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Journal Name	Hydrobiologia	
Corresponding Author	Family Name	<b>Balsamo</b>
	Particle	
	Given Name	<b>Maria</b>
	Suffix	
	Division	Department of Biomolecular Sciences
	Organization	University of Urbino
	Address	Urbino, Italy
	Phone	
	Fax	
	Email	maria.balsamo@uniurb.it
	URL	
	ORCID	<a href="http://orcid.org/0000-0002-7947-0632">http://orcid.org/0000-0002-7947-0632</a>
Author	Family Name	<b>Artois</b>
	Particle	
	Given Name	<b>Tom</b>
	Suffix	
	Division	Centre for Environmental Sciences
	Organization	Hasselt University
	Address	Diepenbeek, Belgium
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	Family Name	<b>Smith</b>
	Particle	
	Given Name	<b>Julian P. S.</b>
	Suffix	III
	Division	Department of Biology
	Organization	Winthrop University
	Address	Rock Hill, SC, USA
	Phone	
	Fax	
	Email	
	URL	

ORCID

---

Author	Family Name	<b>Antonio Todaro</b>
	Particle	
	Given Name	<b>M.</b>
	Suffix	
	Division	Department of Life Sciences
	Organization	University of Modena-Reggio Emilia
	Address	Modena, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

---

Author	Family Name	<b>Guidi</b>
	Particle	
	Given Name	<b>Loretta</b>
	Suffix	
	Division	Department of Biomolecular Sciences
	Organization	University of Urbino
	Address	Urbino, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

---

Author	Family Name	<b>Leander</b>
	Particle	
	Given Name	<b>Brian S.</b>
	Suffix	
	Division	Departments of Botany and Zoology
	Organization	University of British Columbia
	Address	Vancouver, BC, Canada
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

---

Author	Family Name	<b>Steenkiste</b>
	Particle	<b>Van</b>
	Given Name	<b>Niels W. L.</b>
	Suffix	
	Division	Departments of Botany and Zoology
	Organization	University of British Columbia
	Address	Vancouver, BC, Canada
	Phone	

Fax  
Email  
URL  
ORCID

---

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Abstract	<p>Gastrotricha and Platyhelminthes form a clade called Rousphozoa. Representatives of both taxa are main components of meiofaunal communities, but their role in the trophic ecology of marine and freshwater communities is not sufficiently studied. Traditional collection methods for meiofauna are optimized for Ecdysozoa, and include the use of fixatives or flotation techniques that are unsuitable for the preservation and identification of soft-bodied meiofauna. As a result, rousphozoans are usually underestimated in conventional biodiversity surveys and ecological studies. Here, we give an updated outline of their diversity and taxonomy, with some phylogenetic considerations. We describe successfully tested techniques for their recovery and study, and emphasize current knowledge on the ecology, distribution, and dispersal of freshwater gastrotrichs and microturbellarians. We also discuss the opportunities and pitfalls of (meta)barcoding studies as a means of overcoming the taxonomic impediment. Finally, we discuss the importance of rousphozoans in aquatic ecosystems and provide future research directions to fill in crucial gaps in the biology of these organisms needed for understanding their basic role in the ecology of benthos and their place in the trophic networks linking micro-, meio-, and macrofauna of freshwater ecosystems.</p>
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2 **The curious and neglected soft-bodied meiofauna:**  
3 **Rouphozoa (Gastrotricha and Platyhelminthes)**

4 **Maria Balsamo** · **Tom Artois** · **Julian P. S. Smith III** · **M. Antonio Todaro** ·  
5 **Loretta Guidi** · **Brian S. Leander** · **Niels W. L. Van Steenkiste**

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users.

A7 M. Balsamo (✉) · L. Guidi  
A8 Department of Biomolecular Sciences, University of  
A9 Urbino, Urbino, Italy  
A10 e-mail: maria.balsamo@uniurb.it

A11 T. Artois  
A12 Centre for Environmental Sciences, Hasselt University,  
A13 Diepenbeek, Belgium

A14 J. P. S. Smith III  
A15 Department of Biology, Winthrop University, Rock Hill,  
A16 SC, USA

A17 M. Antonio Todaro  
A18 Department of Life Sciences, University of Modena-  
A19 Reggio Emilia, Modena, Italy

A20 B. S. Leander · N. W. L. Van Steenkiste  
A21 Departments of Botany and Zoology, University of British  
A22 Columbia, Vancouver, BC, Canada

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36 Taxonomy · Species diversity

## 37 Introduction

38 Meiofauna constitutes a significant reservoir of biodi-  
39 versity in aquatic ecosystems that is often overlooked.  
40 Sediments and vegetation in freshwater habitats,  
41 including freshwater ponds, lakes, and rivers, but also  
42 mosses, wet soils and semi-aquatic agricultural  
43 ecosystems (e.g., paddy fields), are teeming with  
44 hundreds of thousands, if not millions of poorly known  
45 or completely unrecorded species of these micro-  
46 scopic animals (Giere, 2009).

47 Traditional morphology-based sampling tech-  
48 niques to study biodiversity and ecology of meiofauna  
49 are usually addressed towards ecdysozoan taxa such as  
50 nematodes and copepods, and have so far failed to  
51 account for the sometimes equally abundant and  
52 diverse soft-bodied gastrotrichs and meiofaunal flat-  
53 worms or so-called “microturbellarians” (Martens &  
54 Schockaert, 1986; Nesteruk, 2006; Schockaert et al.,  
55 2008; Giere, 2009). Perhaps more so than other  
56 meiofaunal groups, gastrotrichs and microturbellari-  
57 ans also exemplify the taxonomic impediment, includ-  
58 ing a lack of knowledge on their biodiversity and  
59 organismal biology, a lack of experts and a lack of  
60 taxonomic infrastructure. As such, they have lagged  
61 behind in species discovery and identification, uncov-  
62 ering (cryptic) speciation, biodiversity surveys, pop-  
63 ulation genetics, and phylogeography. Traditional  
64 morphology-based identification methods are often  
65 time-, effort-, and resource-intensive, depend on a  
66 handful of taxonomic experts, and cannot uncover  
67 cryptic diversity. As shown by a few comprehensive  
68 studies of single taxa, the current number of described  
69 species of these two groups in fresh waters is  
70 significantly lower than the estimated species diversity  
71 (e.g., Catenulida: Larsson et al., 2008; *Microstomum*:  
72 Atherton & Jondelius, 2018, 2019; Gastrotricha:  
73 Balsamo et al., 2008).

74 In this contribution, we will focus on the micro- and  
75 meiofaunal freshwater representatives of these taxa  
76 and largely omit the numerous members of the  
77 macrofaunal flatworm group Tricladida, which,  
78 because of their large size, have been rather well  
79 studied and are far better known worldwide.

Freshwater gastrotrichs and microturbellarians not  
only share a number of morphological and biological  
traits, but their parent phyla, Gastrotricha and Platy-  
helminthes, also recently emerged in a monophyletic  
clade within the Spiralia called Rousphozoa (Gr.  
*Rouphao*, ingesting by sucking; Struck et al., 2014),  
which was endorsed by two subsequent, independent  
phylogenomic studies (Egger et al., 2015; Laumer  
et al., 2015a). However, Bleidorn (2019) recovered a  
clade comprising Nemertea and Platyhelminthes sep-  
arate from the clade of Gastrotricha; thus, it is clear  
that further molecular and morphological work is  
needed to test the monophyly of the Rousphozoa. The  
duo-gland adhesive system, recently studied in detail  
for platyhelminthes (Wunderer et al., 2019), was  
proposed as a possible synapomorphy for both taxa  
(Giribet & Edgecombe, 2019). However, studies of the  
gastrotrich duo-gland system are much older (Tyler &  
Rieger, 1980; Ruppert, 1991). Consequently, detailed  
molecular studies of the duo-gland system in Gas-  
trotricha and research to identify other possible  
synapomorphies within Rousphozoa are sorely needed.

Because of their abundance, small body size and  
selective micro- and meiophagous feeding behavior,  
gastrotrichs and microturbellarians most likely play a  
critical role in freshwater trophic networks and  
ecosystem dynamics (Palmer et al., 1997; Balsamo  
& Todaro, 2002; Majdi et al., 2019). However, their  
diversity and ecological roles in aquatic ecosystems  
are still insufficiently known. For freshwater gas-  
trotrichs, the results of the few targeted studies on  
functional ecology appear controversial (Strayer,  
1985; Hummon, 1987; Nesteruk, 1996a, 2007b; Sch-  
mid-Araya, 1997), and for freshwater microturbellari-  
ans no such studies exist to date.

In this study, we aim to give an overview of the  
current state of knowledge on the diversity, distribu-  
tion, and ecology of freshwater rousphozoans. This  
includes an updated census of species in the various  
biogeographical regions, a summary on the impor-  
tance of environmental parameters and biotic interac-  
tions on habitat preferences, spatial and temporal  
distribution, dispersal and trophic ecology of these two  
groups. We also provide recommendations to over-  
come methodological problems and challenges in  
qualitative and quantitative collection and identifica-  
tion of these animals, and discuss future research  
avenues to fill in crucial gaps in our knowledge on  
these important freshwater animals.

## 129 Methodologies for sampling and study

130 It is clear that in studies of freshwater meiofauna,  
 131 Rousphozoa are frequently not considered (Fig. 1). As  
 132 already noted by some authors (e.g., Traunspurger &  
 133 Majdi, 2017), we hypothesize that this is because  
 134 extraction methods used for these soft-bodied organ-  
 135 isms are very different from those used for ecdysozoan  
 136 taxa. This is further supported by historical studies that  
 137 recovered large numbers of rousphozoan taxa using  
 138 extraction methods compatible with their preservation  
 139 (e.g., Strayer, 1985; Robertson et al., 2000). Finally,  
 140 metabarcoding studies in a marine context routinely  
 141 recover rousphozoans thought previously to be rare  
 142 based solely on morphotaxonomic studies (e.g.,  
 143 Rzeznik-Orignac et al., 2017; Leasi et al., 2018).  
 144 Accordingly, we provide up-to-date methods below  
 145 **AQ2** for the collection, preservation, and study of rouspho-  
 146 zoans (Tables 1, 2).

## 147 Sampling and extraction

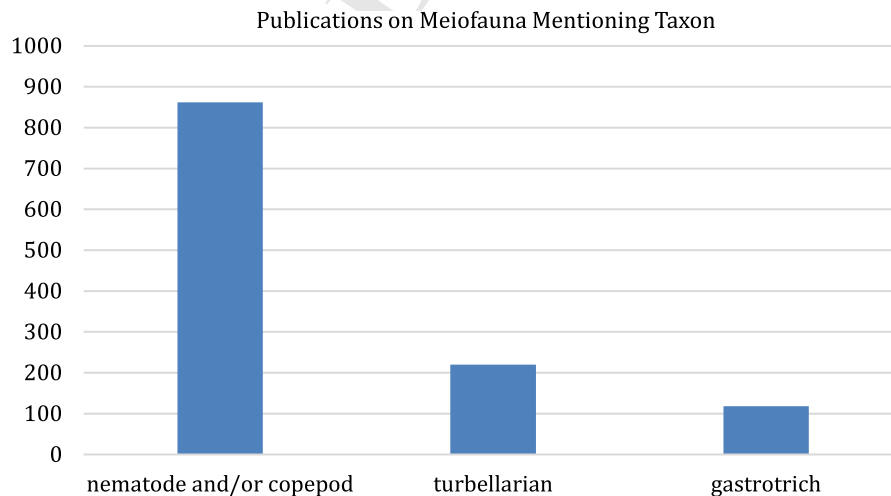
148 Due to the patchy distribution of meiofauna, collec-  
 149 tions of many small samples during different times of  
 150 the year are preferred over a single large sample  
 151 (Giere, 2009). For the same reason the choice of  
 152 sampling sites is also very important, and should touch

all the habitats of a single biotope. (Heitkamp, 1988). 153  
 The main criteria for qualitative/quantitative sampling 154  
 of microturbellarians and gastrotrichs are summarized 155  
 in Table 1. 156

157 Individuals of both groups are more successfully  
 158 studied alive in fresh samples than in preserved  
 159 samples, since their body frailty and strong contrac-  
 160 tility often cause diagnostic morphological characters  
 161 to be distorted after fixation (Balsamo & Todaro,  
 162 2002, Balsamo et al., 2014). In the laboratory,  
 163 collected fresh samples are moved into bowls  
 164 equipped with an aeration system and a lighting neon  
 165 tube if also vegetation is present. The bowls are filled  
 166 with additional filtered water from the sampling site  
 167 (or spring water if necessary) and kept at room  
 168 temperature.

169 A comparative summary of methods for extraction  
 170 and study of freshwater microturbellarians and gas-  
 171 trotrichs is reported in Table 2. Extraction of animals  
 172 from fresh samples implies direct observation of small  
 173 amounts of sediment under a stereomicroscope and  
 174 picking up single living individuals for subsequent  
 175 observation and study under a compound microscope.

176 The extraction of all the animals from a sample is  
 177 clearly critical for quantitative analyses, but regret-  
 178 tably, the techniques currently available are not  
 179 satisfactory for gastrotrichs. A comparative study of



**Fig. 1** Google Scholar entries for meiofaunal studies mentioning a. nematodes and/or copepods; b. micro- or macro-turbellarians; c. gastrotrichs. Methods: Publish or Perish (Harzing, 2007) was used (3.5.20) to search Google Scholar, covering years 1985 through 2020, with the following search strings: **A:** (meiofauna OR meiobenth) AND (freshwater OR lake OR river OR stream)

AND (copepod OR nematod) AND NOT marine; 1985–2020; **B:** (meiofauna OR meiobenth) AND (freshwater OR lake OR river OR stream) AND (turbellaria OR platyhelminthes OR microturbellaria) AND NOT marine; **C:** (meiofauna OR meiobenth) AND (freshwater OR lake OR river OR stream) AND (gastrotrich OR gastrotricha) AND NOT marine

**Table 1** A comparative summary of qualitative and quantitative methods for sampling freshwater gastrotrichs and microturbellarians

	Gastrotrichs	Microturbellarians
Qualitative methods		
N° sampling sites	A sampling site from each habitat of the biotope	A sampling site from each habitat of the biotope
N° samples	At least 3 small samples per site rather than a single large sample	At least 3 small samples per site rather than a single large sample
Frequency of sampling	Seasonal or summer/winter	Bimonthly, monthly, or seasonal
Type of sampling	Dragging the upper sediment surface [epibenthic species] or repeatedly filtering water around aquatic vegetation [periphytic species] with a net with a handle (30 µm mesh)	Hand picking sediments, organic substrate, aquatic vegetation in the littoral zone, coring in deeper zones
	Pushing a manual corer to 5–10 cm into the sediment and taking a core	Plankton tows (63 µm mesh) through water column and in between aquatic vegetation
	Digging a hole in the sandy sediment and filtering the percolating water through the same net cited above [interstitial species]	Digging a hole in the sandy sediment and filtering the percolating water through the same net cited above [interstitial species]
Quantitative methods	Collecting a number of small subsamples in unsieved conditions for a direct counting of individuals	Collecting a number of small subsamples in unsieved conditions for a direct counting of individuals
	Quantitative samples of periphytic species may be obtained by modifying sampling methods for macroinvertebrates (García-Criado & Trigo, 2005), using fine mesh (30 µm) nets for small sample volumes or subsamples	Standardized volumes of sediment, organic debris, aquatic vegetation or water
	True planktonic species can also be collected with the techniques of quantitative plankton sampling (Sandlung, 1982)	Adjust volumes to the size of the water body and number of (micro-)habitats to avoid sample bias in smaller pools
	Collecting and fixing a massive sample in formalin 4%. Not recommended due to the specimens' coarctation	

180	different techniques aimed at this purpose showed that	placed in a tall beaker with clear transparent walls; the	195
181	a rapid forcing of small quantities of sediment through	beaker is then filled with water from the habitat and	196
182	a 130 µm sieve appears to be the most effective way	allowed to stand, creating a vertical cline of dissolved	197
183	for extracting chaetonotid species (Hummon, 1981;	oxygen. Animals are thus forced out of the substrate	198
184	Nesteruk, 1987; Giere, 2009). Details on the methods	and can then be removed from the sides of the beaker	199
185	of sampling, extraction, and study of freshwater	or from the surface film with a pipette.	200
186	gastrotrichs are described in Balsamo et al. (2014).		
187	Recommended methods for extraction and exam-	DNA (meta)barcoding of Rousphozoa	201
188	ination of microturbellarians are described in Schock-		
189	aert (1996). Decantation methods including agitation	DNA extraction and sequencing of taxonomic marker	202
190	of sediment and substrate debris followed by sieving	genes called DNA (meta)barcodes from bulk samples	203
191	(63 µm screen) will dislodge many freshwater flat-	including water, aquatic sediments, and soil (eDNA),	204
192	worms from their substrate. However, the best method	or from pooled individuals separated from the sub-	205
193	for isolating freshwater microturbellarians is oxygen	strate, can reveal the presence of gastrotrichs and	206
194	depletion. A layer of sediment and bottom debris are	platyhelminthes in aquatic environments in	207

**Table 2** A comparative summary of methods for extraction and study of freshwater gastrotrichs and microturbellarians

Gastrotrichs	Microturbellarians
Qualitative methods	
Extraction	
Treatment of the fresh sample with MgCl <sub>2</sub> 1% for relaxing specimens, swirling, stereomicroscopical observation of the supernatant (interstitial species)	Decantation and sieving (63 µm mesh) of sediments, organic substrate, and aquatic vegetation agitated in water
Stereomicroscopical observation of small sediment amounts (2–3 cm <sup>3</sup> ) for search of living specimens (epibenthic and periphytic species)	Hand picking of specimens forced out of organic substrates and aquatic vegetation by oxygen depletion (Schockaert, 1996)
Stereomicroscopical observations of small amounts of plankton net filtrate for search of living specimens (planktonic species)	Stereomicroscopical observations of small amounts of plankton net filtrate for search of living specimens (planktonic species)
Study	
Mounting single live specimens on a slide, observation in vivo under a compound microscope; the use of drops of a 1% aqueous solution of MgCl <sub>2</sub> can help slowing active specimens	Observations of live specimens under a stereomicroscope and mounted on a slide under a compound microscope
Digital imaging and taking measurements under a compound microscope	Digital imaging and taking measurements under a compound microscope
Quantitative methods	
Extraction	
Density gradient centrifugation of small samples or subsamples preserved with formalin 1% then repeatedly washed. Partially useful only for interstitial species. No really efficient technique available for epibenthic, periphytic, and semiplanktonic species (Giere 2009)	No single technique is suitable to extract all microturbellarians. A variety of techniques should be used to obtain representative numbers of different species.
Study	
Mounting specimens on a slide, observation, videorecording, taking measurements and photos. Internal anatomy not yet clearly visible in preserved specimens.	Mounting specimens on a slide, observation, videorecording, taking measurements and photos of diagnostic measurements on live animals and on whole mounts.

208 percentages that would otherwise go unnoticed with  
 209 traditional morphotaxonomic methods (Leray &  
 210 Knowlton, 2015; Arroyo et al., 2016; Martínez et al.,  
 211 2020; Fegley et al., submitted). As such, (meta)bar-  
 212 coding holds great promise to increase our knowledge  
 213 on the diversity, ecology, and role of rousphozoans in  
 214 aquatic ecosystems (Martínez et al., 2020). This  
 215 approach has been reviewed recently (Schenk &  
 216 Fontaneto, 2019): accordingly, we here limit ourselves  
 217 to considering the promises and pitfalls of DNA-based  
 218 methods for evaluation of cryptic diversity and  
 219 community composition among gastrotrichs and  
 220 microturbellarians, including limitations not men-  
 221 tioned in the paper referenced above.

*Choice of amplicon* 222

The ubiquity of MiSeq technology, with up to 300 bp 223  
 paired-end reads, enables useful sequences to be 224  
 recovered for most taxa from the V4/V5 region of 225  
 the 18S rDNA molecule (for Rousphozoa, < 600 bp; 226  
 Hugerth et al., 2014), as opposed to the V9 region 227  
 (~ 120 bp; Amaral-Zettler et al., 2009) or V2/V4 228  
 (~ 400 bp; Creer et al., 2010). The greater taxonomic 229  
 resolution conferred by V4/V5 is also illustrated by the 230  
 fact that a recent metabarcoding trial of this amplicon 231  
 on a well-studied marine beach in North Carolina, 232  
 USA was able to distinguish between congeneric pairs 233  
 of microturbellarian species in three cases, two of 234  
 which had already been documented morphologically, 235  
 and the third documented by 18S rDNA sequencing of 236  
 single individuals (Fegley et al., submitted). The same 237

238 study revealed the existence of numerous separate  
 239 species of both taxa from two beaches in North  
 240 Carolina (Online Resources 1, 2). Accordingly, at least  
 241 for Rousphozoa, V4/V5 might be a better choice over  
 242 the more commonly used COI barcode because of  
 243 poor primer performance with platyhelminthes in  
 244 general (Vanhove et al., 2013) and because COI-based  
 245 species delimitation may inflate actual diversity,  
 246 compared to 18S and 28S rDNA (Van Steenkiste  
 247 et al., 2018). However, the development of nanopore  
 248 sequencing now makes it possible to produce very  
 249 long reads—4 Kb of the rDNA cluster (Kreihenwinkel  
 250 et al., 2019), or individually indexed reads of the full-  
 251 length “Folmer” region of COI (Maestri et al., 2019;  
 252 Kennedy et al., 2020). Because of the increased read  
 253 length, nanopore sequencing is also far more tolerant  
 254 of amplicon read-length variation than the current  
 255 standard of MiSeq 300 bp paired-end sequencing—  
 256 for instance, in the North Carolina study noted above,  
 257 we obtained relatively few OTUs for crustaceans, as  
 258 the V4/V5 region in this taxon is too long for 300 bp  
 259 paired-end reads to overlap. This research area is  
 260 developing rapidly, and because of portability and low  
 261 cost, we urge that MinION sequencing be thoroughly  
 262 tested as a routine method for biodiversity assessment  
 263 of meiofauna in general.

#### 264 *Pitfalls*

265 Although metabarcoding studies have the ability to  
 266 reveal taxa that have not been observed with morpho-  
 267 logical taxonomy (see above), they also are liable to  
 268 miss taxa that are present. For instance, Lindgren  
 269 (1972) reported (“approximately”) 35 species of  
 270 microturbellaria and 20 species of gastrotrichs from  
 271 ISP beach, so the counts of species shown in Online  
 272 Resources 1, 2 are likely an underestimate of actual  
 273 species presence. More directly, a recent study on  
 274 meiofaunal biodiversity along the Pacific and Atlantic  
 275 coast of Panama showed that for all investigated sites,  
 276 the diversity of Gastrotricha, Mollusca, Nemertea, and  
 277 Xenacoelomorpha estimated by metabarcoding the V9  
 278 region of the 18S rRNA was lower than the diversity  
 279 based on morphological taxonomy (Leasi et al., 2018).

280 DNA (meta)barcoding relies completely on metic-  
 281 ulously curated DNA reference databases that link  
 282 sequences to species identified based on morpholog-  
 283 ical characters. DNA extractions of tiny animals such  
 284 as rousphozoans are routinely performed on full

individuals, thereby rendering physical vouchering  
 of morphological characters of the same individual  
 impossible. Live and transparent animals with clear  
 diagnostic features can easily be documented digitally,  
 but opaque animals, (pseudo-) cryptic species, and  
 species groups with uncertain taxonomic features pose  
 more specific challenges, especially when they are rare  
 or are co-occurring in space and time. However, DNA  
 extractions of soft-bodied rousphozoans can be non-  
 destructive, for instance by performing microdissec-  
 tions using the head for DNA extraction and the  
 posterior part for morphological study (e.g., macros-  
 tomids in Schärer et al., 2011; Janssen et al., 2015),  
 and could be a practical solution to incorporate  
 “problem” individuals and species into DNA refer-  
 ence collections.

#### *Promises*

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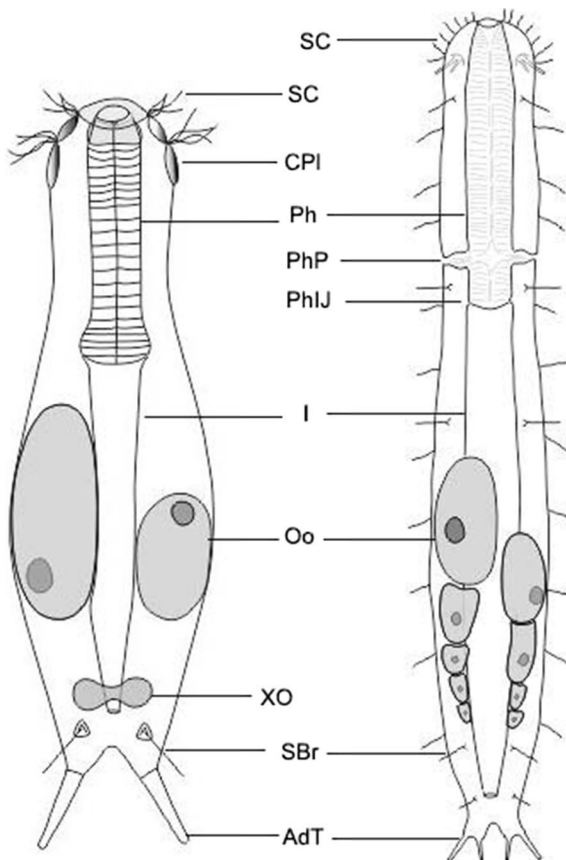
Recently, analysis of marine and freshwater metabar-  
 coding data has shown its potential for DNA-based  
 species discovery and uncovered the existence of two  
 hitherto unknown higher-level flatworm groups in  
 freshwater (Mitsi et al., 2019). Combined with data on  
 abiotic and ecological data, it can provide previously  
 unattainable insights into spatial and temporal changes  
 in species compositions and link environmental  
 parameters with the occurrence of specific taxa  
 (Chariton et al., 2015). This can generate novel  
 ecological information for taxa such as gastrotrichs  
 and microturbellarians that are small, difficult to  
 identify, and may only be present as resting eggs or  
 other propagules during certain times.

However, metabarcoding and its applications in  
 ecology are still in development and need to overcome  
 several challenges, many of which apply to meiofauna  
 in general and rousphozoans in particular (see Ruppert  
 et al., 2019 for a review). DNA reference databases for  
 gastrotrichs and microturbellarians are still poorly  
 populated and need to be strengthened through global  
 collaborations of taxonomic specialists. As this is an  
 ongoing and future effort, students and researchers  
 will need to be trained in fundamental biodiversity  
 research, including careful identification of individu-  
 als selected for building DNA barcode databases.  
 Other well-known issues include PCR primer bias and  
 design, marker choice, standardization of methods,  
 and integration with ecological data (Schenk &  
 Fontaneto, 2019).

## 332 Methods for identification

333 *Gastrotricha*

334 The phylum Gastrotricha currently comprises over  
 335 850 free-living species widespread in aquatic ecosys-  
 336 tems. The division into two classes, Macrodasyoidea  
 337 and Chaetonotidae, each including a single order  
 338 (Macrodasysida and Chaetonotida, respectively) dates  
 339 back to Remane (1925), and follows the evident  
 340 differences in morphology, biology, and ecology  
 341 between the two taxa (Balsamo et al.,  
 342 2009, 2014, 2015; Hummon & Todaro, 2010; Kieneke  
 343 & Schmidt-Rhaesa, 2015) (Fig. 2), which has also  
 344 been confirmed by molecular analyses (Paps &  
 345 Riutort, 2012).



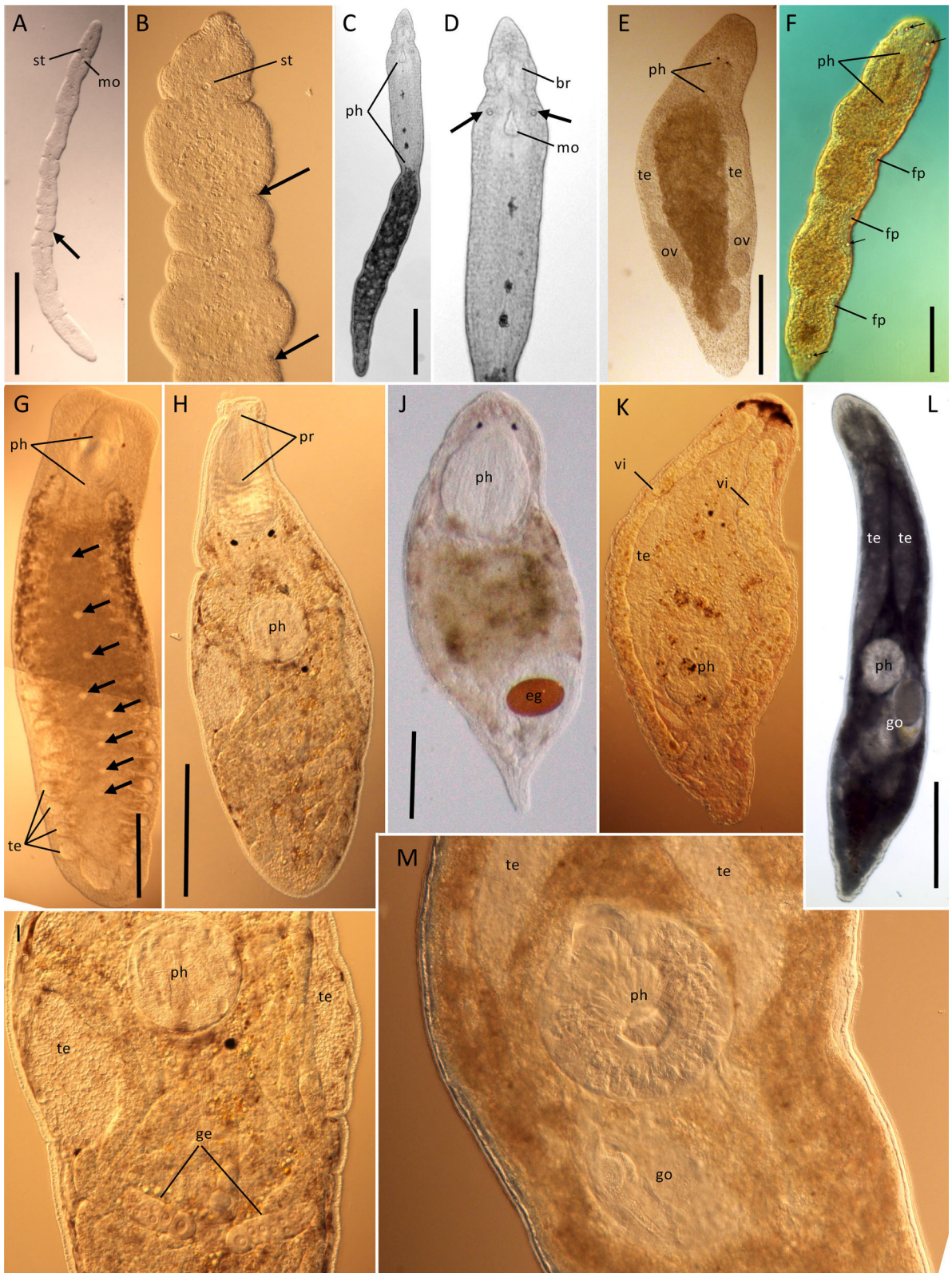
**Fig. 2** Schematic view of freshwater Gastrotricha: **a** Chaetonotida, **b** Macrodasysida. AdT, adhesive tubes; CPI cephalic plates; I intestine; Oo oocyte; Ph pharynx; PhIJ pharyngo-intestinal junction; PhP pharyngeal pores; SBr sensory bristles; SC sensory cilia; XO X-organ

Taxonomy and systematics of Gastrotricha have  
 been traditionally founded on morphological charac-  
 ters, which still represent the basis to systematize  
 species and superspecific taxa (Hochberg & Litvaitis,  
 2000; Kieneke et al., 2008). Diagnostic characters are  
 the general body shape, the morphology of the body  
 cuticle and cuticular elements, the shape and length of  
 the caudal appendages, the arrangement of the ventral  
 ciliation, and the structure of the pharynx. Current  
 taxonomy also makes use of molecular techniques,  
 and has introduced over time several changes and  
 integrations to the traditional classification (e.g.,  
 Kånneby et al., 2013; Todaro et al., 2012, 2015).  
 These suggest that genera including both marine and  
 freshwater species (i.e., *Chaetonotus*, *Aspidiophorus*,  
*Heterolepidoderma*) never form monophyletic clades,  
 but rather cluster according to habitat. It is clear that  
 the intraphylum phylogeny is not yet resolved as are  
 deep ingroup phylogenetic relationships; therefore, a  
 stabilization of gastrotrich taxonomy, especially of  
 Chaetonotida, has not yet been reached.

Details on the anatomy and biology of freshwater  
 gastrotrichs are reported in Balsamo et al. (2014) and  
 Kieneke & Schmidt-Rhaesa (2015). A general key to  
 gastrotrich families and genera was recently published  
 by Todaro et al. (2019). Keys to the freshwater  
 gastrotrich fauna also exist (see Balsamo et al., 2014  
 for a references' summary), but they are generally  
 limited to selected taxa or to limited geographic ranges  
 such as the Neotropics (Garraffoni & Araújo, 2010),  
 the Nearctic (Kånneby, 2016), and the Palearctic  
 (Balsamo et al., 2019). The Gastrotricha Portal (<http://www.gastrotricha.unimore.it>) and the World Register  
 of Marine Species (WoRMS, 2020a) contains lists of  
 marine and freshwater species, but does not provide  
 identification keys.

382 *Platyhelminthes*

The free-living members of the phylum Platy-  
 helminthes comprise ~ 6500 species, of which ~  
 1500 species occur in freshwater or limnoterrestrial  
 environments when also including the macrofaunal  
 triclad. Freshwater microturbellarians can be found in  
 7 flatworm groups: Catenulida, Macrostomorpha,  
 Prorhynchida, Proseriata, Rhabdocoela, Prolecitho-  
 phora, and Bothrioplanida. Given the phylogenetic  
 relationships among and within these 7 major flat-  
 worm groups, incursions of the freshwater



◀ **Fig. 3** Clades of microturbellaria with pharynx simplex and homocellular female gonads (yolk contained in oocytes). **a–d** Catenulida: **a** *Catenula confusa*, showing anterior statocyst (st), mouth (mo), and best-developed fission plane (arrow). Scale = 200 µm. **b** Anterior end of *Catenula lemnae*, a species with consecutive well-developed fission planes (arrows). **c** *Stenostomum* cf. *virginianum*, a genus with a well-developed pharynx simplex (ph). Scale = 200 µm; **d** Enlargement of **c**, to show multilobed brain (br), refractile bodies (arrows), and mouth. **e–f** Macrostomorpha: **e** *Macrostomum* sp., with anterior pigmented eyes and pharynx simplex close behind (ph), paired testes (te), and ovaries (ov). Scale = 500 µm (approximate). **f** *Microstomum* sp. with anterior pharynx simplex (ph), three fission planes (fp) in different stages of development. Scale = 250 µm (approximate). Clades with complex pharynges and heterocellular female gonads (separate yolk cells and oocytes). **g** Prorhynchida: *Geocentrophora* cf. *applanata* with complex pharynx (ph) opening anteriorly, median germovitelarium marked by nuclei of germocytes (arrowheads), and light-colored testes follicles (te) associated with lateral branches of the digestive tract. Scale = 500 µm (approximate). **h–i** Rhabdocoela, Kalyptorhynchia: **h** *Opisthocystis* cf. *goettei*, with anterior cone-shaped muscular proboscis (pr), and median rosulate (wreath-shaped) muscular pharynx (ph). Scale = 500 µm (approximate); **i** enlarged view of mid-body region of **h**, showing pharynx, paired testes, and paired germaria (ge). **j–m** Rhabdocoela, Limnotyphloplanida: **j** Dalyelliidae; cf. *Microdalyellia rossi*, showing anterior doliiform (barrel-shaped) pharynx and mature egg (e.g.). Scale = 500 µm (approximate). **k–l** Typhloplanidae: **k** Typhloplaniid showing lateral rope-like vitellaria (vi), one of two paired testes, and posterior rosulate (wreath-shaped) pharynx with genital region shortly behind pharynx; **l** cf. *Ascophora elegantissima* overview showing paired testes, rosulate pharynx, and genital region (go). Scale = 500 µm (approximate); **m**. enlarged view of **l**, showing testes, pharynx, and genital region

393 environment almost certainly happened multiple times  
394 from different marine and/or brackish water ancestors  
395 (Schockaert et al., 2008; Laumer et al., 2015b).  
396 Conversely, returns to brackish water and marine  
397 environments have also happened (Van Steenkiste  
398 et al., 2013).

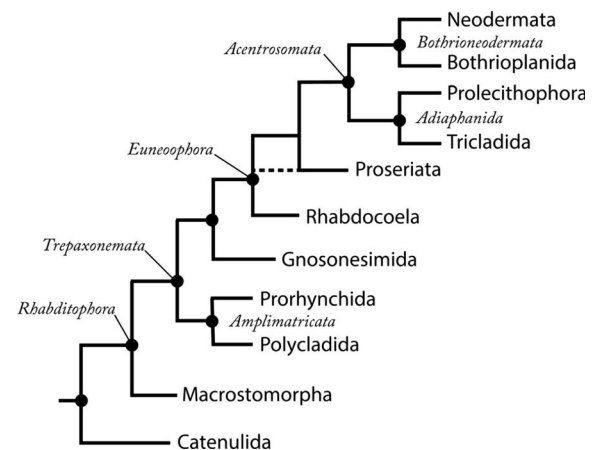
399 It is possible to key most platyhelminthes to family  
400 level based on morphological characters alone (e.g.,  
401 Cannon, 1986; Smith et al., 2020). Useful characters  
402 are the presence/absence of a statocyst, the construc-  
403 tion of the pharynx, the structure of the female gonad,  
404 and the morphology of the male reproductive system  
405 (Fig. 3). The basic anatomy of Platyhelminthes,  
406 including microturbellaria, is covered in detail else-  
407 where (e.g., Rieger et al., 1991).

408 Although DNA taxonomy has been used to trace  
409 species radiations in Gastrotricha (Atherton, 2015), it

has only been employed a few times for (cryptic) 410  
species delimitation in freshwater gastrotrichs (Kån- 411  
neby et al., 2012) and microturbellarians (Larsson 412  
et al., 2008; Atherton & Jondelius, 2018, 2019). This 413  
illustrates the urgency of improving aspects of envi- 414  
ronmental high-throughput sequencing before this 415  
potentially cost-effective approach could be widely 416  
applied for species discovery, biodiversity surveys, 417  
and ecosystem assessments in aquatic ecosystems. 418

Well-supported intraphylum relationships among 419  
most major subtaxa (with the exception of the relative 420  
position of Rhabdocoela and Proseriata) are provided 421  
by two recent transcriptomic studies (Egger et al., 422  
2015, Laumer et al., 2015b—Fig. 4). Recent molec- 423  
ular phylogenies, albeit largely based on only a few 424  
genes, have provided valuable information on rela- 425  
tionships within the major groups, often in conflict 426  
with the traditional morphology-based taxonomy:  
Catenulida (Larsson & Jondelius, 2008), Macrosto- 428  
morpha (Janssen et al., 2015; Atherton & Jondelius, 429  
2019), Rhabdocoela (Willems et al., 2006; Van 430  
Steenkiste et al., 2013; Tessens et al., 2014), and 431  
Proseriata (Laumer et al., 2014; Scarpa et al., 2016). 432  
Accordingly, it has proven to be challenging to 433  
provide morphological apomorphies for many of the 434  
resulting clades. Therefore, phylogenomics based on 435  
much larger molecular datasets and advances in the 436  
study of morphological characters should be inte- 437  
grated to provide a more robust taxonomy for different 438  
microturbellarian groups. 439

A general key for freshwater microturbellarians is 440  
missing at this date, and existing keys focus on specific 441



**Fig. 4** Major higher-level taxa in Platyhelminthes, based on transcriptomic studies (Egger et al., 2015; Laumer et al., 2015b)

442 taxa or regions. A recent key to freshwater Platy-  
 443 helminthes of the Nearctic extends to genus, and  
 444 includes a species list (Noreña et al., 2015). At present,  
 445 there is no genus-level key to the Palearctic, which is  
 446 unfortunate, as the majority of collecting and taxo-  
 447 nomic work has been done there. The Turbellarian  
 448 Taxonomic Database (Tyler et al., 2006–2016) and the  
 449 World Register of Marine Species (WoRMS, 2020b)  
 450 includes worldwide coverage of marine, freshwater,  
 451 and limnoterrestrial Platyhelminthes, but does not  
 452 provide a key.

### 453 Ecology

454 Studies on gastrotrich and microturbellarian autoecol-  
 455 ogy and synecology are not numerous (Schwank,  
 456 1981b, 1982a; Heitkamp, 1982, 1988; Ricci &  
 457 Balsamo, 2000; Kolasa, 2002; Nesteruk, 2016a, b,  
 458 2017). Abiotic and ecological factors define the  
 459 qualitative and quantitative compositions of popula-  
 460 tions, whose mean densities widely vary depending on  
 461 the characteristics of the habitat and seasonal dynam-  
 462 ics, and can range from a few thousand up to 2.6  
 463 million ind/m<sup>2</sup> for both benthic and pelagic gas-  
 464 trotrichs (Nesteruk, 2004a, 2009, 2011) and at least

several thousand ind/m<sup>2</sup> for microturbellarians (Ko-  
 lasa, 2002); however, several studies use different  
 units impeding a reliable comparison of values  
 (Nesteruk, 1993).

### Habitat

Various environmental parameters play an important  
 role in defining the ecological niche of each species of  
 freshwater rousphozoan and thus they determine their  
 small-scale and regional diversity and distribution  
 patterns: these parameters are summarized in Table 3.

Temperature is essential for the colonizing ability  
 of gastrotrich populations and influences the length  
 and intensity of reproductive activity rather than their  
 lifespan (d'Hondt, 1971; Hummon, 1986; Balsamo &  
 Todaro, 1988). Only a few freshwater species, mainly  
 the epibenthic ones, are known to tolerate low oxygen  
 concentrations, unlike some marine species that have  
 well adapted to this particular habitat (Kraus &  
 Colacino, 1984). Grain size, shape and sorting, as  
 well as the amount of organic matter in the substrate  
 determine the interstitial space available to the few  
 interstitial species in coarse to medium-fine sands  
 (Balsamo, 1990; Balsamo & Fregni, 1995; Nesteruk,  
 2007a, b). The pH can vary significantly in fresh

**Table 3** A comparative summary of the main environmental parameters defining the occurrence and distribution of freshwater gastrotrichs and microturbellarians

	Gastrotrichs	Microturbellarians
Temperature	23–28°C (optimum 20–25 °C)	Variable Steno- to eurytherm with variable optimum for reproduction and population growth
Oxygen concentration	High (most species) Low/very low (few tolerant species)	Very high (stream species) High (most species)
pH	4–10 (optimum 6–8)	Unknown for most species 6—(lotic species)
Salinity	Few euryhaline freshwater species	Few euryhaline freshwater species
Water regime	Lentic (most species) Epibenthic/periphytic/planktonic, few interstitial species Lotic (few interstitial species)	Lentic (most species) Mostly epibenthic/periphytic, very few planktonic species Few specialized species in fast-running water
Grain size sediment	Medium-fine (interstitial species) Fine, muddy (epibenthic species)	Variable (lentic species) Optimum of 0.4–0.7 mm for stream species
Organic matter	Oligotrophic (interstitial species) Polytrophic (epibenthic/periphytic species)	Oligotrophic (stream species) Polytrophic (lentic and slow river species)

489 waters; most species live in moderately acidic habi- 538  
 490 tats, but some species can tolerate pH values down to 539  
 491 4, while others live in alkaline water up to pH 10 540  
 492 (Kisielewski, 1981; Nesteruk, 2004a). A few fresh- 541  
 493 water gastrotrich species are able to survive or even to 542  
 494 live in brackish waters. Finally, all freshwater gas- 543  
 495 trotrichs are influenced by the characteristics of the 544  
 496 water column, substrate, and aquatic vegetation. 545

497 Most freshwater chaetonotidans are epibenthic or 546  
 498 periphytic in oxygenated habitats, and more abundant 547  
 499 in eutrophic, standing waters (see Nesteruk, 2017 and 548  
 500 references therein). The epibenthic community is 549  
 501 generally more diverse and is dominated by eurytopic 550  
 502 species of the genera *Chaetonotus*, *Lepidodermella*, 551  
 503 *Heterolepidoderma*, and *Ichthydium*, whereas epi- 552  
 504 phytic assemblages also include semiplanktonic 553  
 505 species of Dasydytidae and Neogosseidae (Nesteruk, 554  
 506 2000; Minowa & Garraffoni, 2017). Sandy sediments 555  
 507 of lentic and running waters host all four freshwater 556  
 508 species of Macrodasysida, but few species of Chaeto- 557  
 509 notida (see Balsamo et al., 2014). Trophic levels and 558  
 510 zonality of water bodies also influence the diversity 559  
 511 and density of gastrotrich populations. Water bodies 560  
 512 with a clear zonality provide a higher habitat diversity 561  
 513 and consequently have a richer and more abundant 562  
 514 fauna, especially in the littoral zone (Kisielewski, 563  
 515 1981; Nesteruk, 2004b, 2005). Compositional differ- 564  
 516 ences also exist between the sublittoral and the deep 565  
 517 zone (Nesteruk, 1996b, 2004b). Alpha-mesotrophic 566  
 518 waters are 26–45% richer in species than waters with a 567  
 519 lower trophic level (Nesteruk, 1996b, 2004a). The few 568  
 520 semiplanktonic or planktonic species preferentially 569  
 521 live in eutrophic ponds, *Sphagnum* bogs, and transi- 570  
 522 tional peat bogs, which appear to have the highest 571  
 523 species richness, independent from altitude, vegeta- 572  
 524 tion, and trophic level (Kisielewski, 1981, 1986, 1991; 573  
 525 Balsamo, 1982; Balsamo & Todaro, 1995). In lotic 574  
 526 habitats, gastrotrichs are mostly present where the 575  
 527 water current is slower, such as vegetated river banks, 576  
 528 bends of the water course, and in small streams 577  
 529 (Kisielewski & Kisielewska, 1986; Kisielewski, 578  
 530 1991). A few interstitial species have been reported 579  
 531 from sediments of springs, rivers, and streams (Ricci 580  
 532 & Balsamo, 2000; Nesteruk, 2008; Garraffoni et al., 581  
 533 2017). Most gastrotrich species are able to colonize 582  
 534 more than a single habitat and can migrate between the 583  
 535 epibenthos, periphyton, and interstitial. 584

536 Very few studies specifically focus on the influence 585  
 537 of abiotic variables on the occurrence and abundance 586

of freshwater microturbellarians. Kolasa (2002) pro- 538  
 vides a brief overview on general preferences and 539  
 tolerance ranges of several abiotic parameters, but 540  
 only for few species tolerance ranges for temperature, 541  
 oxygen, water level, oxygen, pH, and calcium are 542  
 known (Heitkamp, 1982). Most species have an 543  
 optimal temperature range for reproduction and pop- 544  
 ulation growth to occur and temperature can have a 545  
 significant influence on hatching and on the generation 546  
 time (Heitkamp, 1988; Sayre & Wergin, 1994; 547  
 Dumont et al., 2014). Some species are stenotherm, 548  
 while others are eurytherm. Microturbellarians require 549  
 oxygenated layers of water and sediment. Species that 550  
 live in substrates of well-oxygenated, fast-running 551  
 streams are particularly sensitive to low oxygen 552  
 concentrations (Kolasa, 1983). A small number of 553  
 freshwater rhabdocoels are euryhaline and can also be 554  
 found in brackish water habitats (Ax, 2008). However, 555  
 most microturbellarians that occur in brackish water 556  
 are euryhaline marine species or genuine brackish 557  
 water species that do not occur in freshwater habitats. 558  
 Granulometry of freshwater sediments also influences 559  
 species composition and occurrence. Kolasa et al. 560  
 (1987) found higher species richness and abundance 561  
 in stream sediments with a grain size of 0.4–0.7 mm, 562  
 compared to a low species richness and abundance for 563  
 small stones or large gravel. Young (1973) found that 564  
 calcium-rich and calcium-poor lakes each have their 565  
 specific species of microturbellarians, but also share a 566  
 number of species. 567

568 Ecological surveys of microturbellarians associated 569  
 with different freshwater habitats are mostly limited to 570  
 older studies from Central and Southeastern Europe 571  
 (e.g., An der Lan, 1939, 1962, 1967; Mack-Fira, 1974; 572  
 Kolasa, 1979; Schwank, 1981a, b, 1982a, b). More 573  
 recent studies from South America and the Middle 574  
 East provide valuable data on species richness and 575  
 seasonal abundance of microturbellarians in perma- 576  
 nent wetlands and temporary pools (Eitam et al., 2004; 577  
 Braccini & Leal-Zanchet, 2013). Microturbellarians 578  
 are found in almost all types of lentic and lotic 579  
 freshwater habitats. In addition, they also occur in 580  
 limnoterrestrial habitats such as mosses and forest 581  
 soils (Van Steenkiste et al., 2010; Houben et al., 2015). 582  
 Many species are shared between habitats, but some 583  
 species are associated with specific environments. 584  
 Species numbers can be high in both lentic and lotic 585  
 environments with up to 94 and 57 species recorded 586  
 from a single stream and lake, respectively (Kolasa,

587 2000). In large lakes and reservoirs, species richness  
 588 and abundance are significantly higher in sediments  
 589 and aquatic vegetation in the littoral zone, but some  
 590 species have also been found in the limnetic zone as  
 591 part of the pelagic (Dumont et al., 2014). Permanent  
 592 bodies of water are usually dominated by catenulids,  
 593 macrostomids, prorhynchids, and rhabdocoels associ-  
 594 ated with aquatic vegetation, plant roots, and sedi-  
 595 ment, while temporary pools typically harbor species  
 596 with drought resistant resting eggs, such as typhlo-  
 597 planid and dalyelliid rhabdocoels (Artois et al., 2004;  
 598 Eitam et al., 2004). Species compositions in lotic  
 599 systems are highly variable. Mountain springs and fast  
 600 flowing streams or rivers have unique hyporheic and  
 601 psammophilic species or species associated with  
 602 mosses and other vegetation along its course (Sch-  
 603 wank, 1982a, b). The lower courses of rivers systems  
 604 are inhabited by eurytopic species also found in lentic  
 605 habitats. Assemblages of species are further enriched  
 606 by species from habitats at the interface of lotic and  
 607 lentic habitats, including limnoterrestrial, brackish  
 608 water, and groundwater elements (Kolasa  
 609 1983, 2000). A very detailed review on the distribution  
 610 and abundance of microturbellarians in different  
 611 aquatic habitats is given by Young (2001).

#### 612 Spatial and temporal dynamics of rousphozoans' 613 populations

614 Spatial patterns and small-scale horizontal distribu-  
 615 tions of rousphozoans are driven by abiotic and biotic  
 616 factors such as the morphological features of the  
 617 sediment, the heterogeneous distribution of organic  
 618 matter, and bioturbation (Kisielewski, 1974–1999;  
 619 Nesteruk, 1986–2017; Giere, 2009). This leads most  
 620 meiofauna to aggregate in undisturbed sites or in areas  
 621 richer in organic detritus, thus presenting a typical  
 622 patchy distribution. Species composition can differ  
 623 significantly between microhabitats, with adjoining  
 624 patches of gravel, sand, plants, and organic debris  
 625 having distinctive communities at the scale of  
 626 centimeters.

627 The vertical distribution of gastrotrichs is highly  
 628 related to grain size, oxygen concentration, presence  
 629 and velocity of water flow, quantity of organic matter  
 630 present in the interstitial water, predation pressure, as  
 631 well as the tolerating abilities of different species  
 632 (Palmer, 1990; Danielopol et al., 1997). The few  
 633 interstitial freshwater species are mostly found in the

oxygenated upper 5 cm of the sediment, in which  
 about 46–68% of the whole gastrotrich community has  
 been reported. Some species (about 7–10% of the total  
 gastrotrich fauna) can migrate down to 10–15 cm deep  
 (Nesteruk, 1991). Only a few individuals have been  
 found at 30–40 cm deep in lotic gravel habitats where  
 wide interstices allow the penetration of oxygen  
 (Schmid-Araya, 1997).

Temporal patterns of gastrotrich populations and  
 influencing factors are not well known, especially in  
 freshwater environments. Nesteruk (1986, 2007a,  
 2017) reported decreased densities of some freshwater  
 gastrotrich populations during summer and winter,  
 probably related to the seasonal changes in oxygen  
 concentration, water temperature, and food availabil-  
 ity. Periods of drought and freezing in temperate zones  
 strongly influence both the abundance and the struc-  
 ture of communities. In tropical zones, gastrotrichs are  
 present and even abundant in lentic waters throughout  
 the year, with higher abundances during the rainy  
 season. This change in abundance is probably linked to  
 the sediment processes and recirculation of organic  
 matter (Kisielewski, 1991; Zébazé Togouet et al.,  
 2007; Strayer et al., 2010).

Very few studies present data on the vertical  
 distribution of freshwater microturbellarians in the  
 water column and in sediments. Although some lentic  
 microturbellarians have been found in substrates at  
 considerable depths of 20 m or more, most studies  
 show that the largest numbers of species and individ-  
 uals were found in the shallow waters up to 1 m of the  
 littoral zone and then decline with depth. This decline  
 in species richness and abundance is more pronounced  
 in eutrophic lakes than in oligotrophic lakes (Young,  
 2001; Kolasa, 2002). Some pelagic species of *Mesos-  
 toma* follow the diurnal vertical migration of their prey  
 in the water column, rising to the surface at night to  
 feed on cladocerans and copepods (Rocha et al., 1990).  
 Psammic stream-dwelling microturbellarians are most  
 abundant at 20–40 cm deep inside gravel (Schmid-  
 Araya, 1997). Species richness and abundance are,  
 however, mostly a function of the presence of varied  
 microhabitats. Studies on seasonal abundances of  
 freshwater microturbellarians give a mixed image. In  
 Europe and Southern Brazil, different species have  
 different seasonal abundance peaks influencing com-  
 munity compositions throughout the year (Young,  
 2001; Braccini & Leal-Zanchet, 2013). Seasonal  
 occurrence and abundance of microturbellarians also

683 seem to vary according to geographic location and are  
684 most likely linked to the influence of temperature,  
685 food availability, droughts and floods, and several  
686 other abiotic and biotic variables. The scarcity of  
687 studies available on these temporal dynamics high-  
688 lights the need for more research in different parts of  
689 the world.

#### 690 Trophic and biotic interactions

691 Very few studies on gastrotrichs deal with their trophic  
692 ecology, interactions within or among species, com-  
693 petition with and predation by other organisms, or  
694 their symbionts and parasites. Only a few qualitative  
695 experimental studies on mixed cultures of freshwater  
696 species have been done (d'Hondt, 1967; Bennett,  
697 1975, 1979). The primary food seems to be bacteria  
698 and the particulate organic matter on the sediment  
699 surface, in interstitial spaces, and on the microbial  
700 biofilm covering the substrate. Microalgae and other  
701 protists probably supply some nutrients, but may not  
702 be essential (Packard, 1936; Brunson, 1949). As the  
703 interstitial environment is dominated by viscous  
704 forces, all prey capture devices must be adapted to  
705 overcome the functional challenge of feeding at very  
706 low Reynolds numbers. Food uptake and transport are  
707 therefore dependent on two important factors: the  
708 entrance to the pharynx (mouth) and conductance of  
709 the pharyngeal pump. Among meiofauna, only two  
710 taxa rely exclusively on suction for prey capture,  
711 nematodes and gastrotrichs (Ruppert, 1982). Both taxa  
712 have near-identical foreguts (e.g., terminal mouth,  
713 myoepithelial pharynx, triradiate lumen), yet differ in  
714 pharyngeal ultrastructure. Nematodes have strictly  
715 monosarcomeral pharynges that generate strong but  
716 slow contraction. Consequently, nematodes evolved to  
717 feed on different prey through selection on buccal size,  
718 armature, muscle supply, and pharynx shape (Munn &  
719 Munn, 2010). Alternatively, gastrotrichs have 1–12  
720 sarcomeres/myofilament/species (Ruppert, 1982).  
721 More sarcomeres should translate into greater speed  
722 of contraction but with lower force; hence, different  
723 lineages have made an evolutionary tradeoff of force  
724 for speed (or speed for force), depending on ancestry.  
725 An exploration of these tradeoffs should be carried out  
726 by combining molecular diet analysis of selected  
727 species whose diet is already partially known (either  
728 by diagnostic PCR or by parallel sequencing—see  
729 Rubbmark et al., 2019, for comparative review) with a

careful examination of pharynx structure by transmis-  
sion electron microscopy and confocal laser scanning  
microscopy. We predict that species with monosar-  
comeral pharynges will be biofilm feeders, whereas  
species with multisarcomeral pharynges will feed  
primarily on eukaryotes. These studies should deter-  
mine if gastrotrichs form feeding guilds akin to those  
in nematodes (Hochberg, pers. comm.).

Both freshwater and marine gastrotrichs seem to  
have chemotactic abilities to discriminate between  
different bacterial strains (Gray & Johnson, 1970).  
Sporadic observations in laboratory cultures did not  
show apparent reciprocal interactions with conspecific  
individuals (Banchetti & Ricci, 1998). Gastrotricha  
certainly compete with other meiofaunal organisms in  
feeding on bacteria, protists, biofilm, and organic  
detritus. Large protists, cnidarians, flatworms, poly-  
chaetes, and larvae of Diptera have been reported as  
natural predators of Gastrotricha (Strayer & Hummon,  
1991; d'Hondt, pers. comm.). The heliozoon *Acti-  
nophrys sol* Ehrenberg, 1830 and the amoebozoan  
*Amoeba spumosa* Grüber, 1885 were directly observed  
feeding on freshwater chaetonotids, both solitarily and  
cooperatively in samples collected in nature and kept  
under laboratory conditions (Brunson, 1949; Bovee &  
Cordell, 1971). Escape mechanisms of Gastrotricha lie  
in sudden whole body contractions and rapid direction  
changes in locomotion. Most chaetonotids, and  
especially epibenthic or semiplanktonic species, also  
have cuticular scales and/or long, sometimes movable  
spines, and protective cephalic plates that act as  
mechanical barriers against predators.

Individuals of freshwater Chaetonotida have been  
observed containing putative sporozoans in their trunk  
or euglenoids in their intestine, but it is not clear if  
these are food items, endosymbionts or parasites  
(Remane, 1936; Manylov, 1999; Kisielewska et al.,  
2015). Nothing is known about possible epibiotic  
associations between Gastrotricha and other taxa, like  
those observed in other small aquatic micrometazoa  
(i.e., Rotifera, Nematoda) (Bulut & Saler, 2017).

The diet of microturbellarians ranges from ciliary-  
assisted feeding on bacteria and algae (Catenulida) to  
(obligate?) diatomivory (some Macrostromorpha and  
Rhabdozoela) and carnivory on other meiofauna and  
the larvae of macroinvertebrates (see Watzin,  
1983, 1986; Giere, 2009). Catenulids have a disten-  
sible mouth to engulf food and transport it to the  
pharynx simplex through large cilia around the mouth

Predator	215bp amplicon	340bp Amplicon
<i>Cicerina debrae</i>	NEM: <i>Metadesmolaimus</i> sp. JN968218, <i>Daptonema hirsutum</i> AM236231, or <i>Daptonema setosum</i> AM234045 (96%)	NEM: <i>Daptonema hirsutum</i> AM236231 or <i>Daptonema setosum</i> AM234045 (95%)
<i>Prognathorhynchus busheki</i>	Uncultured metazoan clone AF372734 (97%)	NEM: <i>Punctodora ratzeburgensis</i> FJ969138 (78%)
<i>Drepanorhynchides hastatus</i>	NEM: <i>Enoplolaimus</i> sp. HM564464 (99%)	NEM: <i>Enoplolaimus</i> sp. HM564464 (99%)
“EukalyptoRiese”	NEM: <i>Enoplolaimus</i> sp. JN968238 (100%)	NEM: <i>Enoplolaimus</i> HM564473 or Thoracostomopsidae HM564472 (97%)
<i>Cheliplana</i> n.sp.	NEM: <i>Metadesmolaimus</i> sp. JN968218 or <i>Daptonema</i> sp. FJ040463 or <i>D. hirsutum</i> AM236231 or <i>D. setosum</i> AM232405 (76%) (reverse read)	No amplification
<i>Lehardyia alleithoros</i>	Multiple sequences	TURB: Monocelidinae sp. HM026567 (95%)
“ProschizoSpirale”	No amplification	TURB: Coelogyneporidae n. sp.1
<i>Schizochilus</i> "foxi"	NEM: Draconematidae gen.sp. FJ182219, (96%)	No amplification
“ProschizoTertius”	ACOEL: <i>Pseudaphanostoma smithii</i> AY078375 <i>Haplogonaria</i> “schillingi” FR837700 (86%)	No amplification
<i>Paromalostomum</i> "riegeri"	NEM: <i>Metadesmolaimus</i> sp. JN968218 <i>Daptonema hirsutum</i> AM236321 (94%)	No amplification

**Fig. 5** Partial results of PCR amplifications for two primer sets directed against nematodes applied to DNA isolates from single platyhelminth individuals. GenBank accession numbers and

percent sequence identities are listed for each prey species identified by Blastn. NEM nematode; ACOEL acoelomorph, TURB turbellarian. Adapted from Maghsoud et al. (2014)

779 opening. A few species in the catenulid genus  
780 *Paracatenula* are mouthless and maintain symbiotic  
781 bacteria in the gut (Dirks et al., 2011, 2012). Other  
782 microturbellarians use their muscular pharynx for the  
783 capture and uptake of prey items. The pharynx can be  
784 distended to capture and ingest prey as a whole  
785 (Stenostomidae, Dalyelliidae) or protruded to breach  
786 the body wall of larger prey and suck up prey fluids  
787 and tissues (Typhloplanidae, Proseriata). Kalyp-  
788 torhynchs use their anterior proboscis to capture and  
789 possibly envenomate prey and immobilize it while  
790 positioning their pharynx. Some flatworms, such as  
791 prorrhynchids and *Gyratrix hermaphroditus* Ehren-  
792 berg, 1831, use their stylet to stab prey.

793 Feeding strategies of freshwater microturbellarians 793  
794 include mucus trapping, active searching, ambush 794  
795 predation, the use of toxins, and group foraging 795  
796 (Young, 2001; De Roeck et al., 2005; Dumont et al., 796  
797 2014), but comprehensive data on diet composition 797  
798 and prey selection are very limited compared to 798  
799 marine and brackish water microturbellarians (Wat- 799  
800 zin, 1985; Reise, 1988; Menn & Armonies, 1999). 800  
801 Diagnostic PCR was used to reconstruct the diet in 801  
802 individual marine flatworm species (Maghsoud et al., 802  
803 2014; Fig. 5), and could also prove valuable for 803  
804 freshwater microturbellarians. One recent study shows 804  
805 acquired prey selection of toxic and non-toxic ciliates 805  
806 by the catenulid *Stenostomum sphagnetorum* Luther, 806

**Table 4** Feeding guilds in flatworms based in part on pharyngeal structure (from Bilio, 1967; Straarup, 1970)

Taxon	Pharynx type <sup>1</sup>	Ex. FW Genera	Ex. Prey <sup>2,3</sup>	Guild
Catenulida	Simplex, ciliated lumen, ventral ciliated “mustache”	<i>Catenula</i> , <i>Suomina</i>	Unicellular eukaryotes; bacteria?	Ciliary sweep
Catenulida	Simplex, barrel-shaped	<i>Stenostomum</i>	Ciliates, Rotifers, other flatworms	Suction, holozoic <sup>4,5</sup>
Macrostomorpha	Simplex	<i>Macrostomum</i>	Diatoms, nematodes, juvenile mussels, cladocerans	Cilio-muscular, holozoic
Macrostomorpha	Simplex	<i>Microstomum</i>	Diatoms, <i>Hydra</i> tentacles, other FW	Cilio-muscular, holozoic
Proseriata	Plicate, wreath-shaped to tubular	<i>Coelogygnopora</i> ,	Oligochaetes, copepods, carrion	Suction, Holozoic or sucking prey contents
Rhabdocoela	Bulbous, rosulate	<i>Castrada</i>	Diatoms, green algae, rotifers, oligochaetes, cladocerans, copepods, insect larvae, other FW	Suction, holozoic or sucking prey contents
Rhabdocoela	Bulbous, doliiform	<i>Gieysztorja</i>	Diatoms, green algae, rotifers, nematodes, oligochaetes, other flatworms	Suction, holozoic

References: <sup>1</sup>Rieger et al. (1991); <sup>2</sup>Kolasa & Tyler (2010), <sup>3</sup>Young (2001), <sup>4</sup> Nuttycombe & Waters (1935), <sup>5</sup>Smith & Davis (unpublished)

807 1960; this behavior was lost after asexual reproduction  
808 (Buonanno, 2011). Freshwater microturbellarians can  
809 reach high densities and studies have shown that  
810 predation by species of *Mesostoma* and *Phaenocora*  
811 can influence the population dynamics of zooplankton  
812 or benthic communities seasonally (Young, 1977; De  
813 Roeck et al., 2005; Dumont et al., 2014). Larger  
814 microturbellarians that feed on mosquito larvae have  
815 therefore been proposed as biological control agents  
816 (Tranchida et al., 2009). Feeding guilds based in part  
817 on pharyngeal structure have been hypothesized in  
818 flatworms (e.g., Bilio, 1967; Straarup, 1970; Table 4).  
819 Species of *Macrostomum* may be specialist feeders on  
820 diatoms or, alternatively, take any relatively slow-  
821 moving prey small enough to swallow, including  
822 juvenile mussels and cladocerans (Delp, 2002). Prose-  
823 riates with a ventrally directed plicate pharynx and  
824 rhabdocoels with a bulbous rosulate pharynx often use  
825 that to suck out prey contents (Jennings, 1974b; own  
826 observations). Rhabdocoels with an anterior barrel-  
827 shaped (doliiform) pharynx often suddenly dilate the  
828 pharynx, suck in, and swallow fast-moving prey whole  
829 (Bilio, 1967). Rapidly contracting radial muscles  
830 could play a role in overcoming viscous forces and  
831 quickly sucking in smaller prey. This mechanism is  
832 used to capture swimming prey by some members of

the genus *Stenostomum* (Nuttycombe & Waters, 1935) 833  
and confocal microscopy of the head region in 834  
*Stenostomum virginianum* Nuttycombe, 1931 shows 835  
pseudostriation of the radial musculature in the 836  
pharynx—an arrangement that is predicted to increase 837  
contraction velocity (Smith & Davis, unpublished). 838  
Interestingly, pseudostriation has also been observed 839  
in the pharyngeal radial muscles of a Prolecithophoran 840  
(Rieger et al., 1991). Additionally, congenics occur- 841  
ring in the same biotope (e.g., *Catenula lemnae* Duges, 842  
1832 and *Catenula confusa* Nuttycombe, 1956) might 843  
have different diets that are reflected in the structure of 844  
their pharynges—for instance, size-selection between 845  
unicellular algae vs bacteria. In summary, one would 846  
expect to find both convergent and divergent adapta- 847  
tions across the different pharynx types—adaptations 848  
that depend in part on prey mobility, and in part on 849  
prey size (e.g., sucking out the body contents of 850  
oligochaetes and amphipods vs swallowing smaller 851  
prey whole). However, there appear to be no published 852  
studies directed at understanding the biomechanics of 853  
the pharynx in microturbellarians. 854

Microturbellarians are also eaten by other inverte- 855  
brates, small vertebrates, and even protists. Defensive 856  
behavior such as the release of mucous to escape from 857  
predatory ciliates has been observed (Buonanno, 858

859 2009) and rhabdites have long been suggested to be  
 860 defensive, whether or not their primary role is mucus  
 861 production for ciliary gliding (Rieger et al., 1991).  
 862 Both intra- and interspecific predation by other  
 863 microturbellarians have also been recorded (Young,  
 864 2001; own observations). Although the extent and  
 865 impact of predation on microturbellarian populations  
 866 have not been assessed in detail, predator exclusion  
 867 did not produce the expected increase in platy-  
 868 helminthes, suggesting that predation does not regu-  
 869 late flatworm populations except under specialized  
 870 circumstances (Reise, 1979; Giere, 2009).

871 Freshwater microturbellarians, and then predomi-  
 872 nantly rhabdocoels, can be both ectosymbionts on  
 873 other freshwater animals and hosts for other organ-  
 874 isms. Temnocephalids are small freshwater epibionts  
 875 on macroinvertebrates and turtles. They prey on other  
 876 co-symbiotic organisms and feed opportunistically on  
 877 particles of the host's food. The dalyelliid *Varsoviella*  
 878 *kozminskii* Gięsztor & Wiszniewski, 1947 lives on  
 879 the gills of freshwater gammarids (Gięsztor &  
 880 Wiszniewski, 1947). A number of freshwater species  
 881 in the genera *Castrada*, *Dalyellia*, *Gięsztoria*,  
 882 *Phaenocora*, and *Typhloplana* harbor endosymbiotic  
 883 chlorophytes. Little is known about this symbiosis, but  
 884 studies on *Phaenocora typhlops* (Vejdovsky, 1880),  
 885 *Dalyellia viridis* (Shaw, 1791), and *Typhloplana*  
 886 *viridata* (Abildgaard, 1789) suggest that worms could  
 887 benefit from the photosynthate and oxygen produced  
 888 by the algae (Young, 2001 and references therein).  
 889 Kleptoplasty, a form of endosymbiosis where only the  
 890 algal plastids are sequestered and retained, has  
 891 recently been observed in marine and brackish water  
 892 rhabdocoels (Van Steenkiste et al., 2019). Species of  
 893 the genus *Microstomum* often retain nematocysts from  
 894 digested *Hydra* tentacles as kleptocnids (Fig. 3f,  
 895 arrows). Parasites of freshwater microturbellarians  
 896 have occasionally been recorded in older taxonomic  
 897 literature, but very few studies characterize the  
 898 observed parasites in detail. Most of these parasites  
 899 are protists, including apicomplexans, microsporidi-  
 900 ans, ciliates, and euglenozoans. Only a few records  
 901 mention metazoan parasites such as nematodes or  
 902 neodermatan flatworms (for details, see Young, 2001  
 903 and references therein). It is noteworthy that the last  
 904 comprehensive review of microturbellarians as para-  
 905 sites and hosts was published over 100 years ago (von  
 906 Graff, 1903), and less comprehensive modern sum-  
 907 maries are available (Jennings, 1971, 1974a, 1977).

Life strategies

908  
 909 Gastrotrichs have various reproductive modalities.  
 910 While marine Macrodasyida are hermaphrodite with  
 911 cross-fertilization, freshwater Chaetonotida generally  
 912 reproduce by thelytokous parthenogenesis. As a  
 913 consequence, freshwater populations can start from  
 914 any single individual. Many freshwater species can  
 915 also produce resting eggs that can withstand environ-  
 916 mental adverse conditions and act as dispersal propa-  
 917 gules. The factors triggering the production and the  
 918 hatching of the resting eggs are not yet known.  
 919 Parthenogenesis, resting eggs, and short life cycles  
 920 allow gastrotrichs to survive extreme variations in  
 921 environmental conditions (e.g., droughts, floods) and  
 922 colonize challenging habitats such as lotic sediments  
 923 (Ricci & Balsamo, 2000), caves (Vandel, 1964;  
 924 Renaud-Mornant, 1986; Kolicka et al., 2017), high  
 925 mountain biotopes (Baumann, 1910; Tonolli &  
 926 Tonolli, 1951; Gadea, 1988), hot springs (De Guerne,  
 927 1888), and deep crater lakes (Barrois, 1896; R.  
 928 Schabetsberger, unpublished data). In addition, indi-  
 929 viduals might also be able to survive critical condi-  
 930 tions by migrating deeper into the sediment (Nesteruk,  
 931 2007c).

932 Laboratory tests have evidenced the existence of a  
 933 long postparthenogenic phase with production of  
 934 aberrant spermatozoa in Chaetonotida. This suggests  
 935 a possible amphimictic reproduction, and thus the  
 936 existence of two successive reproductive modalities in  
 937 a single lifespan. Such a biphasic reproduction strat-  
 938 egy would allow for a quick increase in population  
 939 numbers through apomictic parthenogenesis followed  
 940 by the introduction of genetic variation through cross-  
 941 fertilization (Balsamo, 1992; Hummon & Hummon,  
 942 1992).

943 Microturbellarians are hermaphrodites and display  
 944 both sexual (cross- and self-fertilization) and asexual  
 945 (paratomy) modes of reproduction (Kolasa, 2000).  
 946 Catenulids and some macrostomids (e.g., *Microsto-*  
 947 *mum*) reproduce asexually, although sexual reproduc-  
 948 tion can also occur. Most other freshwater  
 949 microturbellarians reproduce by internal cross-fertil-  
 950 ization, either by mutual copulation or sometimes by  
 951 hypodermic impregnation. Self-fertilization is rare  
 952 and has only been observed in a few species (Young,  
 953 2001).

954 Life histories of freshwater microturbellarians are  
 955 not well understood and only known for a handful of

species from temperate regions (Cox & Young, 1974; Heitkamp, 1988). Microturbellarians can produce both subitaneous (non-resting) eggs for rapid population growth during their active phase and dormant resting eggs/cocoons enclosed by a thicker, more resistant shell at the end of their active phase to overcome periods of high/low temperature, water level changes, or desiccation (Young, 2001). Life cycles are conditioned by seasonal cycles and droughts or flooding events. As such, many species have flexible life cycles depending on geographic location and habitat. Annual species are active year-round and restricted to permanent water bodies. Reproduction appears in one or more generations throughout the year, often during a specific season and influenced by temperature, food, and the presence of water. Seasonal species only appear in one or more seasons which often overlap with periods of vegetation growth and/or phyto- and zooplankton blooms.

A comparative summary of modes of reproduction of freshwater gastrotrichs and microturbellarians is reported in Table 5.

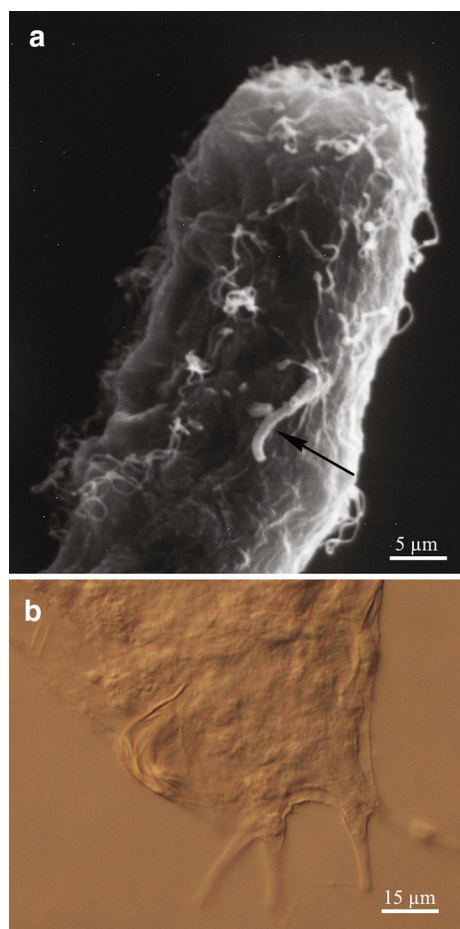
## Global diversity and distribution

The majority of freshwater gastrotrichs are Chaetonotida, with about 350 species in 5 families and 24 genera (72% of total chaetonotidan species). Only four species of Macrodasysida, in one family and one genus (except one species *incertae sedis*), occur in fresh waters (Kisielewski, 1987; Kånneby & Kirk, 2017; Garraffoni et al., 2019). Diversity of freshwater Gastrotricha in different geographic areas is not as well known as that of marine species, and available data are quite heterogeneous. Most research has been carried out in Europe and the Americas. Data on geographic distribution are usually limited to the

sampling sites, especially in older literature, and occasionally include some ecological data (see Balsamo et al., 2014 for previous references). This insufficient knowledge is a direct consequence of technical problems that are common to all soft-bodied meiofaunal animals and concern their collecting and handling, but also to the particular focus of most studies on the epibenthic and periphytic species from standing water bodies. Moreover, the taxonomy—especially of the order Chaetonotida—is still unstable because of the intraspecific variability of many species, the scarcity of diagnostic data in old descriptions, and the increasing evidence of the existence of cryptic species in widespread nominal species (Kieneke et al., 2012; Kånneby et al., 2012, 2013). In Europe, the continent studied most thoroughly, about 250 species have been identified and some countries have been the object of regional ‘faunas’ (Balsamo, 1983; Balsamo & Tongiorgi, 1995; Balsamo et al., 2014 for global references). Of course the effect of sampling effort should be considered in advancing possible scenarios of the global diversity and distribution of the phylum, also because large areas in most other parts of the world have not been explored yet (Balsamo et al., 2008, 2014; Fontaneto et al., 2012). Three out of four species of freshwater Macrodasysida (fam. Redudasyidae) are reported from the Americas (Fig. 6), while the fourth species (*Marinellina flagellata* Ruttner-Kolisko, 1955, *incertae sedis*) is known from Austria (Ruttner-Kolisko, 1955; Schmid-Araya & Schmid, 1995). As for Chaetonotida, three of the five freshwater families, Dasydytidae, Neogosseidae, and especially Chaetonotidae, appear to be cosmopolitan, and most genera and species have been recorded in at least two continents, especially in tropical areas (Figs. 7, 8). Representatives of the rare family Dichaeturidae have occasionally been found in a few European localities and a single Japanese site. Each of

**Table 5** A comparative summary of modes of reproduction of freshwater gastrotrichs (M, Macrodasysida; C, Chaetonotida) and microturbellarians

	Gastrotrichs	Microturbellarians
Asexual reproduction	Thelytokous parthenogenesis in M and C	Paratomy (Catenulida, <i>Microstomum</i> )
Sexual condition	Female only	Hermaphroditic only
Sexual reproduction	Suspected postparthenogenic sexual phase in C	Cross- and self-fertilization
Resting stages	Resting eggs	Resting eggs/cocoons



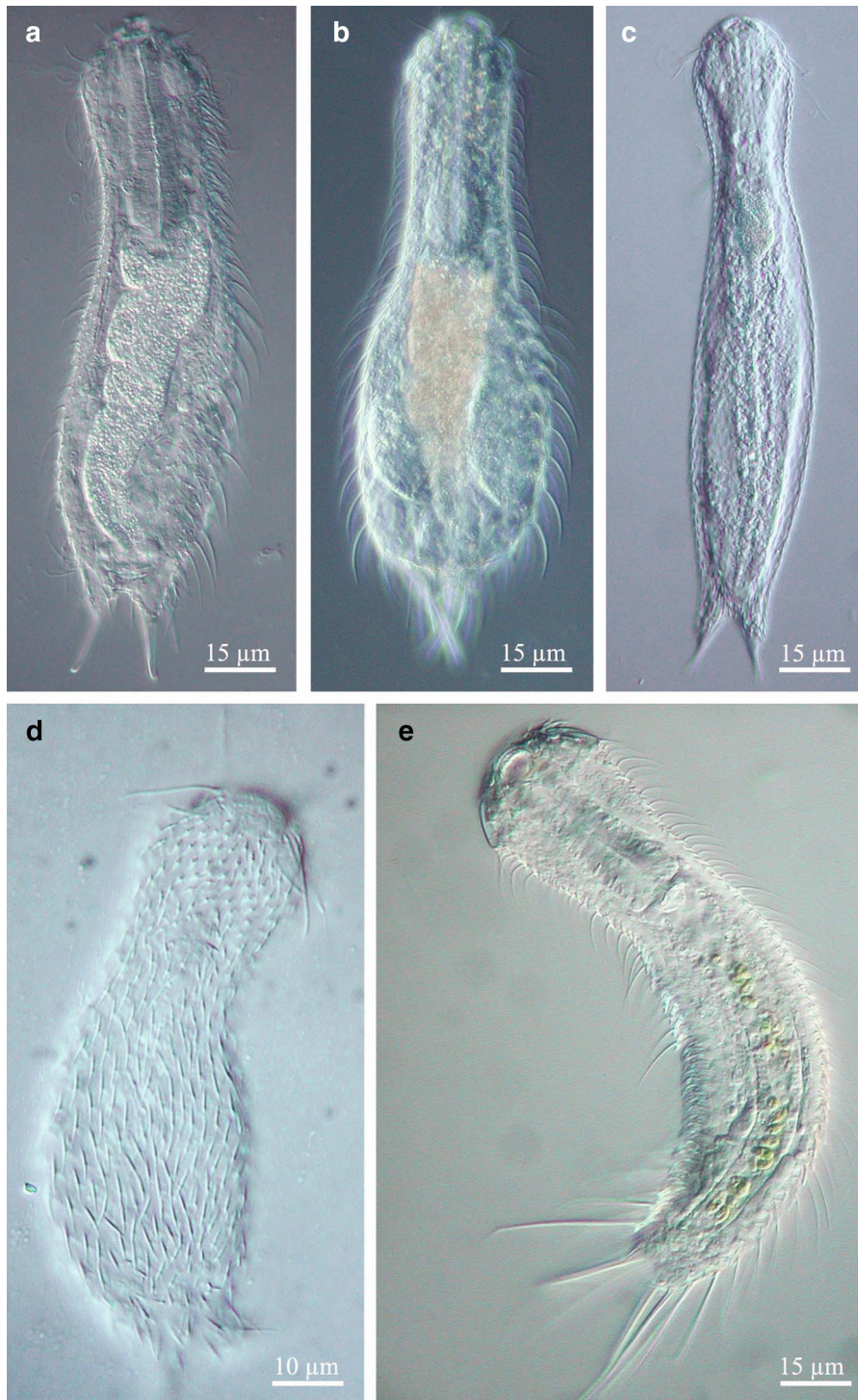
**Fig. 6** A freshwater genus of Macrotrichida, *Redudasys*. **a** Lateral view of the head showing the diagnostic single adhesive tube (SEM); **b** detail of the caudal body end with four adhesive tubes (DIC microscopy)

the two species of the family Proichthyidiidae has only been recorded once in their respective type localities in South America and Asia (see Balsamo et al., 2014 for detailed references). About half of the freshwater genera have an intercontinental distribution; about 1/3 of the European species and 1/3–1/2 of the South American species appear to be cosmopolitan. Tropical areas generally have a high diversity of genera and species. Brazil in particular has many endemic genera, some of which are only known from a single site in Amazonia (e.g., *Undula* in the chaetonotid subfamily Undulinae). There are also numerous other records of species from only one country and often from only one site, but knowledge on gastrotrich diversity in surrounding countries and regions is not sufficient to

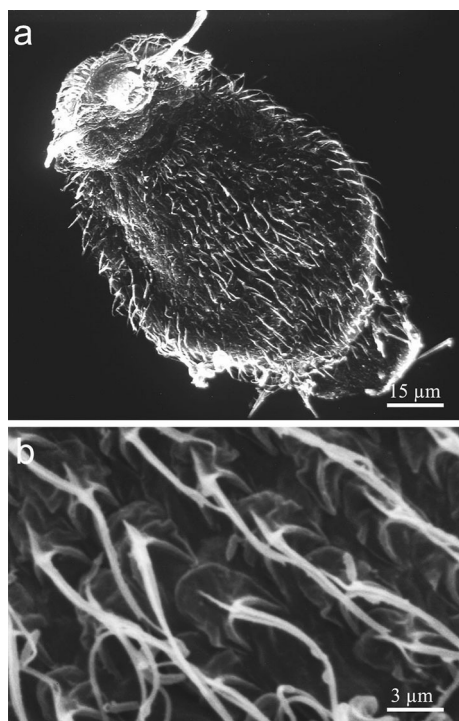
define these species as endemic (Balsamo et al., 2014; Garraffoni & Balsamo, 2017).

An update of the situation reported in Balsamo et al. (2008) highlights the increase in the number of new freshwater species of gastrotrichs recently described, mainly from the Palearctic, but also from the Neotropic and Nearctic (Balsamo et al. 2019; Todaro et al., 2019) (Table 6a; Fig. 9a). This increase is not only related to an increased sampling effort, but also to investigations in environments not yet explored such as Arctic waters and artificial water bodies (greenhouses) (Kolicka et al., 2018; Kolicka, 2019 and references therein).

Global species numbers in the different groups of freshwater microturbellarians amount to the following numbers: Catenulida (95 species), Macrostromorpha (118 species), Prorhynchida (31 species), Proseriata (12 species), Rhabdozoela (739 species), Proleptophora (20 species), and Bothrioplanida (2 species) (Table 6b). The majority of freshwater species belong to three groups within the rhabdozoel clade Limnophloplanida: Temnocephalida (160 species), Dalyelliidae (174 species), and Typhloplanidae (271 species). Knowledge on the diversity and distribution of freshwater microturbellarians in different parts of the world is relatively scarce and, as for most other freshwater meiofauna, reflects the historical efforts and geographical work area of taxonomists rather than actual microturbellarian diversity and distribution. Table 6b and Fig. 9b summarize species numbers for microturbellarians in each biogeographic zone. These numbers are the most current update since the census of freshwater turbellarians in Schockaert et al. (2008). Increased species numbers and distribution records for the Palearctic can largely be attributed to increased taxon sampling of catenulids (Larsson & Willems, 2010), macrostromids (Rogozin, 2012), rhabdozoels (Rogozin, 2011, 2017; Van Steenkiste et al., 2011b; Korgina, 2014; Timoshkin et al., 2014; Houben et al., 2015), and proseriates (Timoshkin et al., 2010), and to the recognition of cryptic species within *Microstromum* (Atherton & Jondelius, 2018). Species numbers and records in the Nearctic have increased slightly due to recent surveys of rhabdozoels in Canada and the USA (Van Steenkiste et al., 2011a; Houben et al., 2014). The largest increase in species numbers and records can be found in the Neotropical, Oriental, and Australian regions thanks to recent studies describing and recording several dozens of rhabdozoels and



**Fig. 7** Representatives of freshwater genera of Chaetonotida: **a, b** *Chaetonotus*; **c, d** *Heterolepidoderma*; **e** *Lepidochaetus* (DIC microscopy)



**Fig. 8** A semiplanktonic genus of Chaetonotida, *Neogossea*. **a** ventral view; **b** detail of cuticular scales and spines (SEM)

macrostomids in South America (e.g., Adami et al., 2012; Martínez-Aquino et al., 2014; Braccini et al., 2016), Southern China (Sun et al., 2015; Lin et al., 2017), and India and Australia (e.g., Van Steenkiste et al., 2012). Only a marginal increase or status quo in species records are shown for the Afrotropical, Pacific, and Antarctic regions, where almost no or very few studies on microturbellarians have been conducted in the past decades. While global species numbers have increased with over 16% in the last 12 years, biodiversity surveys of microturbellarians in some of the world's largest and most diverse freshwater systems, such as the Pantanal or the basins of the Amazon, Congo, and Ganges–Brahmaputra rivers, are still very limited or non-existent. Several freshwater habitats, such as limnoterrestrial habitats are seldom sampled and could contain a hidden reservoir of microturbellarian diversity (Van Steenkiste et al., 2010; Houben et al., 2015). Phreatic aquifers or peat swamp forests remain unexplored altogether. As such, our freshwater microturbellarian census clearly shows a large potential for species discovery in freshwater habitats around the world. Even in the most intensely sampled

biogeographical regions, including the Palearctic, Nearctic, and Neotropics, vast areas and many habitats are still to be surveyed.

As a result of the paucity of data on species diversity in many regions, biogeographic patterns for freshwater microturbellarians are hard to infer. While the majority of species have so far only been recorded from one biogeographic region (“endemic species” in Table 6b), some nominal species of catenulids (e.g., *Stenostomum leucops* (Duges, 1828)), macrostomids (e.g., *Microstomum lineare* (Müller, 1773)), and rhabdocoels (e.g., *Gyratrix hermaphroditus*) seem to have cosmopolitan distributions. Others are widespread, but confined to one or two biogeographic regions. For instance, several nominal species of dalyelliids (e.g., *Microdalyellia armigera* (Schmidt, 1861), *Gieystoria cuspidata* (Schmidt, 1861), *Castrella truncata* (Abildgaard, 1789)) have a Holarctic distribution. One hypothesis is that the widespread distribution of micro-organisms could be the result of long-distance dispersal by long-term resistant dormant stages and the ability to colonize and reproduce quickly (Fontaneto, 2019). In addition, some of these widespread nominal species could be complexes of closely related species, the so-called (pseudo)cryptic species. This has been demonstrated in both marine (Scarpa et al., 2016; Van Steenkiste et al., 2018) and freshwater (Atherton & Jondelius, 2018) microturbellarians, where several nominal species are now considered complexes of different species.

On a superspecific level, distribution patterns of freshwater microturbellarians are even harder to untangle. Most genera have representatives in different biogeographical regions. Some genera or species groups seem confined to certain biogeographical areas and their distribution could possibly be explained by a combination of geological events and dispersal.

## Perspectives

The majority of studies on rousphozoans are mostly conducted by researchers in Europe and the Americas. Programs for taxonomic capacity building in developing countries could benefit biodiversity surveys of freshwater meiofauna in the vastly undersampled but biodiverse freshwater ecosystems of Africa, Southeast Asia, and the Americas. These regions might be of crucial importance for a more realistic biodiversity

**Table 6** Current species numbers for a. Gastrotricha and b. microturbellaria in different biogeographical regions of the world, including numbers of endemic species per region and species numbers from the 2008 census of Balsamo et al. (2008) and Schockaert et al. (2008) for comparison

		PA	NA	NT	AT	OL	AU	PAC	ANT	World
<b>(a) Gastrotricha</b>										
Chaetonotida										
Chaetonotidae	Current census	222	71	78	7	25	8	0	0	296
	2008 census	194	60	76	7	25	8	0	0	281
	<i>increase</i>	28	11	2	0	0	0	0	0	15
Dasydytidae	Current census	21	9	11	0	2	0	0	0	33
	2008 census	21	9	10	0	2	0	0	0	33
	<i>increase</i>	0	0	1	0	0	0	0	0	0
Dichaeturidae	Current census	4	0	0	0	0	0	0	0	4
	2008 census	3	0	0	0	0	0	0	0	3
	<i>increase</i>	1	0	0	0	0	0	0	0	1
Neogosseidae	Current census	4	2	4	4	1	0	0	0	9
	2008 census	4	2	4	3	1	0	0	0	8
	<i>increase</i>	0	0	0	1	0	0	0	0	1
Proichthyidae	Current census	1	0	1	0	0	0	0	0	2
	2008 census	1	0	1	0	0	0	0	0	2
	<i>increase</i>	0	0	0	0	0	0	0	0	0
Macrodasyida	Current census	1	1	2	0	0	0	0	0	4
	2008 census	1	0	1	0	0	0	0	0	2
	<i>increase</i>	0	1	1	0	0	0	0	0	2
Total	Current census	253	83	96	11	28	8	0	0	348
	2008 census	224	71	92	10	28	8	0	0	320
	<i>increase</i>	29	12	4	1	0	0	0	0	28
	Endemic species	84	24	49	3	6	3	0	0	
<b>(b) Microturbellaria</b>										
Catenulida										
	Current census	48	36	49	11	2	1	0	0	95
	2008 census	36	36	45	10	1	1	0	0	90
	<i>increase</i>	12	0	4	1	1	0	0	0	5
Macrostromorpha	Current census	63	30	10	14	15	1	0	0	118
	2008 census	43	26	3	14	2	1	0	0	84
	<i>increase</i>	20	4	7	0	13	0	0	0	34
Prorhynchida	Current census	21	6	5	3	1	3	0	1	31
	2008 census	20	4	4	3	0	3	0	1	31
	<i>increase</i>	1	2	1	0	1	0	0	0	0
Rhabdocoela	Current census	437	97	103	36	38	110	1	1	739
	2008 census	431	86	59	34	9	70	0	1	646
	<i>increase</i>	6	11	44	3	29	40	1	0	93
Proseriata	Current census	7	1	4	0	0	0	0	1	12
	2008 census	5	0	2	0	0	0	0	1	10
	<i>increase</i>	2	1	2	0	0	0	0	0	2
Bothrioplanida	Current census	1	1	1	1	1	0	0	0	2
	2008 census	1	1	1	1	0	0	0	0	1
	<i>increase</i>	0	0	0	0	1	0	0	0	1

Table 6 continued

		PA	NA	NT	AT	OL	AU	PAC	ANT	World
Prolecihoptora	Current census	14	2	1	1	1	1	0	0	20
	2008 census	12	2	0	0	1	1	0	0	12
	<i>increase</i>	2	0	1	1	0	0	0	0	8
Total	Current census	591	173	173	66	58	116	1	3	1017
	2008 census	548	155	114	62	13	76	0	3	874
	<i>increase</i>	43	18	59	4	45	40	1	0	143
	Endemic species	506	95	119	41	41	112	0	3	

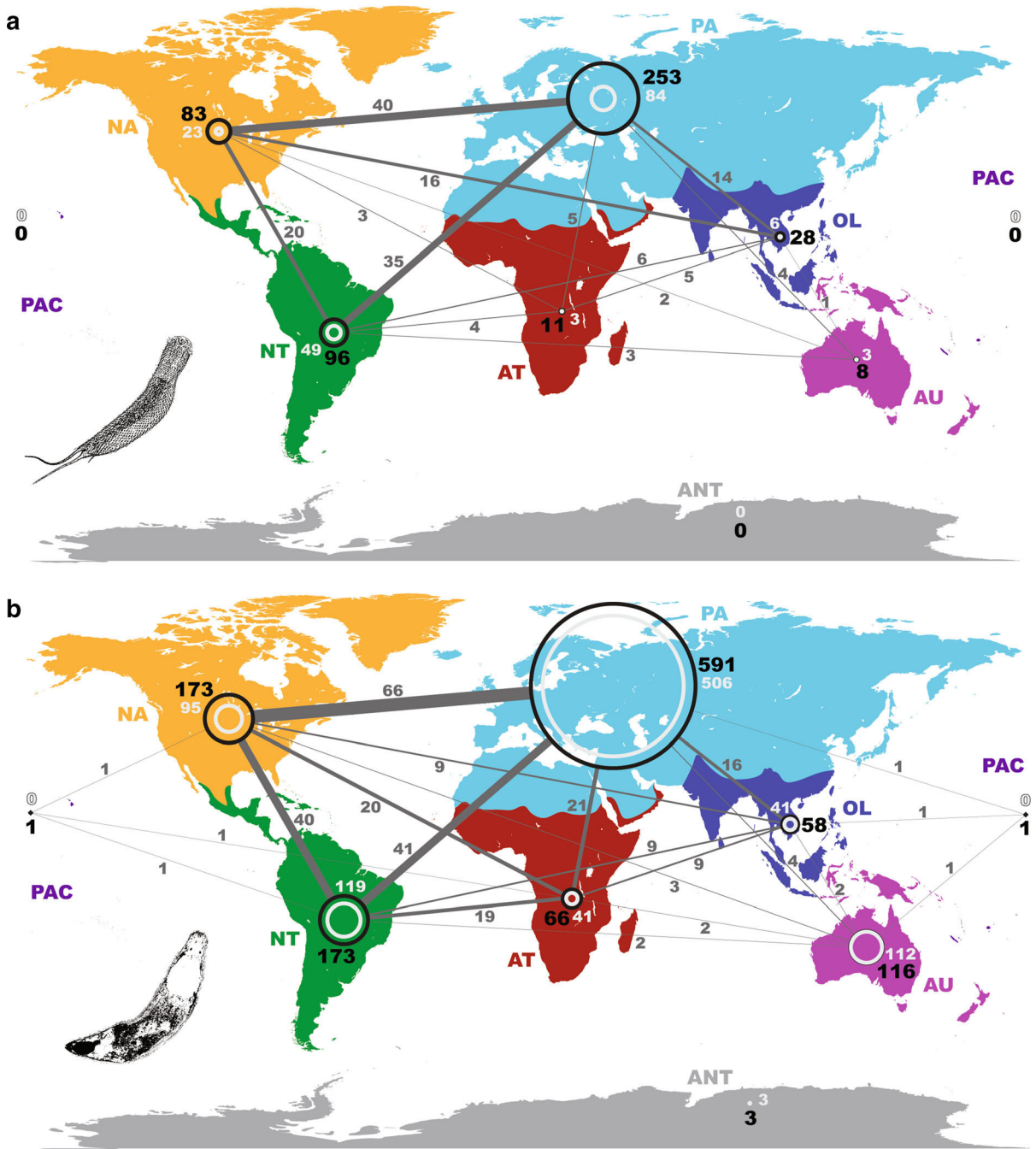
PA Palearctic, NA Nearctic, NT Neotropical, AT Afrotropical, OL Oriental, AU Australian, PAC Pacific, ANT Antarctic

1162 estimation of microturbellarian and gastrotrich species  
1163 diversity, but are at risk because of rapid habitat  
1164 destruction and climate change. Wide-ranging Euro-  
1165 pean research programs on the freshwater animal  
1166 biodiversity have been carried out in the past years  
1167 (2000–2008) leading to the compilation of European  
1168 and global databases of the known biodiversity at the  
1169 time (Fauna.Europaea Project, see de Jong, 2014;  
1170 FADA Freshwater Animal Diversity Assessment  
1171 Project, see Balsamo et al., 2008). Increased species  
1172 discovery should be a concerted effort with expanding  
1173 and updating databases that consolidate existing and  
1174 new taxonomic and biogeographic data. An important  
1175 first step would be the development of regularly  
1176 updated identification keys for freshwater rouspho-  
1177 zoans. This could be part of a broader effort on  
1178 freshwater meiofauna analogous to current efforts for  
1179 marine meiofauna (Schmidt-Rhaesa, 2020). To accel-  
1180 erate biodiversity surveys of rousphozoans, protocols  
1181 for animal collection, vouchering, DNA extraction,  
1182 DNA barcode marker selection, amplification, and  
1183 sequencing should be adjusted to the upcoming and  
1184 promising third-generation sequencing techniques  
1185 (e.g., Nanopore).

1186 A large impediment for future research on taxon-  
1187 omy, biogeography, and phylogeny of Rousphozoa  
1188 (and all other Metazoa for that matter) is the imple-  
1189 mentation of the Nagoya Protocol (NP). Since October  
1190 2014, NP regulates all access to, and benefit sharing of,  
1191 genetic resources worldwide. The protocol was  
1192 designed to ensure fair use of countries' genetic  
1193 resources, including the use of traditional knowledge.  
1194 However, as logical and fair such legislation might

1195 seem, many concerns have been uttered (Deplazes-  
1196 Zemp et al., 2018, and references therein). Whereas  
1197 the NP and resulting legislation is needed to counter  
1198 biopiracy and ensure that countries are not robbed of  
1199 their economically valuable biological and genetic  
1200 resources, it has devastating side effects on (descrip-  
1201 tive) fundamental research. Without any doubt, the NP  
1202 will significantly slow down taxonomic and other  
1203 biodiversity studies just in an era in which such  
1204 projects are much needed. For instance, in our daily  
1205 work on microturbellarians, specimens are exchanged  
1206 between researchers on a very regular base, in several  
1207 cases involving colleagues from developing countries  
1208 with whom we try to build up a structural collabora-  
1209 tion. Because of the regulations of the NP, such  
1210 exchange of material, indispensable for fruitful joint  
1211 scientific activities, is hampered. The administrative  
1212 workload will discourage international collaboration  
1213 between researchers and will cause (and is already  
1214 causing) a bias towards research in countries that did  
1215 not ratify the NP. Moreover, for many biologists and  
1216 institutes, it is not entirely clear (yet) what procedures  
1217 should be followed in practice. We can only hope that  
1218 the regulations of the NP will be revised in the future  
1219 to ensure that at least the much-needed fundamental,  
1220 non-profit research can continue smoothly.

1221 Dispersal abilities of freshwater gastrotrichs and  
1222 microturbellarians and the relationship between dis-  
1223 persal and distribution have not been specifically  
1224 investigated so far. The small size of gastrotrichs and  
1225 microturbellarians and the absence of planktonic  
1226 stages limit active dispersal of live individuals to  
1227 short distances. Wind, running water, and more mobile



**Fig. 9** Current species numbers for **a.** Gastrotricha and **b.** microturbellarians in different biogeographical regions of the world (black numbers and circles), including numbers of endemic species per region (light gray numbers and circles)

and numbers of species shared between regions (dark gray numbers and lines). *PA* Palearctic, *NA* Nearctic, *NT* Neotropical, *AT* Afrotropical, *OL* Oriental, *AU* Australian, *PAC* Pacific, *ANT* Antarctic

1228 animal vectors have all been proposed as passive long-  
 1229 distance dispersal vectors for long-term desiccation-  
 1230 resistant eggs or cocoons of rouphozoans (Gerlach,

1231 1977; Hagerman & Rieger, 1980; Young, 2001; 1231  
 1232 Vanschoenwinkel et al., 2008, 2009; Viana et al., 1232  
 1233 2016). Human-mediated dispersal (aquaculture, 1233

- 1234 ballast waters, etc.) of gastrotrichs, microturbellarians, 1283  
 1235 and many other aquatic micro-invertebrates is likely, 1284  
 1236 but has not yet been the subject of specific studies 1285  
 1237 (Artois et al., 2011). Future studies focusing on the 1286  
 1238 spatial connectivity and gene flow of freshwater 1287  
 1239 gastrotrich and microturbellarian populations are 1288  
 1240 highly needed to support these assumptions. Cerca 1289  
 1241 et al. (2018) have stressed the importance of including 1290  
 1242 ecological and life-history traits, evolutionary history 1291  
 1243 and cryptic speciation, metapopulation dynamics, as 1292  
 1244 well as considering vicariant events and (ancient) 1293  
 1245 dispersal routes on different geographic and temporal 1294  
 1246 scales to explain current-day distribution of marine 1295  
 1247 meiofauna. These are all important considerations to 1296  
 1248 also elucidate recent distribution patterns of freshwa- 1297  
 1249 ter gastrotrichs and microturbellarians. 1298  
 1250 The task of untangling hidden diversity, spatial 1299  
 1251 connectivity, and trophic networks in Rousphozoa will 1300  
 1252 certainly fall to molecular methods. Metagenetic, 1301  
 1253 genomic, and transcriptomic data—when combined 1302  
 1254 and integrated with morphological and ecological 1303  
 1255 data—can also provide new insights into additional 1304  
 1256 properties and patterns such as niche differentiation, 1305  
 1257 differential gene expression, genome duplication or 1306  
 1258 reduction, character evolution, reproduction modes 1307  
 1259 and traits related to sexual selection, origins of 1308  
 1260 symbiotic interactions, co-evolution and host speci- 1309  
 1261 ficity, nutritional strategies, and life cycle modifica- 1310  
 1262 tions. The current lack of such integrated studies 1311  
 1263 impedes our understanding on evolutionary processes 1312  
 1264 within rousphozoans. Many closely related species of 1313  
 1265 freshwater gastrotrichs and microturbellarians occur 1314  
 1266 in sympatry suggesting some kind of ecological 1315  
 1267 differentiation. However, non-ecological speciation 1316  
 1268 in allopatry at some point in the past has been proposed 1317  
 1269 for present-day sympatric organisms (Czekanski-Moir 1318  
 1270 & Rundell, 2019). Species flocks of rhabdocoels in 1319  
 1271 Lake Baikal are the product of spectacular speciation 1320  
 1272 events, but the mechanisms behind these radiations are 1321  
 1273 not known. Revealing these underlying processes 1322  
 1274 remains challenging and will require holistic multi- 1323  
 1275 evidence approaches employing new techniques in 1324  
 1276 high-resolution microscopy and high-throughput 1325  
 1277 sequencing. 1326  
 1278 Finally, there is still a valuable role for functional 1327  
 1279 morphology, specifically, studies with a biomechan- 1328  
 1280 ical approach. From the original analysis of the role of 1329  
 1281 connective tissue in soft-bodied worms (Clark & 1330  
 1282 Cowey, 1958), and subsequent refinements applied to 1331  
 soft-tissue extensible structures more generally (Kier, 1332  
 2010), biomechanical studies in Rousphozoa are rare: 1333  
 proboscis function in *Cheliplana* (Uyeno & Kier, 1334  
 2010) and in Schizorhynchia more generally (Smith 1335  
 et al., 2015), and dynamics of duo-gland adhesion in 1336  
 marine microturbellaria (Wunderer et al., 2019). 1337  
 Accordingly, additional studies directed at a better 1338  
 understanding of rousphozoan biomechanics would 1339  
 provide a much richer context for the evolutionary and 1340  
 ecological work proposed above. 1341
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