of microplasmodia appeared after 74 hours of growth. Additionally, an increased viscosity was visually observed. Protoplasmic streaming, which was observed in dumbbell-shaped microplasmodia, decreased after 74 hours. First microscleorotia were observed after 97 hours indicating the decline of the culture. These observations confirmed the suggestion that axenic shaken cultures should be subcultured after 72 hours.

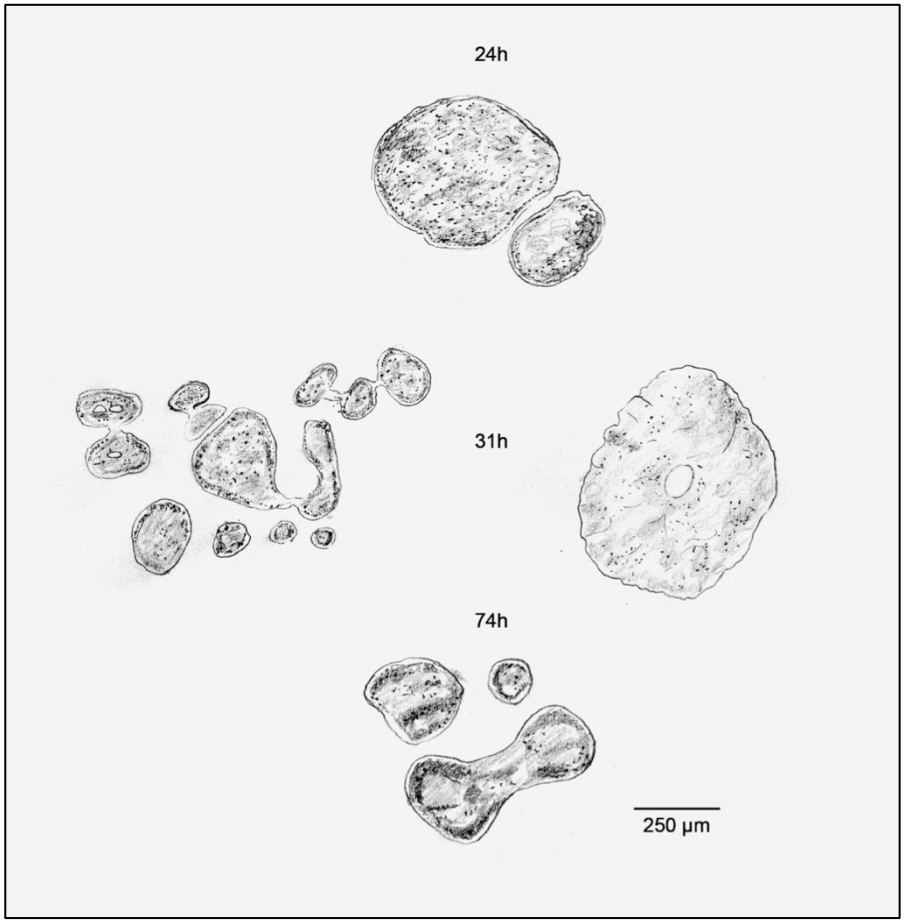

Figure 9: Sketches of *P. polycephalum* microplasmodia. Microplasmodial growth was observed under sterile conditions. Sketches of microplasmodia were taken after 24, 31 and 74 hours. The distinct sizes and shapes of microplasmodia can be seen. After 74 hours a darker color and less protoplasmic streaming was observed.

3.4.2 Growth determination of shaken cultures obtained with different methods

The establishment of sterile shaken cultures of *P. polycephalum* was successful and subcultivation after 72 hours proved to be appropriate. Subsequently, growth was determined by measuring the pigment absorption. Furthermore, the growth was assayed on the basis of wet and dry weight. On the basis of the following figures, the different methods for microplasmodial growth determination can be compared. Boxplots (Figure 10 and Figure 11) show pigment absorption at wavelength E=410nm over the time in two shaken microplasmodial cultures (both eleventh passage) of 50 ml volume. For each point of time, the absorption of a 1-ml sample was measured five times with a spectrophotometer (Implen NanoPhotometer). Samples were taken of the same culture after 0, 24, 31, 48, 52, 57, 74, 81 and 97 hours of growth.

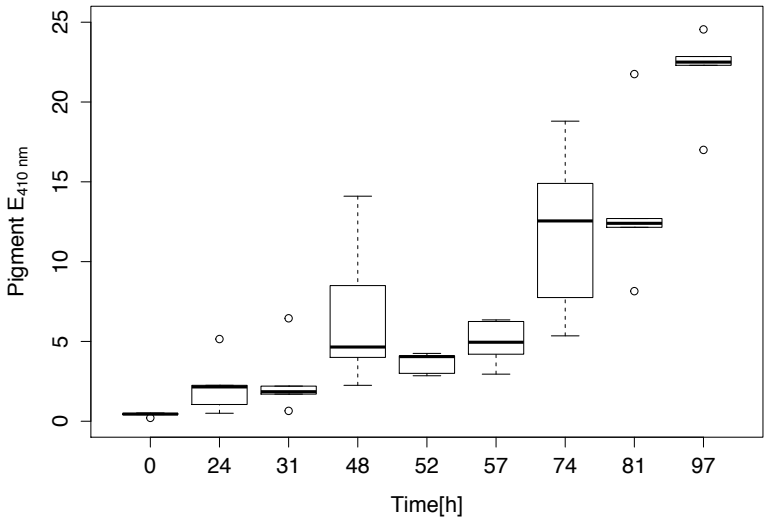

Figure 10: Pigment absorption over time. The absorption of pigments of *P. polycephalum* was measured at wavelength E=410nm over time in a shaken microplasmodial culture of 50ml volume. For each point of time the absorption of extracted pigments from a 1-ml sample was measured five times. Samples were taken of the same culture (eleventh passage).

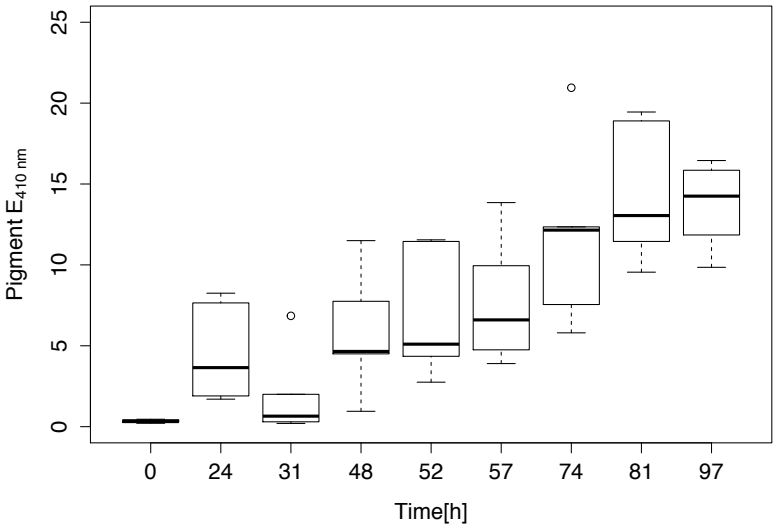

Figure 11: Pigment absorption over time. The absorption of pigments of *P. polycephalum* was measured at wavelength E=410nm over time in a shaken microplasmodial culture of 50ml volume. For each point of time the absorption of extracted pigments from a 1-ml sample was measured five times. Samples were taken of the same culture (eleventh passage).

The pigment extraction protocol of Chapman and Coote (1983) was adjusted regarding the centrifugation rate. They recommended 500 rpm, but particles remained in the supernatant. Thus, the supernatant was again centrifuged at 14.000 rpm. Nevertheless, suspended solids remained in the solution. This seemed to considerably disturb the measurement, which can be seen from the boxplots above. Furthermore it was tried to modify the extraction method by single using TCA or acetone. Unfortunately, this led to insufficient pigment extraction. Therefore growth determination by pigment absorption was regarded as inappropriate under our laboratory conditions.

The plots in Figure 12 and Figure 13 show the wet and dry weight over time of two samples taken from the same shaken microplasmodial culture after 0, 24, 31, 48, 52, 57, 74, 81 and 97 hours of growth. The y-axis shows the growth y(t) in mg/ml. The growth curves were determined with R using the package "grofit" (description on page 25). Dashed lines show the maximal growth rate µ.

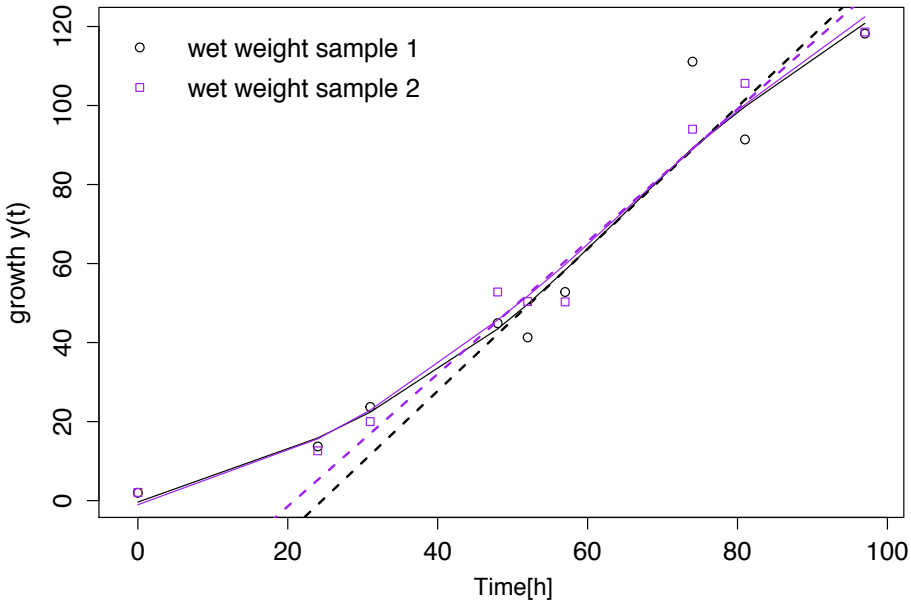

Figure 12: Plot showing the wet weight over time. Two samples were taken of the same shaken microplasmodial culture over time. Growth data were fitted by a smoothed cubic spline fit using the package "grofit" in R. The smoothness of the spline fit (smooth.gc) was set 0.5. The y-axis shows the growth y(t) in mg/ml. The dashed lines show the maximum growth rate µ.

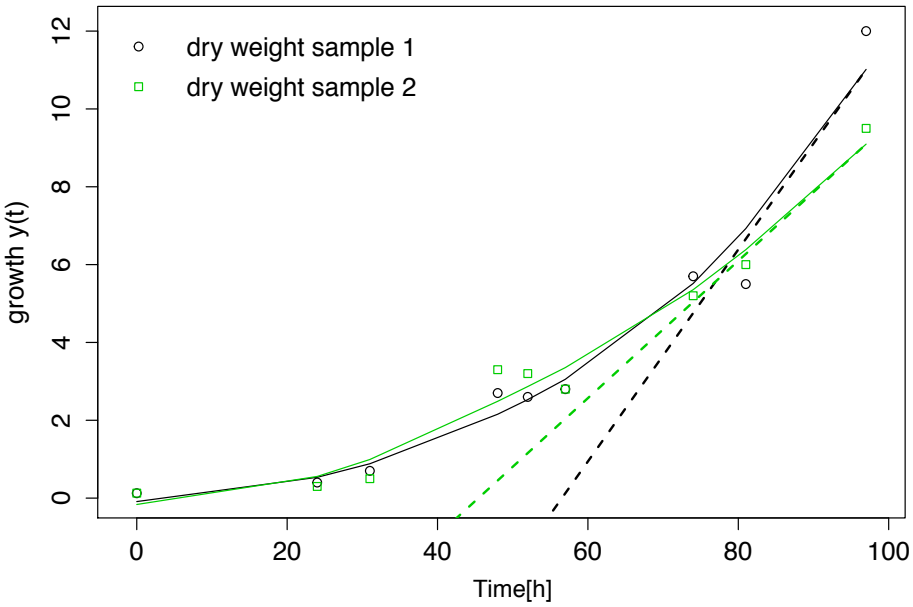

Figure 13: Plot showing the dry weight over time. Two samples were taken of the same shaken microplasmodial culture over time. Growth data were fitted by a smoothed cubic spline fit using the package "grofit" in R. The smoothness of the spline fit (smooth.gc) was set 0.5. The y-axis shows the growth y(t) in mg/ml. The dashed lines show the maximum growth rate μ.

The curves in Figure 12 and Figure 13 resemble growth under our laboratory conditions. It was concluded in which time intervals measurements were required. The first measuring point at 24 hours seems to be appropriate, because little growth occurs within the first 24 hours. A measuring point between 24 and 48 hours was considered to be redundant. After 48 hours three measuring points instead of five were considered to be sufficient.

3.5 Quantification of Microplasmodial Growth with Heat-killed Bacteria

The ability of microplasmodia of *P. polycephalum* to utilize different strains of microorganisms as a food source was investigated by determining the growth of microplasmodia with heat-killed (but undamaged) bacteria and *S. cerevisiae*. *Paenibacillus* sp. was used from the contaminants, since it was the only one that could be adequately grown in liquid medium. Determining growth by wet and dry weight was more accurate (see 3.4.2) and therefore chosen for the observations with heat-killed bacteria. Furthermore, the measuring points were reduced from nine to six (3.4.2). Figure 14 and Figure 15 show the growth of microplasmodia in shaken cultures, when peptone was replaced by heat-killed suspensions (see 2.7) of *S. cerevisiae*, *E. coli*, *P. putida*, *S. plymuthica* and *Paenibacillus* sp.

As controls, growth in semi-defined medium with peptone (positive control) and without peptone (negative control) was conducted. Growth curves were obtained with the package "grofit" in R. The smoothness of the spline fit (smooth.gc) was set 0.5. To compare the growth with different microorganisms non-visually the maximum growth rate μ was derived. Table 8 shows the estimated maximal growth rates from the spline fit.

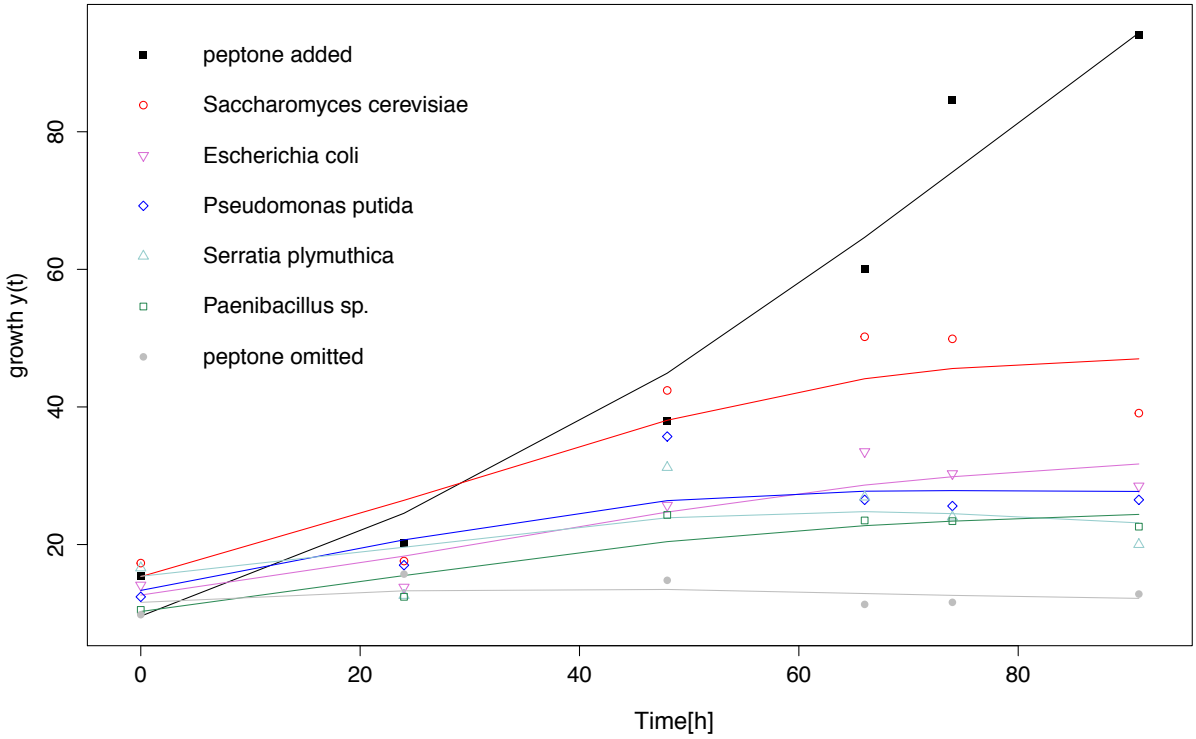

Figure 14: Microplasmodial growth with heat-killed microorganisms determined by wet weight. The growth was determined in shaken cultures with 50 ml semi-defined medium when peptone was replaced by heat-killed suspension of different microorganisms. The growth in SD medium with peptone and without peptone served as controls. 1-ml samples were taken after 0, 24, 48, 66, 74 and 91 hours. Growth data were fitted by a smoothed cubic spline fit using the package "grofit" in R. The smoothness of the spline fit (smooth.gc) was set 0.5. The y-axis shows the growth y(t) in mg/ml.

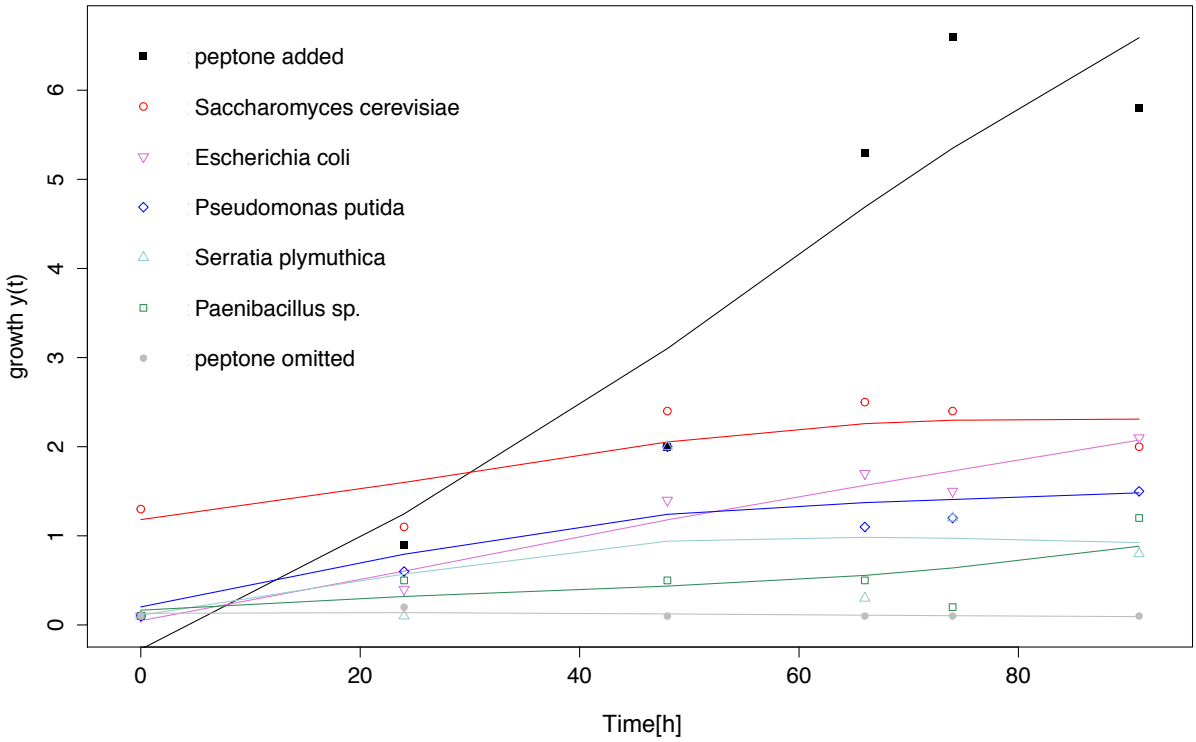

Figure 15: Microplasmodial growth with heat-killed microorganisms determined by dry weight. The growth was determined in shaken cultures with 50 ml semi-defined medium when peptone was replaced by heat-killed suspension of different microorganisms. The growth in SD medium with peptone and without peptone served as controls. 1-ml samples were taken after 0, 24, 48, 66, 74 and 91 hours. Growth data were fitted by a smoothed cubic spline fit using the package "grofit" in R. The smoothness of the spline fit (smooth.gc) was set 0.5. The y-axis shows the growth y(t) in mg/ml.

Table 8: Estimated maximal growth rates (µ). Growth rates were determined in shaken cultures, when peptone was replaced by a heat-killed suspension of different microorganisms.

Microorganisms added	µ, wet weight	µ, dry weight
S. cerevisiae	0.492	0.019
E. coli	0.257	0.024
P. putida	0.313	0.025
S. plymuthica	0.194	0.019
Paenibacillus sp.	0.221	0.016
Controls	µ, wet weight	µ, dry weight
Peptone added, bacteria omitted	1.192	0.086
Peptone omitted, bacteria omitted	0.083	0.001

The highest growth rate was received when peptone was added to the medium (positive control). Barely any growth was observed when both bacteria and peptone were omitted (negative control). The highest growth rates (determined by dry weight), when microorganisms were added, were obtained with heat-killed preparations of *P. putida* and *E. coli* followed from *S. cerevisiae* and *S. plymuthica.* A lower growth rate was obtained with *Paenibacillus* sp.

3.6 Persistence of Applied Bacteria on/in Slime Mold Plasmodia

After the establishment and analysis of sterile shaken cultures of *P. polycephalum* regarding sterility and subcultivation intervals, the ability to utilize different strains of microorganisms (heat-killed) as a food source was determined. The next step was to investigate the persistence of these bacteria (alive) in macroplasmodia and if a correlation with the previous observations exists. Figure 16 shows *P. polycephalum* plasmodia derived from 1 ml of a 72-hour-old microplasmodial culture on a water agar plate with different microorganisms. The microplasmodial suspension was placed in the upper half of the plate. In the lower half, 250 µl of a microorganism suspension was added. The plates, on which *P. putida, E. coli* and *S. cerevisiae* were added, are shown after 24 and 48 hours as exemplary pictures.

After 24 hours, the plasmodium has fed on *E. coli* and *S. cerevisiae*. The microorganism suspensions of *P. putida* and *Paenibacillus* sp. were “tested” but the plasmodium moved away. The plasmodium has not fed on the microplasmodial suspension of *S. plymuthica* after 24 hours. After 48 hours, *P. polycephalum* fed on all of the microplasmodial suspensions. The persistence of bacteria was controlled on NB-2 plates (see 2.8) as well as visually by the appearance of bacterial colonies on the slime traces. Before the first transfer was conducted, the plasmodial front was sampled and plated on NB-2 plates. A dense bacterial lawn has grown out of the plasmodial samples that were in contact with *E. coli*, *S. plymuthica*, *P. putida*, *Paenibacillus* as well as *S. cerevisiae*. Table 9 shows how many transfers microorganisms persisted in the plasmodium and plasmodial traces of *P. polycephalum.*

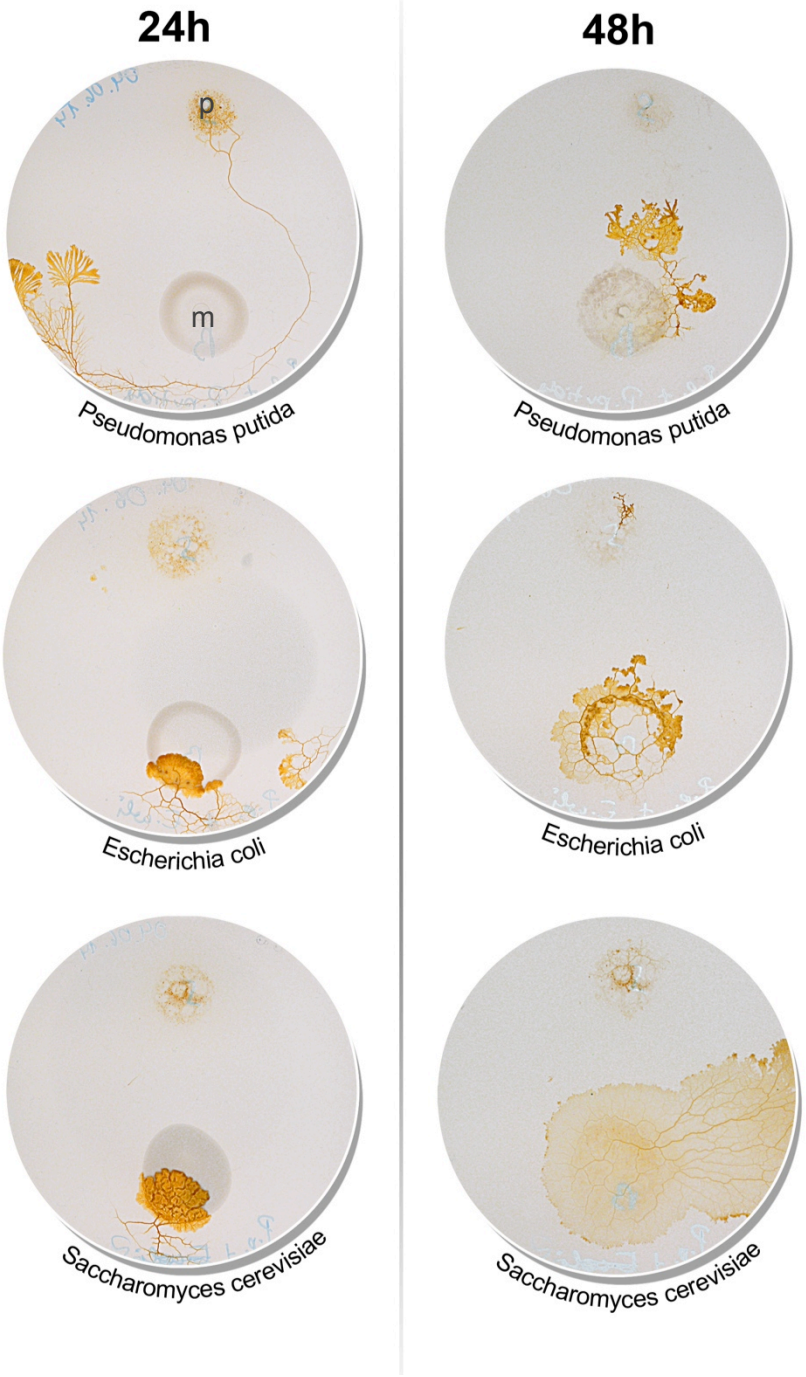

Figure 16: Feeding of *P. polycephalum* on different microorganism suspensions. In the upper half of the agar plates (Ø 90 mm) 1 ml of microplasmodial suspension was placed (p). 250 µl microorganism suspension was placed in the lower half (m). Feeding behavior is shown after 24 hours as well as 48 hours.

Table 9: Persistence of microorganisms over several transfers. The persistence of microorganisms in the plasmodium and plasmodial traces of *P. polycephalum* was determined over several transfers on water agar until bacteria were undetectable. As a control, *P. polycephalum* without bacteria was transferred and sampled.

	S. cerevisiae	*E. coli*	*P. putida*	*S. plymuthica*	*Paenibacillus* sp.	Negative control
Number of transfers detectable in front	1	5	1	1	6	0
Number of transfers visible in trace	3	5	3	3	5	0
Number of transfers until death	4	8	5	5	8	3

In summary, some of the microorganisms were used readily as a food source of the plasmodium after 24 hours (*S.cerevisia*, *E. coli*), whereas the others were tested with following avoidance. Finally, all microorganisms were used as a food source after 48 hours. The initial avoidance with following consumption may be due to the fact that no other (more suitable) food source was present. In general, the plasmodium died, whenever the plasmodium was freed from bacteria. In the case of *Paenibacillus* sp. the growth rate of microplasmodia with heat-killed microorganisms (see 3.5) negatively correlated with the persistence of living bacteria in macroplasmodia. Living *Paenibacillus* sp. persisted the longest in plasmodia whilst giving the lowest growth rate when added as heat-killed suspension in microplasmodial cultures. On the contrary, growth with *E. coli* gave a higher growth rate, although the persistence was long.

4 Discussion

4.1 Identification and Crude Cultures of Different Myxogastrid Species

The identification of the collected plasmodia and keeping them in culture under laboratory conditions was successful. Molecular identification of the unknown species was simplified by searching for universal myxogastrid primers for the amplification of the 18S rDNA. Six out of 35 primers (Fiore-Donno *et al.* 2008) could be assigned to highly conserved regions within several myxogastrids. DNA sequencing allows the identification of slime mold plasmodia, which cannot be identified by morphological characteristics. However, the ITS region of *B. utricularis* was absent from the NCBI database, resulting in a match with *P. polycephalum* (query coverage: 20%, Table 6) and the 18S rDNA did not allow the differentiation between *B. utricularis* and *B.foliicola*. Therefore, taxonomic determination via fruiting body formation was necessary, but the differences between *B. utricularis* and *B.foliicola* were hardly visible.

Furthermore, it was shown that even the distinction between genera is not precise, as some species of the *Physarum* genus have been classified as *Badhamia* genus and vice versa. Phylogenetic studies showed that the genus *Physarum* is polyphyletic consisting of at least three clades. The interspersion of these clades with *Badhamia* isolates suggests the separation between *Physarum* and *Badhamia* to be artificial. Some species of *Physarum* show a tendency toward a capillitium consisting of uniform calcareous tubules, a typical feature for the genus *Badhamia* (Nandipati *et al.* 2012; Novozhilov *et al.* 2013).

In the course of the experiments many differences regarding cultivation conditions have been observed between *P. polycephalum*, *B. utricularis* and *F. septica*. For instance, *F. septica* refused to grow on oat flakes during the first weeks of cultivation, but adaptation occurred gradually from avoiding to fully overgrowing the oat flakes.

All of the plasmodia studied by Cohen (1939) showed several adaptation phenomena. For example, *Badhamia foliicola* was trained to grow at pH 7.4 (usually pH 6.0) by stepwise increase of the pH at each transfer. Cohen also observed that frequent transfers on a new medium increase the vigor of the plasmodium. Therefore, it could be possible that *B. utricularis* and *F. septica*, which have been collected recently in nature, develop to consistent laboratory strains as well over time. Contrary to *B. utricularis, F. septica* is continually changing, because no sclerotia backups were obtained.

The appearance of furrows in the agar and strand-like tracks, on/in which the plasmodium of *F. septica* grows, has not been annotated by now in literature and deserves further investigations. An approach would be to investigate if *F. septica* has the ability to digest the agar itself or if extracellular enzymes, secreted by the slime mold, are responsible for the degradation. It is already known that *P.polycephalum* has the ability to slightly use agar as a carbohydrate source (Knowles & Carlile 1978). The composition of the strand-like tracks should be investigated, as well as the question why the plasmodium grows inside and outside of them. Maybe it uses the traces as a food source and recycles its own deposits. This would explain the longevity of *F. septica* without food for 8 weeks. The mechanisms that are responsible for the "externalized memory", described from Reid *et al.* (2012; 2013), are valid for *P. polycephalum* and *B. utricularis,* but do not hold for *F. septica*. The plasmodium does obviously not avoid areas in which extracellular slime is present.

4.2 Sterile Cultures of Different Myxogastrid Species

The implementation of a shaken culture requires some experience, since there are no exact points of time to transfer the plasmodium during the migration method. Even plasmodia grown out of the same sclerotia backup may have a distinct initial vigor that is important for the success. The starving plasmodium can easily die or the remaining plasmodial mass can be too little for further cultivation in liquid culture. Even if enough plasmodial mass is received, bringing it in liquid culture is a critical step. If the plasmodium sinks to the bottom of the liquid culture during the first 48 hours (without shaking), it dies due to the lack of oxygen. The problem of the lost vigor was partially fixed by transferring the plasmodium after several passages onto a solid SD medium. This process ran the risk of the complete overgrowth with bacteria if the decontamination was insufficient. After the restoration of some plasmodial mass, the transfer to the liquid medium had a higher success rate.

Already Carlile (1971) did not succeed in reproducing the frequently cited SD medium from Daniel and Baldwin (1964). Finally, a modified composition of the Daniel and Baldwin medium by Carlile (1971), which we slightly adjusted, led to successful growth of *P. polycephalum* microplasmodia. There are some crucial differences between the original Daniel and Baldwin medium and the modified medium. Na_2EDTA is included in the medium, whereas manganese is excluded. Furthermore, we modified the medium by using yeast extract instead of biotin and thiamin. According to Daniel and Baldwin (1964), peptone can be re-

placed by casein hydrolysate or amino-acid mixtures. No growth was observed when using casein hydrolysate in our experiments. Carlile (1971) made similar experiences and recommended the use of peptone as well.

Regarding *B. utricularis*, we succeeded in establishing sterile surface cultures. A temporary shaken culture was established, but microplasmodia died after three subcultivations with previous spherule formation, indicating an inappropriate medium. Jahn (1932) observed that *B. utricularis* tolerated a wide pH range (pH 4.0-7.5), similar to *P. polycephalum* (pH 4.0-7.0) (Hok 1950; Scholes 1962). The SD medium had a pH of 4.6, which should have been suitable. No annotations of a specialized semi-defined medium for *B. utricularis* were found in literature. Several tests, in which both pH and composition of the nutrients are modified, should be carried out to possibly adapt the medium for *B. utricularis*. Considering the adaptability of plasmodia in surface cultures (see 4.1) and the temporary success, *B. utricularis* may also be obtained in shaken culture by adaptation if exposed longer to the (unsuitable) medium.

The difficulty to derive constant sterile cultures of *F. septica* was known before. Attempts to create a sterile culture of *F. septica* (as of Scholes (1962)) still remained unsuccessful. Scholes combined the migration method with antibiotic decontamination, resulting in sterile plasmodia that showed slow growth and a lighter color, before dying after a few days. Scholes also expressed reservations about the purity tests of authors who have putatively established axenic cultures with *F. septica* before.

Further microbial investigations were only possible with *P. polycephalum* since it was successfully maintained in axenic shaken cultures.

4.3 Purity Determination of Microplasmodial Cultures and Bacterial Contaminants of (Pure) Cultures

Sterility proofs of putative sterile plasmodia that consider non-cultivable bacteria have never been conducted before. The SSCP can be considered as a suitable technique to investigate the sterility of microplasmodial cultures. The use of more sensitive methods (e.g. pyrosequencing) would have been excessive, since no bacterial communities were analyzed. All excised bands, which were initially derived from microplasmodial cultures of *P. polycephalum* and *B. utricularis*, matched with *P. polycephalum* or *B. utricularis* except one band from *P. polycephalum* that matched with an uncultured alphaproteobacterium. The fact that

no significant match of the bacterial sequence with the mitochondrial DNA of *P. polycephalum* was found, should exclude a mitochondrial origin of this result. Since the sequencing traces showed superimposed signals, it still requires further investigations.

If the existence of an alphaproteobacterium in *P. polycephalum* would be proved, it could be concluded that it acts as an endosymbiont. Free-living amoebae, which probably represent the origin of Myxogastrids, can also be associated with (obligate) bacterial symbionts, for instance alphaproteobacteria (Horn & Wagner 2004). The establishment of synthetic associations between *P. polycephalum* and green algae (Gastrich & Anderson 2002), which can also serve as a food source (Lazo 1961), includes the possible ability of *P. polycephalum* to be associated with bacteria as well. According to the endosymbiotic theory, mitochondria are most closely related to Alphaproteobacteria (Andersson *et al.* 1998; Thrash *et al.* 2011). Therefore it raises the question, whether the alphaproteobacterium could probably represent an earlier stage of mitochondria, which still exists parallel to mitochondria.

Additionally, the SSCP was conducted with the isolated bacterial contaminants (see 2.4) in order to compare the patterns with the microplasmodial cultures. Since none of the contaminants were present in the shaken cultures, it only served as a confirmation of the formerly received sequence results. *Paenibacillus* sp. was found in putative sterile shaken cultures that were plated on NB-2 agar. It is a gram-positive bacterium, which is facultative anaerobic and known for being associated with mycoheterotrophic organisms. Using Fluorescence in situ hybridization (FISH) and Confocal laser scanning microscopy (CLSM) Bertaux *et al.* (2003) detected several *Paenibacillus* sp. inside axenic cultures of *Laccaria bicolor*, a fungi which forms ectomycorrhiza. One of the cultures was kept (putative) axenic for 25 years before the cryptic intracellular bacteria were detected (Boer *et al.* 2005). Moreover *Paenibacillus* sp. can have antifungal activity through extracellular lytic enzymes (Budi *et al.* 2000).

4.4 Observation of Microplasmodial Growth under Sterile Conditions

Microscopic observations confirmed that axenic shaken cultures should be subcultured after 72 hours. This proof was necessary since the growth rate of the subculture depends on the amount and age of the initial inoculum (Daniel & Baldwin 1964). The growth determination by pigment absorption (suggested by the protocol of Chapman and Coote (1983)) was regarded as inaccurate in our case. Although the protocol was adjusted by increasing the centrifugation rate, solid particles remained in the solution; this considerably disturbed the meas-

urement. The protocol of Chapman and Coote (1983) lacks the examination of the accuracy of this method.

The growth determination by wet and dry weight gave more accurate results, although differences between two measurements taken from the same culture at the same time were observed. Due to the irregular distribution and sizes of microplasmodia (despite shaking well before sampling), the sample must have been at least 1 ml to determine the weight. At the same time, the volume of the entire culture should remain nearly constant to obtain an accurate growth curve. For this reason, only two measurements at each point of time could be taken. In summary, it was shown that growth determination by wet and dry weight was the most accurate method and should therefore be chosen for experiments.

4.5 Quantification of Microplasmodial Growth with Heat-killed Bacteria

Microplasmodial growth with heat-killed bacteria (as replacement for peptone) was successfully quantified by the determination of wet and dry weight over time. Due to the higher accuracy of dry weight determinations (Carlile 1971) the discussion is restricted to these results. Both gram-negative (*Escherichia, Serratia, Pseudomonas*) and gram-positive (*Paenibacillus* sp.) served as food source. Therefore, no general conclusions can be drawn regarding the difference between gram-negative and gram-positive bacteria as a food source. However, feeding on *Paenibacillus* sp. led to the lowest growth rate. The feeding preference on distinctive types of bacteria is debated in literature and it seemed as if other factors (e.g. slimy colonies) were more important than the Gram reaction (Cohen 1941). Chapman and Coote (1983) also pointed out the importance of the bacterial surface structure and observed that gram-negative bacteria were more readily utilized as a food source than gram-positive ones, except slime-forming gram-negative bacteria.

It has to be mentioned that growth determination by weight involves disadvantages in this case. The addition of bacterial suspension changes the initial weight and volume. Regarding this, growth determination by pigment absorption would have been more ideal since it does not interfere with the amount of added bacteria, but suffers from the disadvantages mentioned above. The fact that *Paenibacillus* sp. and *S. plymuthica* could not reach the same OD_{600} as the other microorganisms should be mentioned as well, although Chapman and Coote (1983)

claimed that the doubling time of microplasmodial cultures was independent from the amount of added bacterium.

4.6 Persistence of Applied Bacteria on/in Slime Mold Plasmodia

The establishment of verifiable sterile shaken cultures of *P. polycephalum* allowed persistence experiments with living microorganisms in macroplasmodia. This criterion was definitely not fulfilled in former bacterial nutrition studies with plasmodia (Cohen 1939; Scholes 1962; Chapman & Coote 1983). Although the plasmodia initially avoided bacteria, the following consumption may be due to the fact that no other (more suitable) food source was present. The ability from *P. polycephalum* to make selective nutritional decisions has been shown before (Bonner 2010; Dussutour *et al.* 2010).

On the contrary, the plasmodium of *P.polycephalum* fed on *S.cervisiae* and *E. coli* from the beginning. *S.cervisiae* is known to be a food source in two-membered cultures and is also used for the enrichment method (Cohen 1939; Daniel & Baldwin 1964). Therefore, it is not surprising that it was readily used. The persistence in the plasmodium was however not given (only one passage), resulting in early death of the plasmodium. *E. coli*, which has also been proved to be a suitable food source in plasmodial agar cultures (Haskins & de Basanta 2008), persisted remarkably long (five passages).

Cohen (1939) came to the conclusion: "[…] wherever bacterial contamination occurred, growth took place." This is consistent with our results. In general, the plasmodium died, whenever it was freed from bacteria, confirming the difficulties that are connected with the establishment of axenic shaken cultures. It is necessary to catch the plasmodium in a bacteria-free state, but alive, to subsequently transfer it to a shaken culture.

Authors that have established sterile surface cultures of *P. polycephalum* with the migration method (Cohen 1939; Sobels 1950; Daniel & Baldwin 1964) did not use more than three transfers, because *P. polycephalum* has a highly motile plasmodium. Therefore the longer persistence of *E. coli* and *Paenibacillus* sp. (Table 9) should be noticed. Furthermore it appears that the growth rate negatively correlated with the persistence, due to the fact that heat-killed suspensions of *Paenibacillus* sp. gave the lowest growth rate in microplasmodial cultures. The disability to feed on *Paenibacillus* sp. could explain its longer persistence. This does not hold for growth with *E. coli*, which gave a higher growth rate, but persisted long.

The fact that *Paenibacillus* sp. is a persistent and perseverant contaminant can explain its appearance in shaken cultures. *Paenibacillus* sp. did not visibly overgrow microplasmodial cultures and its occurrence could not be confirmed by SSCP results. This suggests its occurrence in smaller amounts. The fact that the DNA of some bands was not isolated (due to the low concentration), does not completely exclude the possibility that *Paenibacillus* sp. was present in shaken cultures. *Paenibacillus* sp. also persisted inside axenic fermentor cultures of *Laccaria bicolor* in small amounts, but rarely appeared (Bertaux *et al.* 2003).

The long persistence may also be explained by the cultivation on non-nutrient water agar, since the probability for temporary associations on non-nutrient agar is increased. Kubo *et al.* (2013) showed that a synthetic obligate bacteria–eukaryote mutualism (SOBEM) can be constructed between organisms that are usually in predator-prey relationship. Mixing *E. coli* and *D. discoideum* and exposing them to conditions unfavorable for monoculture have led to a synthetic mutualistic relationship. Studies with two-membered cultures (*P. polycephalum* and bacteria) were always conducted on low-nutrient agar, but not on non-nutrient agar (Carlile 1971). This could explain why a longer persistence was previously not observed.

A longer persistence or temporary association could be interpreted as a basis for an enforced symbiosis. It seems to be easier to arrange with organisms, which cannot be digested, instead of combating them. This may not be valid for *P. polycephalum* in nature, which can migrate rapidly to a more favorable environment. Moreover the distinction between food source and symbiont can be easily switched, as shown with *D. discoideum.* Stallforth *et al.* (2013) found a single point mutation in the bacterial *gacA* gene of *Pseudomonas fluorescens* that causes the switch-over from a symbiotic to a food source bacterium. The resulting changes of the bacterial secondary metabolism explain the feeding of *D. discoideum* on some bacteria whilst keeping some.

4.7 Conclusion

This is the first report investigating the sterility of *P. polycephalum* shaking cultures regarding non-cultivable bacteria. Sterility was not complete as a putative alphaproteobacterium was found in shaken cultures of *P. polycephalum*. Further investigations have to confirm this result. Moreover, a sterile surface culture and a temporary shaken culture of *B. utricularis* have been established. Further adjustment of the semi-defined growth medium may lead to successful growth of *B. utricularis* in shaken cultures as well. *F. septica* could not be freed from contaminants, the methods suffered from the same problems as of Scholes (1962). We observed different characteristics of *F. septica*, which were contrary to the behavior of the other two species and not described in current literature. For instance, the growth on/in left-behind tracks shows that *F. septica* does not avoid areas in which extracellular slime is present. With the collection of *B. utricularis* and *F. septica*, two more strains (in addition to *P. polycephalum*) are available for further experiments.

Bacterial contaminants of crude and axenic cultures of *P. polycephalum* were identified, whereas one species (*Paenibacillus* sp.) was used for the following experiments in addition to bacterial laboratory strains and *S. cerevisiae*. It cannot be concluded from the experiments that *P. polycephalum* was associated with any of the used microorganisms, but the persistence of two bacteria (*E. coli, Paenibacillus* sp.) was longer than the previously mentioned three passages to free *P. polycephalum* plasmodia from bacteria. The fact that synthetic associations have successfully been established for *E. coli* and *D. discoideum* (Kubo *et al.* 2013), as well as for *P. polycephalum* and *Chorella pyrenoidosa* (Gastrich & Anderson 2002) does raise the question that *P. polycephalum* can be associated with bacteria as well. *Paenibacillus* sp. did not visibly overgrow microplasmodial cultures, but it was shown that it can occur invisibly in axenic fermentor cultures (Bertaux *et al.* 2003). In the case of *Paenibacillus* sp. the plasmodial growth rate correlated negatively with the persistence of living bacteria indicating that less digestible bacteria remain longer in the plasmodium.

In conclusion, the methods used in this study expanded investigations on the microbial ecology of myxogastrids to the molecular level and can serve as the basis for further experiments.

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Appendix A

```
#####################################################################
#                                                                   #
#    Microplasmodia growth curves, created with package grofit      #
#                            Katrin Helmel                          #
#                                                                   #
#####################################################################


##########################################################
#  Growth curves obtained by pigment absorption over time   #
##########################################################

#Import data for pigment absorption
pigment = read.table("Pigment_3.csv", sep=";", header = TRUE, dec = ".")

#Create boxplots
par(mar = c(5.1, 4.5, 3.1, 1.1))

boxplot(pigment$Pigment2 ~pigment$Time, ylim= c(0,25), xlab = "Time[h]",
ylab=expression("Pigment"~ E[410~nm]))
title("a")

boxplot(pigment$Pigment1 ~pigment$Time, ylim= c(0,25), xlab = "Time[h]",
ylab=expression("Pigment"~ E[410~nm]))
title("b")


#####################################################
#  Growth curves obtained by wet weight over time   #
#####################################################

#Load grofit package
library(grofit)

par(xpd=FALSE)

#Create time matrix
timepoints <- c(0, 24, 31, 48, 52, 57, 74, 81, 97)
time <- t(matrix(rep(timepoints, 2), c(9, 2)))
head(time)

#Import data for dry weight
data.dry = read.table("Gewicht_3_grofit_dry.csv", sep=";", header = FALSE, dec =
".")

#Settings
MyOpt1 <- grofit.control(smooth.gc = 0.5, parameter = 28, interactive = FALSE)

#Run grofit dry weight
Run1 <- grofit(time, data.dry, TRUE, MyOpt1)

#Define graphical parameters
colData <- c("black", "green3")
colSpline  <- c("black", "green3")
pch <- 21:22
dev.new(width = 8, height = 3)
par(mar = c(5.1, 4.1, 3.1, 1.1))

#Plot dry weight
plot.gcFit(Run1$gcFit, opt="s", colData=colData, pch=pch, colSpline=colSpline,
slope=TRUE)
title("c")

#Summary statistics dry weight
Run1$gcFit$gcFittedSplines[[1]]$parameters
Run1$gcFit$gcFittedSplines[[2]]$parameters
```

```
#####################################################
#  Growth curves obtained by dry weight over time   #
#####################################################

#Load grofit package
library(grofit)
par(xpd=FALSE)

#Create time matrix
timepoints <- c(0, 24, 31, 48, 52, 57, 74, 81, 97)
time <- t(matrix(rep(timepoints, 2), c(9, 2)))
head(time)

#Import data for wet weight
data.wet = read.table("Gewicht_3_grofit_wet.csv", sep=";", header = FALSE, dec =
".")

#Settings
MyOpt1 <- grofit.control(smooth.gc = 0.5, parameter = 28, interactive = FALSE)

#Run grofit
Run2 <- grofit(time, data.wet, TRUE, MyOpt1)

#Define graphical parameters
colData <- c("black", "purple")
colSpline  <- c("black", "purple")
pch <- 21:23
dev.new(width = 8, height = 3)
par(mar = c(5.1, 4.1, 3.1, 1.1))

#Plot
plot(Run2$gcFit, opt="s", colData=colData, pch=pch, colSpline=colSpline,
slope=TRUE)
title("d")

#Summary statistics
TestRun1$gcFit$gcFittedSplines[[1]]$parameters
TestRun1$gcFit$gcFittedSplines[[2]]$parameters


#####################################################################
#  Growth curves obtained by wet weight over time, bacteria added   #
#####################################################################

#Load grofit package
library(grofit)
par(xpd=FALSE)

#Create time matrix
timepoints <- c(0, 24, 48, 66, 74, 91)
time <- t(matrix(rep(timepoints, 7), c(6, 7)))
head(time)

#Import data for wet weight
data.wet2 = read.table("MO_grofit_wet.csv", sep=";", header = FALSE, dec = ".")

#Settings
MyOpt1 <- grofit.control(smooth.gc = 0.5, parameter = 28, interactive = FALSE)

#Run grofit
Run3  <- grofit(time, data.wet2, TRUE, MyOpt1)

#Define graphical parameters
colData <- c("black", "red", "orchid", "blue", "paleturquoise3", "seagreen",
"grey")
colSpline  <- c("black", "red", "orchid", "blue", "paleturquoise3", "seagreen",
"grey")
pch <- c(15,21,25,23,24,22,16)
dev.new(width = 8, height = 3)
par(mar = c(5.1, 4.1, 3.1, 1.1))

#Plot
plot(Run3$gcFit, opt="s", colData=colData, pch=pch, colSpline=colSpline)
```

```
#Summary statistics
Run3$gcFit$gcFittedSplines[[1]]$parameters;
Run3$gcFit$gcFittedSplines[[2]]$parameters;
Run3$gcFit$gcFittedSplines[[3]]$parameters;
Run3$gcFit$gcFittedSplines[[4]]$parameters;
Run3$gcFit$gcFittedSplines[[5]]$parameters;
Run3$gcFit$gcFittedSplines[[6]]$parameters;
Run3$gcFit$gcFittedSplines[[7]]$parameters


######################################################################
#  Growth curves obtained by dry weight over time, bacteria added   #
######################################################################

#Load grofit package
library(grofit)
par(xpd=FALSE)

#Create time matrix
timepoints <- c(0, 24, 48, 66, 74, 91)
time <- t(matrix(rep(timepoints, 7), c(6, 7)))
head(time)

#Import data for wet weight
data.dry2 = read.table("MO_grofit_dry2.csv", sep=";", header = FALSE, dec = ".")

#Settings
MyOpt1 <- grofit.control(smooth.gc = 0.5, parameter = 28, interactive = FALSE)

#Run grofit
Run4  <- grofit(time, data.dry2, TRUE, MyOpt1)

#Define graphical parameters
colData <- c("black", "red", "orchid", "blue", "paleturquoise3", "seagreen",
"grey")
colSpline  <- c("black", "red", "orchid", "blue", "paleturquoise3", "seagreen",
"grey")
pch <- c(15,21,25,23,24,22,16)
dev.new(width = 8, height = 3)
par(mar = c(5.1, 4.1, 3.1, 1.1))

#Plot
plot(TestRun4$gcFit, opt="s", colData=colData, pch=pch, colSpline=colSpline)

#Summary statistics
Run4$gcFit$gcFittedSplines[[1]]$parameters;
Run4$gcFit$gcFittedSplines[[2]]$parameters;
Run4$gcFit$gcFittedSplines[[3]]$parameters;
Run4$gcFit$gcFittedSplines[[4]]$parameters;
Run4$gcFit$gcFittedSplines[[5]]$parameters;
Run4$gcFit$gcFittedSplines[[6]]$parameters;
Run4$gcFit$gcFittedSplines[[7]]$parameters
```

Deutsche Fassung:
Beschluss der Curricula-Kommission für Bachelor-, Master- und Diplomstudien vom 10.11.2008
Genehmigung des Senates am 1.12.2008

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Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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