Zoological Studies **57:** 9 (2018)
doi:10.6620/ZS.2018.57-09

Zoological Studies

Open Access

The Efficiency of Cultivation Media in Recovering Naked Lobose Amoebae from Freshwater Environments

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(Received 19 May 2017; Accepted 2 March 2018; Published 8 March 2018; Communicated by Benny K.K. Chan)

Citation: Mrva M, Garajová M. 2018. The efficiency of cultivation media in recovering naked lobose amoebae from freshwater environments. Zool Stud **57:**9. doi:10.6620/ZS.2018.57-09.

Martin Mrva and Mária Garajová (2018) This paper deals with the efficiency of cultivation media in recovering the species diversity of naked lobose amoebae. A total of 24 species belonging to ten families were isolated with six enrichment media (two liquid and four agar media) during a two-year study on naked lobose amoebae in a branch of the Danube River in Bratislava (Slovak Republic). The highest efficiency was seen in the grass-seed infusion, grass-seed agar, and non-nutrient agar, with 79%, 67%, and 58% of species recovered, respectively. The grass-seed infusion yielded the highest numbers of Thecamoebidae, Vannellidae, Mayorellidae and Dermamoebidae species and is likely the most suitable medium for their recovery. The most effective media were based on grass-seed infusions, which are easy to prepare and suitable for studying the diversity and ecology of naked amoebae from freshwater samples.

Key words: Amoebae cultures, Enrichment media, Environmental samples, Naked lobose amoebae, Species recovery.

BACKGROUND

Naked amoebae diversity in various habitats remains poorly studied, in spite of various studies across the years. The study of species spectra and ecology in freshwater and soil samples requires the use of appropriate cultivation methods that reveal as many amoebae species from sampled material as possible. Identification to the species level requires examining higher numbers of specimens at all stages of their life cycles (Smirnov and Brown 2004); this is usually possible only if clonal cultures are available to provide sufficient quantities of material for light and electron microscopic analyses and molecular analyses.

The first real culture media used for naked amoebae were probably infusions that supported the growth of organisms accompanying amoebae in samples. They were prepared by adding boiled hay (Schaeffer 1916), wheat grains (Taylor 1924) or rice grains (Brandwein 1935) into collected water; alternatively, sterile infusions prepared by boiling of this material were inoculated with amoebae (Doflein 1916; Kudo 1966). These liquid media were frequently converted into a solid form by adding a small amount of gelatine or agar (Jollos 1916). To obtain denser cultures, some authors modified the culture media by adding several nutrients: *e.g.*, beef bouillon, lettuce extract and peptone (Doflein 1916; Schoenichen 1927; Jírovec et al. 1953). On the other hand, Severtzoff (1922) proposed that non-nutrient agar is the most appropriate medium for amoebae, and this remains the most commonly used recipe up to the present

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time. As the culture medium is basically a nutritive base for organisms consumed by amoebae, some authors concentrated on finding appropriate food for organisms in cultures. Although most of amoebae species are polyphagous and able to feed on bacteria or other protists frequently accompanying the cultivated species from a sample (Kalinina and Page 1992), selecting an appropriate food organism is essential for establishing and maintaining cultures. The primary food source for naked amoebae are bacteria, which was noted early and proved experimentally (Mouton 1902; Cutler and Crump 1927). The preference of bacteria as food organisms was studied experimentally by Singh (1941 1945), who detected that some bacterial species inhibit the multiplication of amoebae and the species producing pigments can even have toxic effect on the cultures. These results were later supported by Weekers et al. (1993), who considered pigment producing bacteria inappropriate for cultures and recommended *Escherichia coli* and *Klebsiella aerogenes* from ten species of tested bacteria. Several common bacterial species - such as *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* - have been routinely used in laboratory conditions (*e.g.*, Page 1976 1991; Upadhyay 1968; Červa 1971; Ciarkowska and Metryka 1979; Baldock et al. 1980). However, we do not know the percent recovery of amoebae species from the samples using these bacteria and therefore the food preference of particular amoebae species is not fully understood. Several soil species of amoebae are specialised to fungi and can be cultivated on them (Chakraborty et al. 1985, Page 1991), although *Mucor*, *Penicillium*, *Aspergillus*, and *Botrytis* inhibit multiplication and enhance encystment of amoebae in cultures (Heal 1962). In rare cases, some amoebae species have been shown to be specialised to algae (Smirnov et al. 2011a). On the other hand, small protists have to be used to maintain cultures of large amoebae like *Amoeba* spp. and *Chaos* spp. The ciliate *Colpidium* and flagellate *Chilomonas* were used as food organisms for *Amoeba proteus* by Mast (1939) and later the ciliate *Tetrahymena pyriformis* was successfully used as a food organism by Prescott and James (1955) in routine mass cultures of *A. proteus*. For *Chaos carolinense* cultures, the ciliate *Paramecium* was used by Andresen (1956). Alternatively, small amoebae (of the genera *Acanthamoeba*, *Rosculus*, *Vannella*, and *Vexillifera*) are recommended as food organisms for larger species of amoebae (Page 1991).

The appropriate culture media for freshwater and soil naked amoebae were initially selected and summarized by Page (1976 1988 1991) in his classic monographs and in a subsequent paper by Kalinina and Page (1992), which also emphasized that different genera of naked amoebae have different food requirements. Basic media suitable for the recovery of soil amoebae were later included in an article by Smirnov and Brown (2004) and specialised media needed for cultivation of pathogenic free-living amoebae were re-evaluated by Schuster (2002).

Recently, research on freshwater and soil naked amoebae has made continuous progress: old taxa have been reclassified based on molecular data and morphodynamic organization of locomotive forms and new taxa have been described. Naked lobose amoebae are classified in the subphylum Lobosa, which comprises two classes: Tubulinea and Discosea. Tubulinea includes amoebae with tubular or subcylindrical pseudopodia and monoaxial flow of cytoplasm. The class Discosea includes flattened amoebae that never produce tubular or subcylindrical pseudopodia and typically have polyaxial flow of cytoplasm (Smirnov et al. 2011b).

Up to the present time, culture methods have changed minimally and it is not clear how reliable and efficient the media are in recovering amoebae from environments. For this reason, various formulas have been used to examine naked amoebae diversity and ecology (*e.g.* Butler and Rogerson 2000; Smirnov and Brown 2004; Anderson 2006; Mrva 2006; Shmakova et al. 2013; Geisen et al. 2014). Attempts to compare media efficiency in the recovery of amoebae from soil (Ciarkowska and Metryka 1979) and freshwater samples (Smirnov 2003) are very rare, and the suitability of particular culture media for various groups of amoebae in long term studies has not yet been checked. The present article compares the efficiency of several types of enrichment media for naked lobose amoebae in a two-year continuous study.

MATERIALS AND METHODS

Samples of gravel and muddy sediment with water were collected from the littoral zone of Karloveské rameno - a branch of the Danube River (17°02'E, 48°09'N) - in 0.3 l sterile glass vessels. In total, 33 samples were taken bimonthly from three sites over two years. Immediately after

transporting to the laboratory, subsamples were inoculated on six enriched culture media (two liquid and four agar media) in sterile Petri dishes with a diameter of 50 mm. For each medium, two dishes were inoculated with about 0.2 ml of sediment. The following media were used: 1. Prescott-James (PJ) medium with two rice grains per dish (Prescott and James 1955). 2. Grass-seed infusion (GS) prepared from 2 g of millet grains in 1litre of sterile tap water. Grains were boiled in the water for 10 min and dispensed into Petri dishes at 2 grains per dish. This medium was prepared one day before inoculation. 3. Grass-seed agar (GSA) made from 1 litre of GS and 15 g of non-nutrient agar (Page 1988). 4. GSA with an overlay of water from the sample (GSA + W). 5. Non-nutrient agar (NNA) prepared from 1 litre of modified Neff's amoeba saline (AS) and 15 g of non-nutrient agar (Page 1988). 6. NNA with an overlay of water from the sample (NNA + W). For detailed information on recipes, see Page (1988 1991). Cultures were incubated at 22°C in indirect light.

Thorough examination of cultures was performed with Nikon Labophot light microscope with phase contrast optics during a one month incubation period. Keys by Page (1988 1991) and Smirnov and Brown (2004) were used to identify species. The classifications by Smirnov et al. (2011b) and Adl et al. (2012) were used. To evaluate the efficiency of culture media, hierarchical cluster analysis applying the complete linkage method was performed, using Sørensen similarity index. Ordination was carried out with non-metric multidimensional scaling (NMDS) using Sørensen similarity index of species presence. Statistical analysis was made with programs Statistica 7.0 (StatSoft CR Ltd., Prague, Czech Republic) and Syntax (Podani 1994).

RESULTS

A total of 24 species of naked lobose amoebae belonging to ten families were isolated in Petri dishes using enrichment media. The efficiency of media to recover the amoebae was uneven. GS medium had the highest efficiency, with 19 species recovered; GSA medium recovered 16 species; NNA recovered 14 species; PJ medium recovered 13 species; and GSA + W recovered 12 species. NNA + W had the lowest yield, recovering only 7 species of amoebae (Table 1).

The hierarchical classification and multi-dimensional scaling were used to evaluate the efficiency of enrichment media in recovering amoebae species. The dendrogram based on qualitative representation (Sørensen similarity index) (Fig. 1) is composed of one outlier (NNA + W) and a cluster of all other media at a

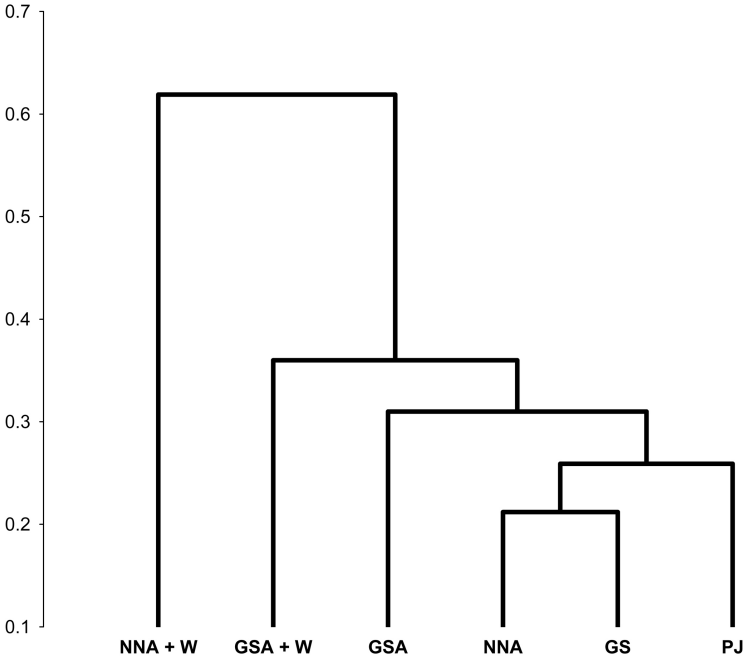

Fig. 1. Hierarchical cluster analysis of recovered amoebae species in enrichment media (Sørensen similarity index, complete linkage Method). Vertical axis represents dissimilarity (for media abbreviations see Materials and methods).

dissimilarity level of 0.62. The outlier medium is typical with above mentioned lowest number of recovered species. Further separation begins at a dissimilarity level of 0.36 (GSA + W was separated from the rest of used media). Culture media NNA, GS and PJ, which had the highest similarity in groups of recovered species, form a cluster at a dissimilarity level of 0.26. This cluster is also distinctly supported by results of non-metric multidimensional scaling (NMDS) ordination showing the formation of three distinct groups of media, where NNA + W is significantly separate from the other media (Fig. 2).

From a systematics viewpoint, the GSA medium recovered the highest number of families in subphylum Lobosa (9 families). Further, species from 7 Lobosa families were recorded in GS and NNA and species from 6 families were detected for PJ, GSA + W and NNA + W media (Table 1; Fig. 3). The efficiency of enrichment media is also demonstrated by the number of recovered species within particular families. The GS, NNA, and GSA + W media recovered the highest number of species in family Thecamoebidae (4 species); GS and PJ recovered the highest number in Vannellidae (3 species); GS and NNA recovered the highest number in Mayorellidae (3 species); and PJ, GS and NNA recovered the highest number in Dermamoebaidae (3 species). One to two species were detected from other families (Table 1).

DISCUSSION

It is generally important to use a wide spectrum of media for maximum recovery of naked amoebae from environmental samples. On one hand, a higher number of media increases the probability of recovery (Smirnov 2003). On the other hand, having to make and frequently examine multiple culture plates increases laboriousness of laboratory work. Therefore, for studies of amoebae diversity and ecology, it is essential to define the efficiency of particular media in order to select the limited set of most appropriate ones.

As noted in Mrva (2006), enrichment media with grass-seed infusion recovered the most amoebae species: 79% for GS and 67% for GSA in the present study. This correlates with an experimental comparison of four types of agar media by Ciarkowska and Metryka (1979), who detected highest numbers of soil amoebae on an agar medium with sterilised grass infusion. Similarly, Smirnov (2003) found that the CPA medium (Cerophyl-Prescott agar) was the most productive of five tested agar media. CPA is

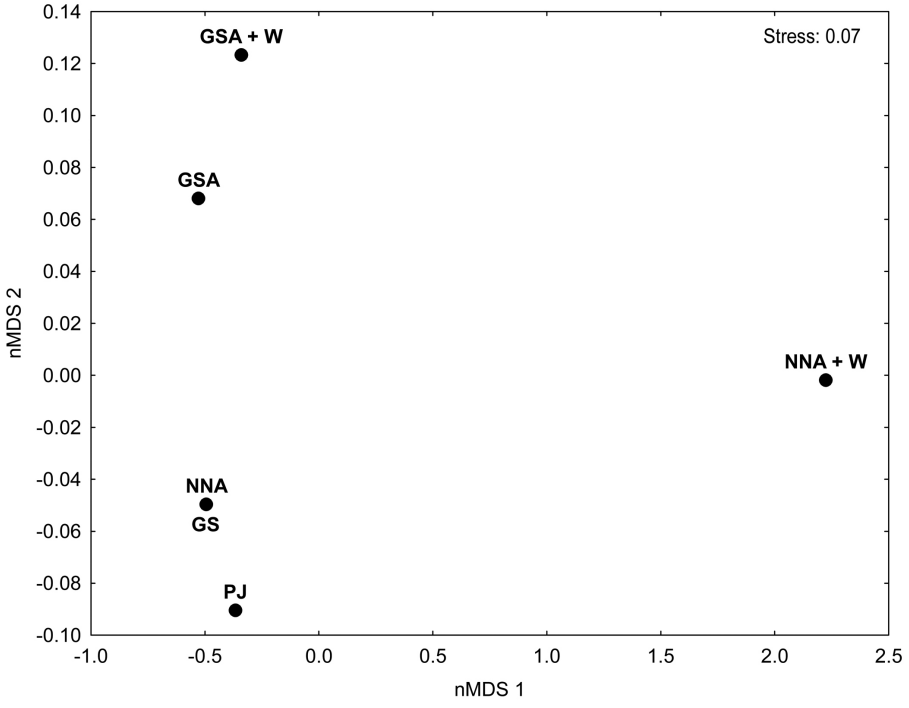

Fig. 2. Non-metric multidimensional scaling (NMDS) ordination for recovered amoebae species in enrichment media based on Sørensen similarity index of species presence (for media abbreviations see Materials and methods).

made of Cerophyl, an extract of cereal leaves, boiled in PJ solution. All these results, along with the present study, suggest that agar media with grass infusion or grass-seed extract are very suitable for the recovery of naked amoebae from environmental samples.

In the present study, the NNA medium also obtained very good results, with 14 species recovered. However, NNA + W - which contained a liquid overlay - was the least appropriate, with only 7 species detected. Media with the water overlay (NNA+W, GAS+W) yielded fewer species than media without the water overlay. However, the difference between GSA and GSA + W was smaller than the difference between NNA and NNA + W (16 vs. 12 and 14 vs. 7, respectively). There are several reasons for the lower numbers of species in the media with liquid overlay: an unsuitable composition of the medium, overgrowth of bacteria inhibiting amoebae growth (Smirnov 2003), or the accumulation of bacterial waste and progressive oxygen deficit (Page 1976).

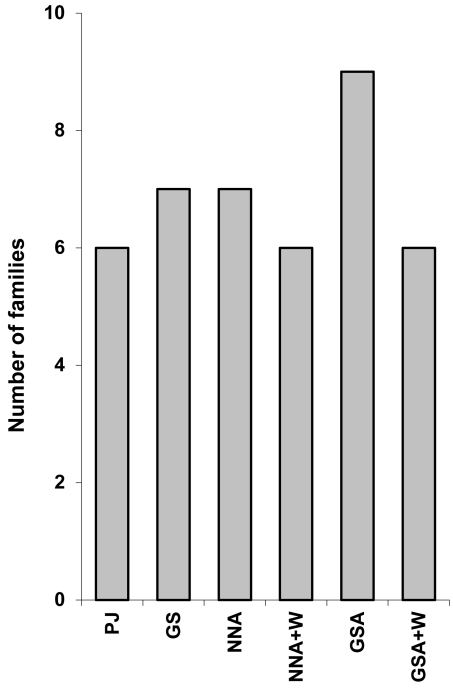

Fig. 3. The numbers of families recovered using different culture media (for media abbreviations see Materials and methods).

Bacteria, cyanobacteria, algae, flagellates, small ciliates and other protists were food sources found in all media used. Though broad in spectrum, these enrichment media selectively affect the food sources by enabling certain organisms to multiply and suppressing others. Singh (1941 1945) demonstrated that the composition of food organisms in amoebae cultures is important by observing that inappropriate bacteria cause encystment or can have toxic effects on amoebae. Similarly, Heal (1962) proved that only some fungi are suitable for cultivations. The life conditions of some amoebae species may be more suitable in culture media than the others. The selective effects of media on accompanied food organisms thus leads to different results in studies on naked amoebae diversity.

The results obtained in this study show how effective some media are at cultivating certain groups of gymnamoebae. For species in family

Table 1. Numbers of Lobosa species grown on culture media (for media abbreviations see Materials and Methods)

Family	Culture medium					
	PJ	GS	NNA	NNA+W	GSA	GSA+W
Amoebidae					1	
Leptomyxidae		2			2	1
Vermamoebidae			1		1	
Paramoebidae	1	2	1	1	1	2
Vexilliferidae				1		
Vannellidae	3	3	1	1	2	
Cochliopodiidae	2	2	1	2	2	2
Mayorellidae	1	3	3	1	2	2
Dermamoebidae	3	3	3		2	1
Thecamoebidae	3	4	4	1	3	4
Number of species	13	19	14	7	16	12
Percent of the recovered species	54%	79%	58%	29%	67%	50%

Thecamoebidae, a relatively large spectrum of media is appropriate, with the exception of NNA + W. For species in family Dermamoebidae, the most suitable are GS, PJ and NNA; for species in family Mayorellidae, it is GS and NNA; and for species in family Vannellidae, the most suitable are GS and PJ. The best enrichment medium in the present study was GS, which yielded highest numbers of species in Thecamoebidae, Vannellidae, Mayorellidae and Dermamoebidae. GS infusion, which is a liquid medium with low amount of nutrients, does not have such a strong influence on the accompanied organisms as agar media do, and it enables the growth of rich communities of microorganisms from the original sample. This feature is important for members of families Thecamoebidae, Mayorellidae and Dermamoebidae which, besides bacteria, feed on small flagellates and ciliates (Page 1991). The results show that members of these related families of subclass Longamoebia (Smirnov et al. 2011b) also have similar food requirements. For subclass Flabellinia (Smirnov et al. 2011b) - including the families in this study Paramoebidae, Vexilliferidae, Vannellidae, and Cochliopodiidae - is it hard to conclude which medium is best, although, again, GS recovered the highest number of species. However, the sole medium that produced members of all mentioned Flabellinia families was NNA + W, which generally was the most ineffective in terms of total number of recovered species.

CONCLUSIONS

In conclusion, the most effective enrichment media of the six tested in this study were the ones based on grass-seed infusion. These media are easy to prepare and useful for the study of diversity and ecology of gymnamoebae from freshwater samples. High numbers of specimens are also needed for detailed light microscopy and electron microscopy examination as well as for molecular analyses in taxonomic studies, and the present study shows which media may yield these numbers. This information could be useful for researchers who study free-living naked amoebae.

In the future, research should also focus on finding appropriate media for particular groups of naked amoebae, as the species in individual families have similar food requirements. New information on enrichment media selectivity may subsequently bring more light to knowledge of the cryptic diversity of gymnamoebae in the environment.

Acknowledgments: The study was supported by the grant VEGA 1/0365/16. The work was supported by the Slovak Research and Development Agency under the contract No. APVV-15-0123. The authors highly acknowledge the assistance of Prof. Milada Holecová in statistical evaluations.

Authors' contributions: MM and MG contributed equally to this work. MM and MG wrote the paper and read and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

Availability of data and materials: The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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