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Tips and tricks how to culture water bears: simple protocols for culturing eutardigrades (Tardigrada) under laboratory conditions

M. ROSZKOWSKA ^{1,2}, D. WOJCIECHOWSKA ², H. KMITA ², S. CERBIN ³,
M. K. DZIUBA³, E. FIAŁKOWSKA ⁴, R. SOBKOWIAK ⁵, W. SZYDŁO ^{6,7}, &
Ł. KACZMAREK ^{1*}

¹Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland, ²Department of Bioenergetics, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland, ³Department of Hydrobiology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland, ⁴Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Krakow, Poland, ⁵Department of Cell Biology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland, ⁶Center for Advanced Technology, Adam Mickiewicz University, Poznań, Poland, and ⁷Population Ecology Lab, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland

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Abstract

Tardigrades are microinvertebrates inhabiting almost all aquatic and terrestrial ecosystems throughout the world. They are known for their ability to enter into cryptobiosis and to survive extreme environmental conditions (e.g. lack of water, very high and low temperatures, high doses of radiation, vacuum space). Thanks to these abilities, tardigrades are excellent model organisms for various types of studies, e.g. ecological, ethological, physiological, astrobiological, biotechnological or medical, or even in integrative taxonomy. For most of these studies well-established tardigrade cultures are essential. Here we present a review of methods/protocols used in tardigrade culturing in the past. Based on this data and on our several years of experience in tardigrade culturing, we tried different methods and developed new ones that seem to be optimal. Here, we propose our own simple protocols for culturing herbivorous, omnivorous and carnivorous eutardigrade species in environmental chambers as well as in room conditions. We also describe methods for culturing rotifers, nematodes and algae, used as food sources for tardigrades. Moreover, many years of tardigrade culturing allowed us to describe the problems that may occur during culturing, explain their causes and propose solutions. We believe that these simple protocols will be very useful for many scientists planning tardigrade applications in their studies.

Keywords: Eutardigrade cultures, culture problems and solutions, rearing methodology, tardigrades

1. Introduction

There is at present no standard method for tardigrade culturing, and such a standard, unified approach for all tardigrade taxa will never be proposed. This is due to the fact that different tardigrade species have different environmental requirements. In the past many different culture protocols were proposed by different authors. The first information on tardigrade culturing (probably of *Pseudobiotus* species), based on keeping them in an aquarium, was published by Von Wenck (1914) and Marcus (1929). Nowadays, different authors use different culture methods not only for different species, but also for the same species (for a review see Altiero et al. 2018). The

main differences concern the walking substrate, type of food, ambient temperature and photoperiod (for more details see Table I and the Discussion section).

Such a diversity of applied protocols, for e.g. culturing of the same species, could be potentially problematic for comparing the results and conclusions of different experiments focused on different aspects of tardigrade biology. Therefore, here we propose rearing protocols for stock cultures of five eutardigrade species (terrestrial and freshwater) with different food preferences, i.e. herbivorous, omnivorous and carnivorous. The protocols were developed and, based on our observations, constantly applied and continually improved by us over the past several

*Correspondence: Ł. Kaczmarek, Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland
Email: kaczmar@amu.edu.pl

Table I. Summary of the different freshwater and terrestrial eutardigrade species (in taxonomic order) and culture conditions used by different authors (species names according to Degma et al. 2009–2020; genus abbreviations according to Perry et al. 2019; some species names were updated according to modern literature, i.e. *Hys. dujardini*/*Hys. exemplaris*, *Mil. tardigradum*/*Mil. inceptum*, *Pam. cf. richtersi*/*Pam. faurbanki* and *Mil. cf. alpigenum*/*Mil. alpigenum*).

Author	Species	Walking substrate/container	Medium	Temp.	Photoperiod	Food
Kosztyla et al. (2016)	<i>Mil. alpigenum</i>	Petri dish scratched with fine sandpaper	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Rotifers (<i>Lecane inermis</i>)
Sugiura et al. (2020b)	<i>Mil. pacificum</i>	1.2% agar/plastic dish (32 mm)	Spring water (Volvic)	20°C	ND	Rotifers (<i>L. inermis</i>)
Morek et al. (2020)	<i>Mil. eurystomum</i>	Petri dish scratched with fine sandpaper	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	10°C	Dark	Rotifers (<i>L. inermis</i>) and tardigrades (<i>Hys. exemplaris</i>)
Suzuki (2003)	<i>Mil. inceptum</i>	2% agar/plastic culture dish (30 or 60 mm)	Water (Milli-Q, Millipore)	ND	ND	Rotifers (<i>L. inermis</i>)
Hengherr et al. (2008)	<i>Mil. inceptum</i>	3% agar/plastic plates	Water, not specified	ND	ND	Rotifers (<i>Philodina citrina</i>) and algae (<i>Chlorogonium elongatum</i>)
Schill and Fritz (2008); Schill (2013)	<i>Mil. inceptum</i>	3% agar/plastic plates	Spring water (Volvic)	20°C	12/12 L/D	Rotifers (<i>P. citrina</i>)
Baumann (1964)	<i>Mil. tardigradum</i>	Algae and plants/Petri dish (50 mm)	Water, not specified	15–22°C	ND	Rotifers (<i>Philodina rosola</i>)
Horikawa et al. (2006)	<i>Mil. tardigradum</i>	1.5% agar/plastic dish (24 mm)	Water, not specified	ND	ND	Unidentified bdelloid rotifers
Sugiura et al. (2020b)	<i>Mil. tardigradum</i>	1.2% agar/plastic dish (32 mm)	Spring water (Volvic)	20°C	ND	Rotifers (<i>L. inermis</i>)
Morek et al. (2016)	<i>Mil. variegatum</i>	2% agar/24-well plastic plate	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Rotifers (<i>L. inermis</i>)
Kosztyla et al. (2016)	<i>Dip. higginsii</i>	Petri dish scratched with fine sandpaper	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.)
Baumann (1961)	<i>Hys. convergens</i>	Algae and agar/glass dish or bottles	Sterile water	ND	Daylight	Algae (<i>Chlorella pyrenoidosa</i>)
Ammermann (1962)	<i>Hys. dujardini</i>	Algae	Water, not specified	ND	ND	Algae (Chlorococcales)
Gabriel and Goldstein (2007)	<i>Hys. exemplaris</i>	Algae/glass Petri dish (60 mm)	Spring water (Crystal Geyser or Deer Park)	10–20°C	Shaded place	Algae (<i>Chlorococcum</i> sp.)
Gabriel et al. (2007)	<i>Hys. exemplaris</i>	Algae/250 mL Erlenmeyer flasks	Chalkley's medium with 2% soil extract	10–18°C	14/10 L/D	Algae (<i>Chlorococcum</i> sp.)
Arakawa et al. (2016)	<i>Hys. exemplaris</i>	2% agar/plate	Spring water (Volvic)	18°C	Dark	Algae (<i>C. vulgaris</i>)
Hyra et al. (2016)	<i>Hys. exemplaris</i>	1.5% agar/24-well plastic plate	Spring water (Żywiec Zdrój) mixed with distilled water (1:1)	16°C	ND	Algae (<i>Chlorococcum</i> sp.)

(Continued)

Table I. (Continued).

Author	Species	Walking substrate/container	Medium	Temp.	Photoperiod	Food
Koszytka et al. (2016)	<i>Hys. exemplaris</i>	Petri dish scratched with fine sandpaper	Spring water (Żywic Zdroj) mixed with ddH ₂ O (1:3)	16°C	Dark	Algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.)
Erdmann et al. (2017)	<i>Hys. exemplaris</i>	Algae/250 mL plastic bottle	Distilled water	ND	ND	Algae (<i>Chlorella</i> sp. and <i>Chlorococcum</i> sp.)
McNuff (2018)	<i>Hys. exemplaris</i>	Algae/250 mL Erlenmeyer flasks or plastic Petri dish (60 mm)	Chalkley's medium with soil extract	10–20°C	14/10 L/D or shaded place	Algae (<i>Chlorococcum</i> sp.)
Gross et al. (2018)	<i>Hys. exemplaris</i>	Algae/plastic Petri dish	Spring water (Volvic)	21°C	ND	Algae (<i>Chlorococcum</i> sp.)
Altiero and Rebecchi (2001)	<i>Adr. cf. scoticum</i>	1.2% agar/plastic dish (15 mm/7 mm)	Spring water (San Benedetto)	14 and 20°C	12/12 L/D	Algae (<i>Scenedesmus acutus</i>)
Tumanov (2020)	<i>Not. pallidoides</i>	Scratched plastic Petri dish	Distilled and filtered tap water (3:1)	16°C	ND	Algae (<i>Chlorella</i> sp.)
Dougherty et al. (1961) and Dougherty (1964)	<i>Not. arcticus</i> (probably <i>Acu. antarcticus</i>)	Watch glass and screw-capped tubes/xenic algal fragments	Distilled water	4–7°C	ND	Xenic algal fragments or blue-green algae
Kagoshima et al. (2013)	<i>Acu. antarcticus</i>	1.8% agar, 1 × Bold Modified Basal Freshwater Nutrient media and 5 µg/mL cholesterol/plate (100 mm)	Distilled water	4 and 10°C	12/12 L/D	Cyanobacteria and green algae from the natural environment
Tsujimoto et al. (2015)	<i>Acu. antarcticus</i>	1.5% agar/plastic plates (35 mm)	Spring water (Volvic)	15°C	Dark	Algae (<i>Chlorella</i> sp.)
Altiero et al. (2015)	<i>Acu. antarcticus</i>	1.2% agar/plastic box (15 mm/7 mm) and flask	Spring water	14°C	12/12 L/D	Algae (<i>Chlorococcum</i> sp.)
Tsujimoto et al. (2020)	<i>Acu. antarcticus</i>	1.5% agar/12-well culture plate	Spring water (Volvic)	ND	ND	Algae (<i>Chlorella</i> sp.)
Baumann (1966)	<i>Ram. oberhauseri</i>	Algae and agar/glass dish	Water, not specified	ND	ND	Algae (<i>Pseudochlorella aquatica</i>)
Horikawa et al. (2008)	<i>Ram. varicormatus</i>	1.5% agar/plastic Petri dish (35 mm)	Distilled water	25°C	Dark	Algae (<i>Chlorella vulgaris</i>)
Hashimoto et al. (2016), Yoshida et al. (2017)	<i>Ram. varicormatus</i>	2% agar/culture plate	Spring water (Volvic) with hypochlorite	22°C	Dark	Algae (<i>C. vulgaris</i>)
Yoshida et al. (2019)	<i>Ram. varicormatus</i>	2% agar/plastic dish	Spring water (Volvic)	ND	ND	Algae (<i>C. vulgaris</i>)
Sayre (1969)	<i>Gre. myriops</i>	Sphagnum/culture dish (80 × 100 mm)	Demineralsised water	ND	ND	Nematodes (<i>Panagrellus redivivus</i>)
Ito et al. (2016)	<i>Gre. myriops</i>	1.2% agar/plate (90 mm)	Spring water (Volvic)	22–23°C	ambient photoperiod	Algae (<i>Parachlorella beijerinckii</i>) and rotifers (<i>L. inermis</i>)
Altiero and Rebecchi (2001)	<i>Gre. monoicus</i>	1.2% agar/plastic dish (15 mm/7 mm)	Spring water (San Benedetto)	4 and 14°C	12/12 L/D	Natural sediment with undetermined algae

(Continued)

Table I. (Continued).

Author	Species	Walking substrate/container	Medium	Temp.	Photoperiod	Food
Koszytła et al. (2016)	<i>Gre. pushkini</i>	Petri dish scratched with fine sandpaper	Spring water (Zywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.)
Koszytła et al. (2016)	<i>Thu. ruffoi</i>	Petri dish scratched with fine sandpaper	Spring water (Zywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.)
Janelt et al. (2019)	<i>Thu. ruffoi</i>	Algae/plastic Petri dish	Spring water (Zywiec Zdrój) mixed with distilled water (1:1)	19°C	12/12 L/D	Algae (<i>Chlorella</i> sp. and <i>Chlorococcum</i> sp.)
Janelt and Poprawa (2020)	<i>Thu. ruffoi</i>	Scratched plastic Petri dish and plastic 24-well plates	Spring water (Zywiec Zdrój) mixed with distilled water (1:1)	19 and 25°C	ND	Algae (<i>Chlorella</i> sp. and <i>Chlorococcum</i> sp.)
Kondo et al. (2020)	<i>Thu. ruffoi</i>	2% agar/plate	Spring water (Volvic)	15°C	ND	Algae (<i>C. vulgaris</i>)
Hejmol and Schnabel (2005)	<i>Thu. stephaniae</i>	ND	Water, not specified	15–25°C	ND	Algae
Bingemer et al. (2016)	<i>Iso. dasychi</i>	2% agar/microscope slides (1-Slide '2 Well', Ibidi) in small chambers	Defaunated tap water	ND	ND	Algae (<i>C. vulgaris</i>)
Bartel and Hohberg (2020)	<i>Iso. dasychi</i>	2% agar/culture plate	Tap water	20°C	ND	Algae (<i>C. vulgaris</i>)
Baumann (1970)	<i>Mac. hufelandi</i>	Algae/glass dish	Water, not specified	20°C	ND	Algae (<i>Pseudochlorella</i> sp.)
Altiero and Rebecchi (2001)	<i>Mac. joannae</i>	1.2% agar/plastic dish (15 mm/7 mm)	Spring water (San Benedetto)	14 and 20°C	12/12 L/D	Nematodes (<i>Pristionchus ihertneri</i> , <i>Panagrolaimus rigidus</i> , <i>Caenorhabditis elegans</i>)
Stec et al. (2015)	<i>Mac. paulinae</i>	Petri dish scratched with fine sandpaper	Spring water (Zywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.) and rotifers (<i>L. inermis</i>)
Poprawa et al. (2015a)	<i>Mac. polonicus</i>	2% agar/Petri dish	Tap water mixed with distilled water (1:1)	Room temp.	Shaded place	Nematodes (<i>C. elegans</i>) and algae
Hengherr et al. (2008)	<i>Mac. sapiens</i>	3% agar/plastic plates	Water, not specified	ND	ND	(<i>Chlorella</i> sp.) Rotifers (<i>P. citrina</i>) and algae (<i>C. elongatum</i>)
Lemloh et al. 2011	<i>Mac. sapiens</i>	Agar/multi-well culture plates	Spring water (Volvic)	20°C	12/12 L/D	Algae (<i>C. elongatum</i>)
Sugiura et al. (2019)	<i>Mac. shonaticus</i>	1.2% agar/plastic dish (90 mm)	Spring water (Volvic)	20°C	Dark	Rotifers (<i>L. inermis</i>) and algae (<i>C. vulgaris</i>)
Sugiura et al. (2020a)	<i>Mac. shonaticus</i>	1.2% agar/plastic dish (32 and 90 mm)	Spring water (Volvic)	22°C	ND	Rotifers (<i>L. inermis</i>) and algae (<i>C. vulgaris</i>)
Guidetti et al. (2020)	<i>Meb. joenssoni</i>	3% agar/plastic culture dish	Spring water (Volvic)	20°C	12/12 L/D	Algae (<i>C. elongatum</i>)

(Continued)

Table I. (Continued).

Author	Species	Walking substrate/container	Medium	Temp.	Photoperiod	Food
Itang et al. (2020)	<i>Meb. ditlmanensis</i>	2% agar and moss/plastic Petri dish (60 mm)	Water (ddH ₂ O)	ND	ND	Algae (<i>Chlorella</i> sp.)
Hengherr et al. (2008)	<i>Pam. richtersi</i> group 1 and 2	3% agar/plastic plates	Water, not specified	ND	ND	Rotifers (<i>P. citrina</i>) and algae (<i>C. elongatum</i>)
Suma et al. (2020)	<i>Paramacrobiotus</i> sp.	2% agar/plastic culture dish (35 mm)	KCM solution in water	20°C	Dark	Nematodes (<i>C. elegans</i>)
Sugiura et al. (2019)	<i>Paramacrobiotus</i> sp. TYO strain	1.2% agar/plastic dish (90 mm)	Spring water (Volvic)	20°C	Dark	Rotifers (<i>L. inermis</i>) and algae (<i>C. vulgaris</i>)
Kaczmarek et al. (2020)	<i>Pam. experimentalis</i>	Petri dish scratched with fine sandpaper	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	20°C	ND	Rotifers (<i>L. inermis</i>)
Altiero and Rebecchi (2001)	<i>Pam. fairbanksi</i>	1.2% agar/plastic dish (15 mm/7 mm)	Spring water (San Benedetto)	14 and 20°C	12/12 LD	Nematodes (<i>P. itheriteri</i> , <i>P. rigidus</i> , <i>C. elegans</i>)
Altiero et al. (2006, 2010)	<i>Pam. fairbanksi</i>	1.2 and 2% agar/plastic dish (15 mm/7 mm)	Spring water	14°C	12/12 LD	Bacteriophagous nematodes
Kosztyła et al. (2016)	<i>Pam. fairbanksi</i>	Petri dish scratched with fine sandpaper	Spring water (Żywiec Zdrój) mixed with ddH ₂ O (1:3)	16°C	Dark	Rotifers (<i>L. inermis</i>) and algae (<i>Chlorococcum</i> sp. and <i>Chlorella</i> sp.)
Schill and Fritz (2008); Schill (2013)	<i>Pam. kenianus</i>	3% agar/plastic plates	Spring water (Volvic)	20°C	12/12 LD	Rotifers (<i>P. citrina</i>)
Schill and Fritz (2008); Schill (2013)	<i>Pam. palaui</i>	3% agar/plastic plates	Spring water (Volvic)	20°C	12/12 LD	Rotifers (<i>P. citrina</i>)
Hohberg (2006)	<i>Pam. richtersi</i>	2% agar and soil particles/glass Petri dish (70 mm)	Tap water	16°C	Dark	Nematodes (<i>Pelodera teres</i> and <i>Acroboloides nanus</i>)
Hengherr et al. (2008)	<i>Pam. richtersi</i>	3% agar/plastic plates	Water, not specified	ND	ND	Rotifers (<i>P. citrina</i>) and algae (<i>C. elongatum</i>)
Hengherr et al. (2008)	<i>Pam. tonolii</i>	3% agar/plastic plates	Water, not specified	ND	ND	Rotifers (<i>P. citrina</i>) and algae (<i>C. elongatum</i>)
Lemloh et al. 2011	<i>Pam. tonolii</i>	Agar/multi-well culture plates	Spring water (Volvic)	20°C	12/12 LD	Rotifers (<i>P. citrina</i>) and algae (<i>C. elongatum</i>)
Węglarska (1957)	<i>Dactylobiotus dispar</i>	Limestone with green algae <i>Chlorosphaera</i> sp. and moss <i>Fontinalis</i> sp./10 × 10 × 8 glass containers	Tap water	ND	ND	Algae (<i>Chlorosphaera</i> sp.) and diatoms
Kihm et al. (2020)	<i>Dac. ovimutans</i>	1.5% agar/plate	Spring water (Volvic)	11°C	ND	Rotifers and algae from King George Island
Bertolani and Buonagurelli (1975)	<i>Dac. parthenogeneticus</i>	Undetermined algae/plastic dish	Water, not specified	19°C	Daylight	Undetermined algae from environment
Poprawa et al. (2015b)	<i>Dac. parthenogeneticus</i>	Algae/plastic Petri dish	Spring water (Żywiec Zdrój) mixed with distilled water (2:8)	20°C	ND	Algae (<i>Chlorella</i> sp. and <i>Chlorococcum</i> sp.).

years. We describe useful tips and tricks for eutardigrade culturing and discuss possible problems that may arise during such laboratory culturing, as well as propose possible solutions. We think that the presented protocols can be useful for different types of studies, e.g. ecological, ethological, physiological, astrobiological, biotechnological or medical, or even in integrative taxonomy.

2. Establishment of cultures

2.1. Origin of the cultured species

The commercial culture of parthenogenetic *Hys. exemplaris* Gąsiorek, Stec, Morek and Michalczyk, 2018 found in a benthic sample with type locality in a pond in Darcy Lever, Bolton, Lancashire, England (53°33'32"N, 2°23'48"W; 75 m asl) was provided by Sciento (Manchester, UK) (under catalogue number Z151). Specimens of bisexual *Mac. polypiformis* Roszkowska, Ostrowska, Stec, Janko and Kaczmarek, 2017 were extracted from a moss sample collected from a concrete wall in a tropical rainforest, in the type locality next to E15 road, ca. 3.5 km west of San Lorenzo, Manabí Province, Ecuador (1°04'06"S, 89°52'18"W; 370 m asl). Specimens of facultatively parthenogenetic/bisexual *Mil. inceptum* Morek, Suzuki, Schill, Georgiev, Yankova, Marley & Michalczyk, 2019 were extracted from a moss sample on a concrete wall next to Przybyszewskiego street in Poznań, Poland (52°24'15"N, 16°53'18"E; 87 m asl). Specimens of two populations of bisexual *Pam. experimentalis* Kaczmarek, Mioduchowska, Poprawa and Roszkowska, 2020 (i.e. MAD-TAR-11 and MAD-TAR-9) were extracted from a sample of mosses from soil collected near Ambavaniasy, Toamasina Province, Madagascar (8°56'37"S, 48°30'52"E, 717 m asl) and near Fort-Voyron, Antananarivo, Antananarivo Province, Madagascar (18°55'35"S, 47°31'23"E, 1 340 m asl), respectively. Specimens of parthenogenetic *Par. fairbanksi* Schill, Förster, Dandekar and Wolf, 2010 (strain Pam. fai_I_PL.018) were kindly provided to us by the Michalczyk Lab; they were collected in Jagiellonian University Botanical Garden, Kopernika 27 Street in Kraków, Poland (50°03'44"N, 19°57'26"E, 205 m asl) from a moss sample taken from a tree. Another parthenogenetic *Pam. fairbanksi* population (WS-MN01) was collected from a moss sample on a rocky hill in Töv Province, Mongolia (47°49'57.0"N, 107°31'26.8"E, 1432 m asl).

Tardigrades were extracted from the samples and studied following the standard methods as described in Dastyk (1980). Tardigrade taxonomy follows Bertolani et al. (2014) and later

updates for Isohypsibiidae (Gąsiorek et al. 2019). Genus abbreviations follow Perry et al. (2019).

2.2. Equipment for keeping, cleaning and feeding eutardigrade cultures

Two climatic chambers were used: a POL EKO KK 115 TOP+ (POL-EKO Aparatura) climatic chamber with controlled temperature and relative humidity (RH) (climatic chamber 1) (Figure 1), and a POL ST1 BASIC (POL-EKO Aparatura) climatic chamber with controlled temperature and photoperiod (climatic chamber 2). The tardigrade cultures were also maintained under room conditions (see section 2.3).

All species were reared on plastic vented Petri dishes with one of three combinations of diameters (\emptyset) and heights: 35 mm and 11 mm, 55 mm and 13 mm or 90 mm and 13 mm, respectively. The bottom of each Petri dish was scratched with sand paper (grid size from P-100 to P-240) to allow tardigrade locomotion. Each Petri dish was filled with a culture medium containing spring water (Żywiec Zdrój: bicarbonates: 121.06 mg/L, fluorides: 0.07 mg/L, Mg^{2+} : 5.37 mg/L, Ca^{2+} : 36.39 mg/L, Na^{+} : 7.79 mg/L) mixed with double distilled water (ddH₂O) in a 1:3 ratio (hereafter referred to simply as culture medium). Sterile plastic disposable (Pasteur) pipettes (3 mL) were applied to exchange culture medium, and automatic pipettes (20–200 μ L) were used to add food and extract tardigrades and/or their eggs.



Figure 1. Climatic chamber containing tardigrade cultures on Petri dishes.

2.3. Details on culture conditions for each eutardigrade species

As detailed in Table II, in chamber 1 (see section 2.2) the cultures were performed in complete darkness, at 17°C and at 40% RH (defined as culture regime CR 1), whereas in chamber 2 they were performed at 20°C with 12 h/12 h (L/D) photoperiod, at an RH of ca. 50% (defined as CR 2). It is also possible to grow this culture at room temperature if there is no access to a climatic chamber. We reared tardigrades at room temperature, which ranged from 21 to 24°C, with RH estimated at 40–60% and light conditions naturally regulated (by seasonal changes) in a day/night cycle (defined as CR 3). However, we do not recommend this third method because these conditions are very variable. Table II summarises all culture conditions for each tardigrade species that we tested.

Macrobiotus polypiformis was cultured in Ø 55 mm Petri dishes under CR 2 and 3 with similar rates of success. Initially the species was cultured under CR 3 during autumn, winter and spring, whereas during summer (due to high temperatures in the laboratory) specimens were transferred to the chamber 2 (under CR 2 conditions), and since that time we have continued to culture them successfully using that regime. Algae, rotifers and nematodes were used as food, as we noticed that juveniles fed on algae and that adults had a mixed diet.

Paramacrobrotus experimentalis (MAD-TAR9 and MAD-TAR11), *Pam. fairbanksi* (PL-01) and *Mil. inceptum* were cultured in Ø 35, 55 or 90 mm Petri dishes under CR 1. Rotifers and nematodes were used as food.

Hypsibius exemplaris, *Pam. experimentalis* (MAD-TAR11) and *Pam. fairbanksi* (WS-MN01) were cultured in Ø 55 mm Petri dishes under CR 2. Algae

(in the case of *Hys. exemplaris*) and rotifers and nematodes (in the case of *Paramacrobrotus* species) were used as food.

3. Protocols to obtain different types of food for cultured eutardigrades

3.1. Algae

The green alga *C. vulgaris* strain SAG211-11b was obtained from the culture collection of algae (Sammlung von Algenkulturen (SAG)) at the University of Göttingen, Germany. Algae were cultivated under sterile conditions in Wright's Cryptophytes (WC) medium (Guillard & Lorenzen 1972) containing CaCl₂, MgSO₄, NaHCO₃, K₂HPO₄, NaNO₃, Na₂SiO₃, a mixture of micronutrients, vitamins (thiamin HCl, biotin) and N-[Tris (hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES) (Sigma-Aldrich, CAS Number 7365-44-8) for buffering and pH adjusted to 7.6–7.8 with NaOH before autoclaving. Stock axenic algae cultures were reared on solid WC medium (with 1.5% agarose) and refreshed monthly by transferring a portion of algae with a flame-sterilised inoculation loop onto sterilised (by autoclaving) solid WC medium under the laminar flow hood. These stock cultures were further used to inoculate (under a laminar flow hood) autoclaved 250 mL Erlenmeyer flasks filled with 150 mL of liquid WC medium. Such cultures, after reaching the stationary growth phase, were used to initiate continuous cultures in turbidostats (Figure 2a-b). Each turbidostat comprises a 2 L bottle filled 2/3 full with autoclaved liquid WC medium, two inflow channels and a culture excess outflow. One of the inflows was used to supply fresh WC medium by a peristaltic pump at rate of 800 mL/day; filtered (with 0.2 µm filter) air was pumped, by an air pump at the bottom of the chemostat, through the other inflow to mix the culture and

Table II. Culture conditions for studied tardigrade species. CR = culture regimes, T = temperature (°C), RH = relative humidity (%), LC = light conditions, PDD = Petri dish diameter (mm).

CR	Species	T	RH	Location	LC	PDD
1	<i>Paramacrobrotus fairbanksi</i> (PL-01) <i>Milnesium inceptum</i> <i>Paramacrobrotus experimentalis</i> (MAD-TAR-9 and MAD-TAR-11)	17	40	Chamber 1	Darkness	Ø 35, 55, 90
2	<i>Hypsibius. exemplaris</i> <i>Paramacrobrotus experimentalis</i> (MAD-TAR-11) <i>Paramacrobrotus fairbanksi</i> (WS-MN01) <i>Macrobiotus polypiformis</i>	20	Not regulated, ca. 50	Chamber 2	12 h/12 h (L/D) photoperiod	Ø 55
3	<i>Macrobiotus polypiformis</i>	20–24	ca. 40–60	Room conditions	Shaded place with seasonal changes	Ø 55

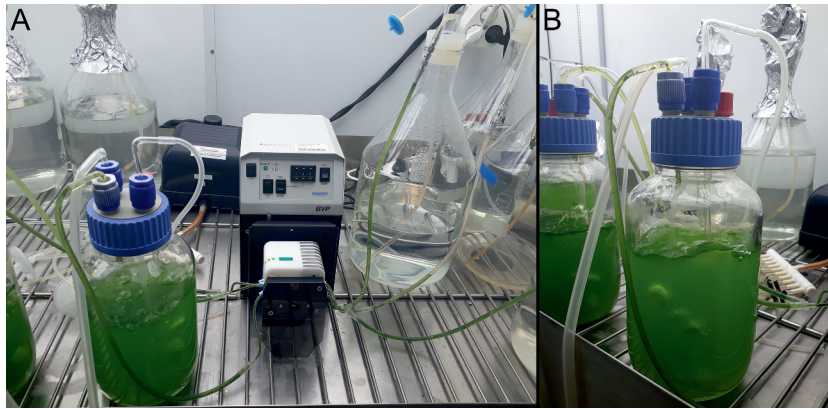


Figure 2a-b. *Chlorella vulgaris* cultures performed in turbidostats.

supply it with CO₂. When the medium was pumped into the turbidostat, the excess culture was pushed out (due to the overpressure induced by air pumped into the bottle), and collected in another flask. The turbidostats were held in a walk-in phytotron (Conviron), with a constant temperature of 20°C, 160 μmol photons per m²/s light intensity and a 16 h/8 h (L/D) photoperiod. To concentrate the algae in the outflow flask, they were left to settle for 2 days, and the excess medium was decanted afterwards. The settled algae were then transferred without a sterile regime to glass bottles (not completely twisted off) and left for 2–3 weeks so that the algae began to aggregate on the bottom of the bottle. This stock of aggregated algae was further used for feeding the tardigrades.

3.2. Rotifers

The rotifer *L. inermis* strain 1.A2.15 originates from a wastewater treatment plant (WWTP) located in southern Poland and was obtained from a sludge sample derived from the WWTP aeration chamber. The sample was divided into 1 mL portions that were poured into a 24-well tissue culture plate and kept in a SANYO MLR-350 Versatile Environmental Test Chamber (Sanyo Electric Co Ltd) at 15°C for 3 weeks. Then, single rotifers were individually transferred with a micropipette into 24-well tissue culture plates filled with spring water (Żywiec Zdrój), and 10 μL of 2% NOVO (nutrition powder, patent Pajdak-Stós et al. 2017) suspension was added as food source. The plate was transferred to a walk-in climatic chamber with a constant temperature of 20°C and kept in darkness. The strains were controlled every few days and the best proliferating strains were then selected for further culturing.

The selected strain 1.A2.15 was transferred into Ø 55 mm plastic Petri dishes filled with spring water and fed weekly with a pinch of NOVO powder. Since then

the cultures have been constantly incubated in the same walk-in climatic chamber in darkness at 20°C. When the density of cultures reached ca. 5000 individuals/mL, the cultures were transferred to Ø 90 mm Petri dishes. Once a week the cultures were checked, half the culture volume from each dish was removed and made up with the fresh spring water, and they were fed with NOVO powder. Strain 1.A2.15 is now constantly cultured in the laboratory of the Aquatic Ecosystems Group, Institute of Environmental Sciences, Jagiellonian University in Kraków.

3.3. Nematodes

The wild-type Bristol N2 strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (Duluth, Minnesota, USA). Standard methods are used for the maintenance and manipulation of the strain under sterile conditions (Brenner 1974). Briefly, animals were grown monoxenically on solid nematode growth medium (NGM; NaCl 50 mM, peptone 0.25%, CaCl₂ 1 mM, cholesterol 5 μg/mL, KH₂PO₄ 25 mM, MgSO₄ 1 mM and agar 1.7%) using *Escherichia coli* Migula, 1895 (strain OP50) as the food source. The *E. coli* strain was grown in liquid Luria broth (LB) medium. An overnight LB culture of *E. coli* was used to seed the lawn on NGM prepared on Ø 100 mm plates. The plates were incubated overnight at 37°C, allowed to cool before animals were transferred and then incubated at room temperature in the dark. Specimens of *C. elegans* reproduce quickly and in large numbers. A sterilised spatula was used to move a chunk of agar with animals onto freshly seeded NGM plates, and after 4–5 days animals were harvested by washing them off the plate with the tardigrade culture medium and used as food for tardigrades.

4. Tardigrade feeding and culture cleaning

All the tardigrade cultures were cleaned, and tardigrades were fed, once per week. This schedule seems to be optimal because at shorter intervals it was observed that not all food was consumed and the medium was still fresh and relatively clean. Tardigrades were fed (with algae and/or rotifers and nematodes) according to the quantities presented in Table III, after cleaning of cultures. As the rotifers tend to concentrate on the bottom of vials, before feeding tardigrades the rotifer cultures were thoroughly mixed. Culture medium was poured onto plates with nematodes (ca. 20–30 mL, depending on the number of tardigrade cultures that needed to be fed) and medium with suspended nematodes was transferred to a 50 mL falcon tube prior to feeding.

Some differences concerning food intake were observed among taxa. For example, *Pam. experimentalis* specimens consumed food much faster than did specimens of other species, even when cultured at the same density as two *Pam. fairbanksi* populations. Moreover, some examples of cannibalism were sometimes observed (most often, larger specimens fed on juveniles). A different situation was observed in the case of *Mac. polytipiformis*, which almost never consumed all food offered during the week. In the cultures of herbivorous species (especially in darkness), it was observed that after a week some algae were faded (yellow-brown in colour) and the fading process seemed to occur faster when the number of specimens in culture was higher. This indicates that the tardigrades were at least partly responsible for this process, i.e. by sucking/killing algae cells.

Usually, after a week, a thin iridescent film – probably the accumulation of metabolic wastes and bacteria – was observed on the surface of the water in almost all dishes (Figure 3a-b). This thin film was always firstly removed during the cleaning procedure. It was removed delicately using the Pasteur pipette, along with the upper part of the culture medium, with special attention paid to not removing the animals or their eggs. Later, fresh culture medium was added and the cultures were then strongly mixed using the pipette. After the animals and their eggs fell to the bottom, floating food remains, old exuviae and dead animals were removed. During this procedure a portion of empty eggs (completely transparent) from the cultures of *Macrobiotus* and *Paramacrobiotus* species were also removed. At the same time, if necessary, eggs of *Paramacrobiotus* species were collected to establish new cultures.

The procedure for establishing new cultures from the eggs begins with transferring eggs to Ø 55 mm Petri dishes with a smooth bottom (not scratched), with a small amount of rotifers as food for hatching juveniles. In these Petri dishes eggs continued to hatch, and once per week juveniles were transferred to new culture Petri dishes (that were scratched on the bottom). Hatching carried out in smooth dishes makes it easier to catch juveniles and transfer them to new cultures.

5. Possible problems and solutions

Table IV presents a list of the most frequent problems we observed in our cultures, as well as their possible solutions.

Table III. Feeding schedule.

Species	Food	Amount of food (per Ø 55 mm dish)	Number of adult individuals per dish (Ø 55 mm)*
<i>Hypsibius exemplaris</i>	Algae	40–50 µL of aggregated algae	1500–2000
<i>Macrobiotus polytipiformis</i>	Algae, nematodes, rotifers	40–50 µL of aggregated algae, 200 µL of nematodes (ca. 3500 specimens in 1 mL) and 2 mL of rotifers (ca. 7500 specimens in 1 mL)	200–300
<i>Paramacrobiotus experimentalis</i>	Nematodes, rotifers	200 µL of nematodes (ca. 3500 specimens in 1 mL) and 2 mL of rotifers (ca. 7500 specimens in 1 mL)	ca. 200
<i>Paramacrobiotus fairbanksi</i>	Nematodes, rotifers	200 µL of nematodes (ca. 3500 specimens in 1 mL) and 2 mL of rotifers (ca. 7500 specimens in 1 mL)	200–300
<i>Milnesium inceptum</i>	Nematodes, rotifers	200 µL of nematodes (ca. 3500 specimens in 1 mL) and 2 mL of rotifers (ca. 7500 specimens in 1 mL)	300–400

*These are values that we find optimal per dish, i.e. animals do not show any signs of population overcrowding and remain active. Smaller and larger numbers of animals per dish are also possible, but in that case dishes should be checked more frequently to detect problems (such as anoxia, lack of food, cessation of reproduction, etc.) in time.

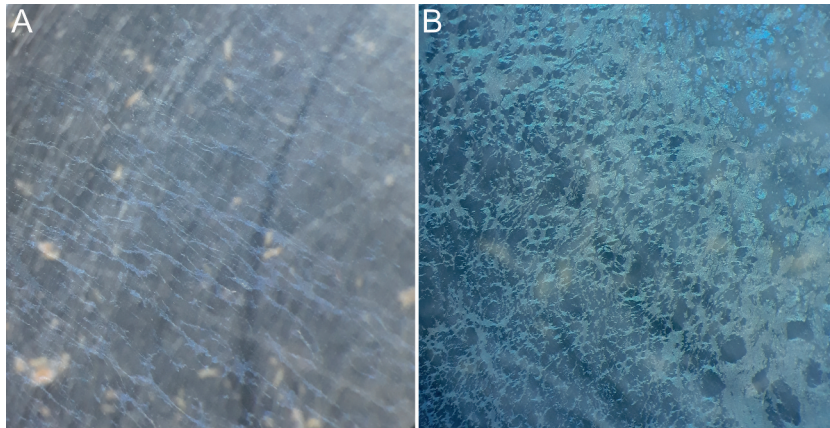


Figure 3a-b. A thin iridescent film (most likely concentrated metabolic wastes and bacteria) that may appear on the water surface of the culture dishes.

6. Discussion

The available culture protocols concerning Eutardigrada species differ in detail regarding medium, walking substrate, food, culture containers, environmental conditions (e.g. ambient temperature or photoperiod) and cleaning/feeding schedule. The majority of cultured species are freshwater or terrestrial taxa from the superfamilies Hypsibioidea Pilato 1969, Isohypsibioidea Sands et al. 2008 or Macrobiotioidea Thulin 1928. However, one of the most commonly reared taxa is *Milnesium* Doyère, 1840, belonging to the family Milnesiidae Ramazzotti 1962. As shown in Table I, the number of successfully cultured taxa is high, but it is even higher taking into account all the other species to which protocols used for one species have been later adapted. For example, the protocol proposed by Stec et al. (2015) for *Mac. paulinae* Stec, Smolak, Kaczmarek & Michalczyk, 2015 was later used with success for cultures of *Mac. caelestis* Coughlan, Michalczyk and Stec, 2019, *Mac. canaricus* Stec, Krzywański and Michalczyk, 2018, *Mac. hanna* Nowak and Stec, 2018, *Mac. kamilae* Coughlan and Stec, 2019, *Mac. noongaris* Coughlan and Stec, 2019, *Mac. papei* Stec, Kristensen and Michalczyk, 2018, *Mac. scoticus* Stec, Morek, Gąsiorek, Blagden and Michalczyk, 2017, *Meb. radiatus* (Pilato, Binda and Catanzaro, 1991) and *Pam. lachowskae* Stec, Roszkowska, Kaczmarek & Michalczyk, 2018 (Stec et al. 2017, 2018a, 2018b, 2018c, 2018d; Nowak & Stec 2018; Coughlan & Stec 2019; Coughlan et al. 2019). Also, the protocol suggested for *Hys. exemplaris* was used later for *Mac. shonai-cus* Stec, Arakawa and Michalczyk, 2018 (Stec et al. 2018e). This clearly suggests that for species with similar food and environmental requirements, culturing methods can be considered more or less universal.

Carnivorous species are fed mainly with small rotifers or/and nematodes of a few species, whereas herbivorous tardigrades are fed mainly with different species of unicellular freshwater algae (for more details see Table I). Our observations indicate that some species (like *Mac. polypiformis*) may have a mixed diet, which was also observed for *Mac. shonai-cus* Stec, Arakawa and Michalczyk, 2018, for example (Sugiura et al. 2020a). Although a few different species of algae, nematodes and rotifers have been proposed as food for different Eutardigrada species, the most often used were algae: *Chlorococcum* sp. or *Chlorella vulgaris* Beijerinck 1890; nematodes: *Caenorhabditis elegans* (Maupas, 1900) and rotifers: *Lecane inermis* (Bryce, 1892). Other types of food proposed for some tardigrade species were bacteria, diatoms, cyanobacteria, other tardigrades or even fungi (Nelson et al. 2010; Guidetti et al. 2012; Roszkowska et al. 2016; Morek et al. 2020; Bryndová et al. 2020). Moreover, the same type of food (the same prey species or algae) has been proposed for different tardigrade taxa, which shows that tardigrades are not monophagous and some are even omnivorous, which was also previously suggested by several authors (e.g. Schill et al. 2011; Kosztyła et al. 2016; Bryndová et al. 2020; Kihm et al. 2020). However, it should be mentioned that lifespan and reproductive success can be strongly affected by food type (Bryndová et al. 2020).

The majority of cultures are maintained on plastic Petri dishes with a thin layer of agar on the bottom of the dish. Some authors used other plastic containers, or glass, such as watch glasses, bottles or Erlenmeyer

Table IV. Possible problems with cultures, their causes and proposed solutions.

Problem	Reason	Solution
Tardigrades are not moving, they lie still and are crescent shaped (Figure 4a)	- Too many tardigrades on one plate	- Divide the animals into two dishes; exchange culture medium for fresh
	- There was a shortage of oxygen - Wrong diet - Animals brought into light condition sometimes do not move for a while	- Exchange culture medium for fresh - Try a different type of food - Wait a few minutes
Tardigrades' bodies are straight and transparent; animals are not moving (Figure 4b)	- Critical shortage of oxygen (anoxibiosis)	- Immediately exchange culture medium (it may take up to several days for the animals to regenerate, and some of them may not survive; if necessary change the medium once a day)
The largest animals are alive but they are not walking on the dish, only lying and moving (Figure 4c)	- Animals are dead - Animals ate too much	- No action required
Tardigrades are becoming more transparent, not milky-white as they should be (Figure 4d)	- Animals are not eating	- Change the food (try different type of food) or exchange the medium; sometimes it is good to change the dish to a new one or try to add a photoperiod if the animals are constantly in the dark
Tardigrades eat smaller individuals	- More food is needed	- Give more food per dish, or transfer adult specimens to a separate dish
Tardigrades do not reproduce	- The species may be dioecious - Complete darkness is not optimal for the species - Temperature is not optimal	- More specimens should be placed in one dish - Add a photoperiod or place the culture in light conditions - Try increasing/decreasing the temperature by a few degrees (similar to the natural environmental conditions of the species)
Some tardigrades are in the shape of a tun (Figure 4e)	- Inappropriate conditions in the chamber	- Try increasing/decreasing the temperature, or add a photoperiod if the animals are constantly in the dark - Try a different type of food - Wrong diet - Change the dish to a new one - Dish too "old" with some fungi appearing
Some tardigrades form larger groups on the dish (Figure 4f)	- Animals are likely mating	- No action required

flasks. Because tardigrades are unable to crawl on smooth glass or plastic surfaces, different researchers use agar or algae as a walking substrate, or scratch the bottom of dishes with sand paper (Table I).

The agar solution (which ranges from 1.2% to 3%), container type and size of the Petri dishes probably are not very important factors in the success or failure of tardigrade cultures. According to our observations, the agar layer is not a perfect solution because it dissolves in water (used as culture medium) after some time; it also peels off from the dishes and falls apart into smaller fragments, which makes cleaning and observing tardigrades very difficult. The other popular walking substrate, i.e. algae, used also as food, is employed only for herbivorous species. Again, according to our observations, it is not a good solution, especially if the algae are

planktonic and not overgrowing the bottom, because when a tardigrade accidentally falls down to the bottom of the dish it most likely will die because of its inability to return to the algae.

As mentioned, some authors use scratched Petri dishes, the same method as used in the present study. This is a very useful method because it is free from the disadvantages of both approaches described above, i.e. it never encounters the problems caused by fragmented agar and it avoids the death of tardigrades due to their inability to return to algae.

The most often used media are different types of spring water or a mixture of spring water and ddH₂O; however, some authors used tap water or distilled water only. In other cases the type of water used was not specified.

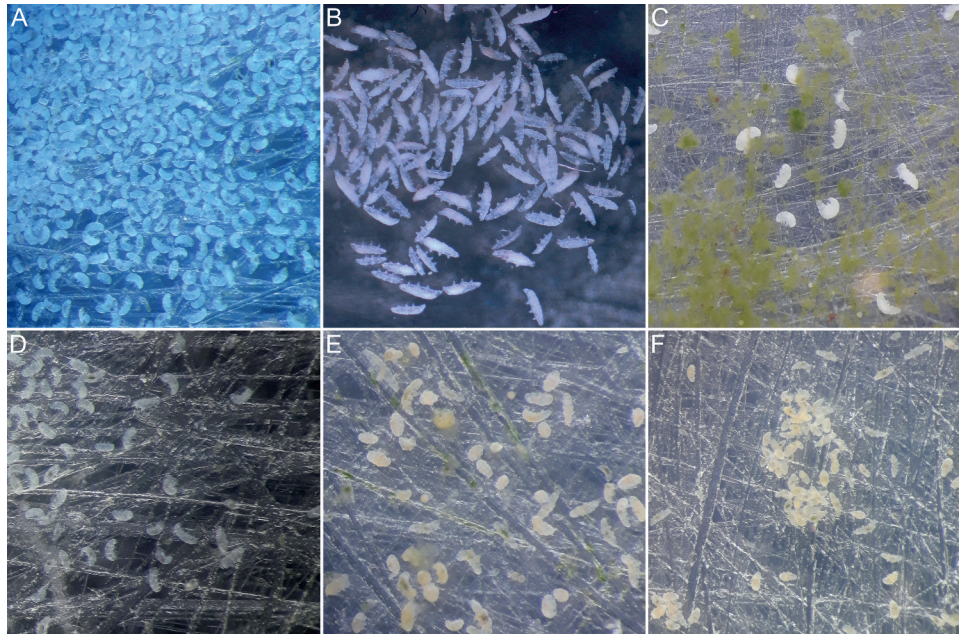


Figure 4a-f. Problems that may arise with tardigrades in the cultures: (a) tardigrades curled into crescent shapes, not moving (*Hys. exemplaris*); (b) tardigrades straight and transparent, not moving (*Pam. fairbanksi*); (c) the biggest animals are alive, lying and moving but not walking on the dish (*Pam. experimentalis*); (d) tardigrades are becoming transparent (*Hys. exemplaris*); (e) some of the tardigrades start forming “tuns” in the water (*Mac. polypiformis*); (f) tardigrades form larger groups (*Mac. polypiformis*).

The culture conditions varied in different studies, with ambient temperature being the main controlled factor. Temperatures used in different culture protocols varied from very low (4–7°C) to quite high (25°C); the most frequently applied temperatures were in the range of 14°C to 20°C. The lowest temperatures were used in cultures of the Antarctic species *Acutuncus antarcticus* (Richters, 1904) or the high mountain species *Grevenius monoicus* (Bertolani, 1982), in line with the natural conditions under which these species live. In contrast, the highest temperatures were used in cultures of terrestrial *Milnesium* species and *Ramazottius varieornatus* Bertolani & Kinchin, 1993 and of freshwater *Thulinus ruffoi* (Bertolani, 1982), *Thu. stephaniae* (Pilato, 1974), *Hys. exemplaris* and *Gre. myrops* (du Bois-Reymond Marcus, 1944). Species of *Milnesium* and *Ramazottius* Binda & Pilato, 1986 are known to inhabit dry and hot (i.e. xerothermic) habitats (e.g. Dastyh 1988), so it is not surprising that the temperature of their cultures was the highest. Freshwater species of the genus *Thulinus* Bertolani, 2003 and species *Hys. exemplaris* and *Gre. myrops* can be found in shallow freshwater sediments or even in sediments of WWTPs, where the water temperature can be very high and the O₂ concentration very low (e.g. Utsugi 2001; Sobczyk et al. 2015; Jakubowska-Krępska et al. 2018).

But, of course, the impact of temperature is strongly species-dependent, which has been confirmed by other authors (e.g. Sømme & Meier 1995; Ramlov & Westh 2001; Li & Wang 2005a, 2005b; Hengherr et al. 2009; Zawierucha et al. 2018; Neves et al. 2020). Nevertheless, as shown in Table I, some tardigrade species have been cultured with success at different temperatures. The same tardigrade species have also been developed properly at different temperature and/or food regimes, although some morphological structures were larger at lower temperatures and smaller at higher temperature (Koszyła et al. 2016). According to our observations, controlling the ambient temperature is essential, and at higher temperatures (above 25°C) tardigrades cease activity (probably due to anoxia) whereas at lower temperatures (below 16°C) they reproduce much slowly. These aspects certainly require more detailed studies in the future.

In turn, according to our observations, photoperiod and relative humidity seem not to be crucial for tardigrade cultures. The same can be inferred based on the literature (see Table I and papers cited herein) because humidity values are almost never reported, and the photoperiod is also not reported or different regimes are proposed. This is understandable especially in the case of humidity, because

all of the cultures are maintained permanently in a liquid medium. The photoperiod seems not to be very important because many tardigrades are blind. However, a positive or negative phototaxis was observed for some species, e.g. by Marcus (1929), Baumann (1961) and Beasley (2001), and also by our team in the case of *Mac. polypiformis*.

It is also very important, and should be considered before establishing tardigrade cultures, that some tardigrades can be cultured as isogenic strains because they are parthenogenetic while others need to be cultured in pairs, in small groups or in dense populations because they are dioecious.

In our study we used simple methods of mass culturing of herbivorous, omnivorous and carnivorous tardigrades, allowing us to avoid the problems, discussed above, with walking substrate (agar or algae). We also discussed in detail the amount of food which is necessary and a quite simple schedule of cleaning and feeding. Moreover, we listed possible problems that may occur when culturing some taxa (e.g. dioecious species, anoxia, cannibalism, etc.) and proposed easy solutions. The culturing of terrestrial Heterotardigrada is still a challenge, as a good protocol has not been established yet; this is because tardigrades are usually cultured in a liquid medium, but most terrestrial heterotardigrades prefer very dry and sunny conditions. Nevertheless, we hope that our simple methods will help beginners to establish tardigrade cultures. This simple approach for culturing many different taxa of tardigrades can accelerate more advanced ecological, ethological, physiological, astrobiological, biotechnological, taxonomical and molecular studies on tardigrades.

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ORCID

M. Roszkowska  <http://orcid.org/0000-0003-1640-8142>
 D. Wojciechowska  <http://orcid.org/0000-0003-1587-9153>
 H. Kmita  <http://orcid.org/0000-0001-5018-5224>
 S. Cerbin  <http://orcid.org/0000-0002-2800-0390>
 E. Fiałkowska  <http://orcid.org/0000-0002-6330-4787>
 R. Sobkowiak  <http://orcid.org/0000-0002-8928-3865>
 W. Szydło  <http://orcid.org/0000-0001-5990-5611>
 Ł. Kaczmarek  <http://orcid.org/0000-0002-5260-6253>

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