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2 The curious and neglected soft-bodied meiofauna: 3 Rousphozoa (Gastrotricha and Platyhelminthes)

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Introduction

Meiofauna constitutes a significant reservoir of biodiversity in aquatic ecosystems that is often overlooked. Sediments and vegetation in freshwater habitats, including freshwater ponds, lakes, and rivers, but also mosses, wet soils and semi-aquatic agricultural ecosystems (e.g., paddy fields), are teeming with hundreds of thousands, if not millions of poorly known or completely unrecorded species of these microscopic animals (Giere, 2009).

Traditional morphology-based sampling techniques to study biodiversity and ecology of meiofauna are usually addressed towards ecdysozoan taxa such as nematodes and copepods, and have so far failed to account for the sometimes equally abundant and diverse soft-bodied gastrotrichs and meiofaunal flatworms or so-called “microturbellarians” (Martens & Schockaert, 1986; Nesteruk, 2006; Schockaert et al., 2008; Giere, 2009). Perhaps more so than other meiofaunal groups, gastrotrichs and microturbellarians also exemplify the taxonomic impediment, including a lack of knowledge on their biodiversity and organismal biology, a lack of experts and a lack of taxonomic infrastructure. As such, they have lagged behind in species discovery and identification, uncovering (cryptic) speciation, biodiversity surveys, population genetics, and phylogeography. Traditional morphology-based identification methods are often time-, effort-, and resource-intensive, depend on a handful of taxonomic experts, and cannot uncover cryptic diversity. As shown by a few comprehensive studies of single taxa, the current number of described species of these two groups in fresh waters is significantly lower than the estimated species diversity (e.g., Catenulida: Larsson et al., 2008; *Microstomum*: Atherton & Jondelius, 2018, 2019; Gastrotricha: Balsamo et al., 2008).

In this contribution, we will focus on the micro- and meiofaunal freshwater representatives of these taxa and largely omit the numerous members of the macrofaunal flatworm group Tricladida, which, because of their large size, have been rather well 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 Platyhelminthes, also recently emerged in a monophyletic clade within the Spiralia called Rousphozoa (Gr. *Rousphao*, 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 separate 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 Gastrotricha 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 gastrotrichs, the results of the few targeted studies on functional ecology appear controversial (Strayer, 1985; Hummon, 1987; Nesteruk, 1996a, 2007b; Schmid-Araya, 1997), and for freshwater microturbellarians no such studies exist to date.

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

Methodologies for sampling and study

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

Sampling and extraction

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

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

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

A comparative summary of methods for extraction and study of freshwater microturbellarians and gastrotrichs is reported in Table 2. Extraction of animals from fresh samples implies direct observation of small amounts of sediment under a stereomicroscope and picking up single living individuals for subsequent observation and study under a compound microscope.

The extraction of all the animals from a sample is clearly critical for quantitative analyses, but regrettably, the techniques currently available are not satisfactory for gastrotrichs. A comparative study of

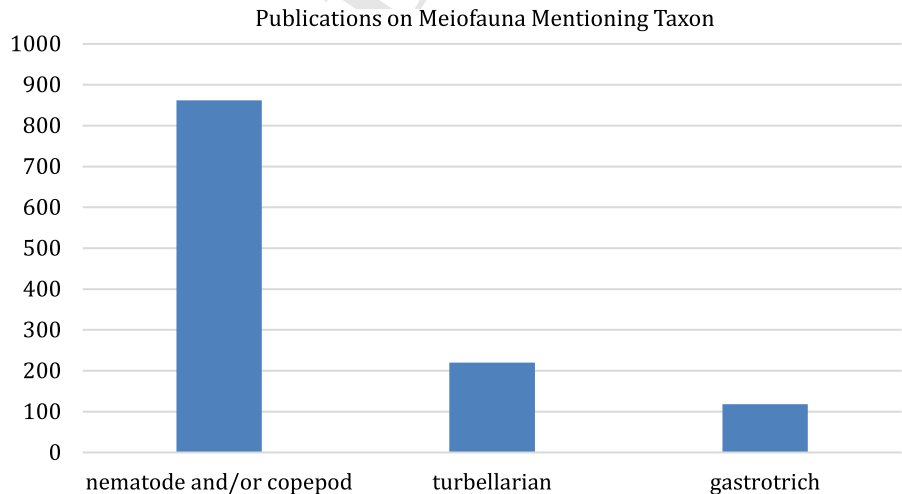


Fig. 1 Google Scholar entries for meiofaunal studies mentioning a. nematodes and/or copepods; b. micro- or macro-turbellarians; c. gastrotrichs. Methods: Publish or Perish (Harzing, 2007) was used (3.5.20) to search Google Scholar, covering years 1985 through 2020, with the following search strings: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

different techniques aimed at this purpose showed that a rapid forcing of small quantities of sediment through a 130 µm sieve appears to be the most effective way for extracting chaetonotid species (Hummon, 1981; Nesteruk, 1987; Giere, 2009). Details on the methods of sampling, extraction, and study of freshwater gastrotrichs are described in Balsamo et al. (2014).

Recommended methods for extraction and examination of microturbellarians are described in Schockaert (1996). Decantation methods including agitation of sediment and substrate debris followed by sieving (63 µm screen) will dislodge many freshwater flatworms from their substrate. However, the best method for isolating freshwater microturbellarians is oxygen depletion. A layer of sediment and bottom debris are

placed in a tall beaker with clear transparent walls; the beaker is then filled with water from the habitat and allowed to stand, creating a vertical cline of dissolved oxygen. Animals are thus forced out of the substrate and can then be removed from the sides of the beaker or from the surface film with a pipette.

DNA (meta)barcoding of Rousphozoa 201

DNA extraction and sequencing of taxonomic marker genes called DNA (meta)barcodes from bulk samples including water, aquatic sediments, and soil (eDNA), or from pooled individuals separated from the substrate, can reveal the presence of gastrotrichs and 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 ₂ 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 ³) 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 ₂ 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.

percentages that would otherwise go unnoticed with traditional morphotaxonomic methods (Leray & Knowlton, 2015; Arroyo et al., 2016; Martínez et al., 2020; Fegley et al., submitted). As such, (meta)barcoding holds great promise to increase our knowledge on the diversity, ecology, and role of rouphozoans in aquatic ecosystems (Martínez et al., 2020). This approach has been reviewed recently (Schenk & Fontaneto, 2019): accordingly, we here limit ourselves to considering the promises and pitfalls of DNA-based methods for evaluation of cryptic diversity and community composition among gastrotrichs and microturbellarians, including limitations not mentioned in the paper referenced above.

Choice of amplicon

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

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

Pitfalls

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

DNA (meta)barcoding relies completely on meticulously curated DNA reference databases that link sequences to species identified based on morphological characters. DNA extractions of tiny animals such as rousphozoa 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 microdissections using the head for DNA extraction and the posterior part for morphological study (e.g., macrostomids in Schärer et al., 2011; Janssen et al., 2015), and could be a practical solution to incorporate “problem” individuals and species into DNA reference collections.

Promises

Recently, analysis of marine and freshwater metabarcoding 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 individuals 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).

Methods for identification

Gastrotricha

The phylum Gastrotricha currently comprises over 850 free-living species widespread in aquatic ecosystems. The division into two classes, Macrodasypoidea and Chaetonotidae, each including a single order (Macrodasypoda and Chaetonotida, respectively) dates back to Remane (1925), and follows the evident differences in morphology, biology, and ecology between the two taxa (Balsamo et al., 2009, 2014, 2015; Hummon & Todaro, 2010; Kieneke & Schmidt-Rhaesa, 2015) (Fig. 2), which has also been confirmed by molecular analyses (Paps & Riutort, 2012).

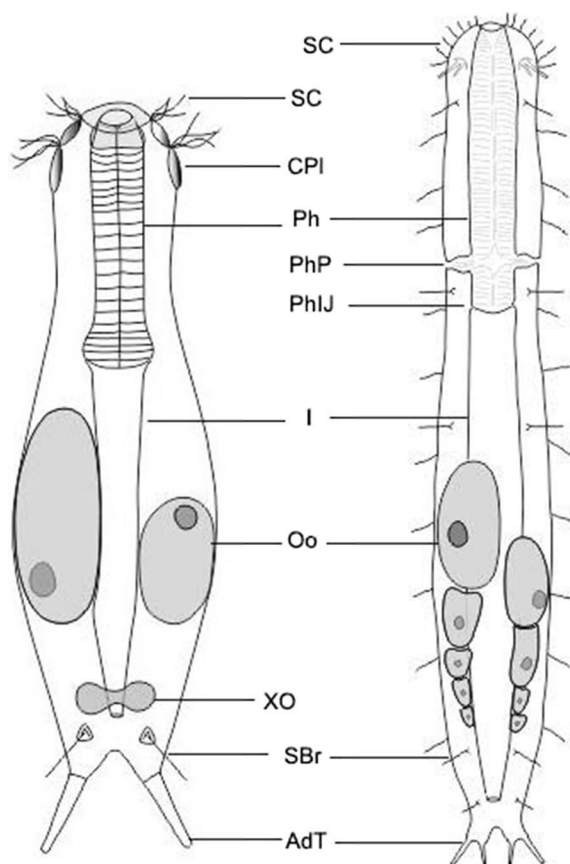


Fig. 2 Schematic view of freshwater Gastrotricha: **a** Chaetonotida, **b** Macrodasypoidea. AdT, adhesive tubes; CPI cephalic plates; I intestine; Oo oocyte; Ph pharynx; PhIJ pharyngo-intestinal junction; PhP pharyngeal pores; SBr sensory bristles; SC sensory cilia; XO X-organ

Taxonomy and systematics of Gastrotricha have been traditionally founded on morphological characters, 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.

Platyhelminthes

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

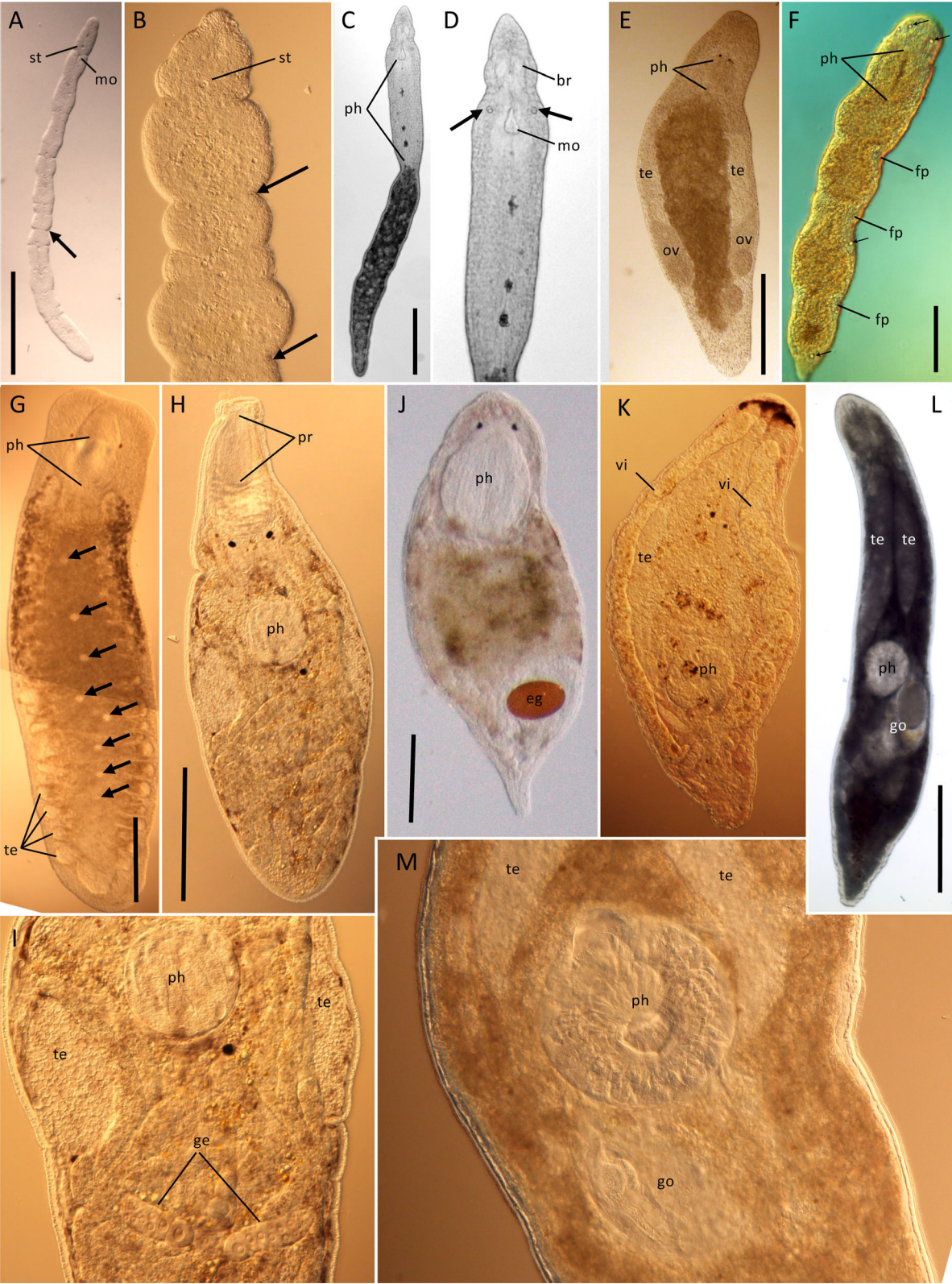


Fig. 3 Clades of microturbellaria with pharynx simplex and homocellular female gonads (yolk contained in oocytes). **a–d** Catenulida: **a** *Catenula confusa*, showing anterior statocyst (st), mouth (mo), and best-developed fission plane (arrow). Scale = 200 µm. **b** Anterior end of *Catenula lemnae*, 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

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

It is possible to key most platyhelminthes to family level based on morphological characters alone (e.g., Cannon, 1986; Smith et al., 2020). Useful characters are the presence/absence of a statocyst, the construction of the pharynx, the structure of the female gonad, and the morphology of the male reproductive system (Fig. 3). The basic anatomy of Platyhelminthes, including microturbellaria, is covered in detail elsewhere (e.g., Rieger et al., 1991).

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

has only been employed a few times for (cryptic) species delimitation in freshwater gastrotrichs (Kånneby et al., 2012) and microturbellarians (Larsson et al., 2008; Atherton & Jondelius, 2018, 2019). This illustrates the urgency of improving aspects of environmental high-throughput sequencing before this potentially cost-effective approach could be widely applied for species discovery, biodiversity surveys, and ecosystem assessments in aquatic ecosystems.

Well-supported intraphylum relationships among most major subtaxa (with the exception of the relative position of Rhabdocoela and Proseriata) are provided by two recent transcriptomic studies (Egger et al., 2015; Laumer et al., 2015b—Fig. 4). Recent molecular phylogenies, albeit largely based on only a few genes, have provided valuable information on relationships within the major groups, often in conflict with the traditional morphology-based taxonomy: Catenulida (Larsson & Jondelius, 2008), Macrostomorpha (Janssen et al., 2015; Atherton & Jondelius, 2019), Rhabdocoela (Willems et al., 2006; Van Steenkiste et al., 2013; Tessens et al., 2014), and Proseriata (Laumer et al., 2014; Scarpa et al., 2016). Accordingly, it has proven to be challenging to provide morphological apomorphies for many of the resulting clades. Therefore, phylogenomics based on much larger molecular datasets and advances in the study of morphological characters should be integrated to provide a more robust taxonomy for different microturbellarian groups.

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

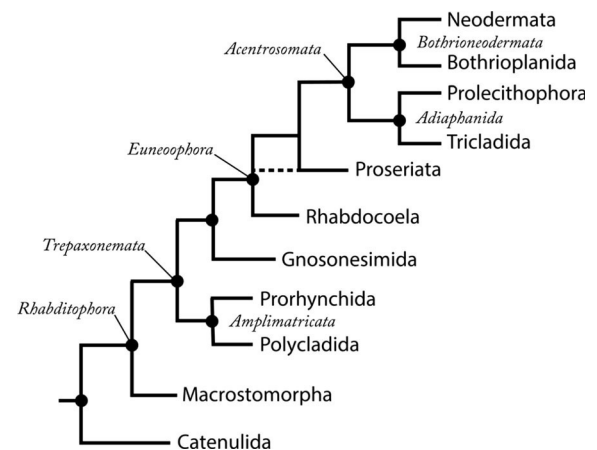


Fig. 4 Major higher-level taxa in Platyhelminthes, based on transcriptomic studies (Egger et al., 2015; Laumer et al., 2015b)

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

Ecology

Studies on gastrotrich and microturbellarian autoecology and synecology are not numerous (Schwank, 1981b, 1982a; Heitkamp, 1982, 1988; Ricci & Balsamo, 2000; Kolasa, 2002; Nesteruk, 2016a, b, 2017). Abiotic and ecological factors define the qualitative and quantitative compositions of populations, whose mean densities widely vary depending on the characteristics of the habitat and seasonal dynamics, and can range from a few thousand up to 2.6 million ind/m² for both benthic and pelagic gastrotrichs (Nesteruk, 2004a, 2009, 2011) and at least

several thousand ind/m² for microturbellarians (Kolasa, 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)



waters; most species live in moderately acidic habitats, but some species can tolerate pH values down to 4, while others live in alkaline water up to pH 10 (Kisielewski, 1981; Nesteruk, 2004a). A few freshwater gastrotrich species are able to survive or even to live in brackish waters. Finally, all freshwater gastrotrichs are influenced by the characteristics of the water column, substrate, and aquatic vegetation.

Most freshwater chaetonotidans are epibenthic or periphytic in oxygenated habitats, and more abundant in eutrophic, standing waters (see Nesteruk, 2017 and references therein). The epibenthic community is generally more diverse and is dominated by eurytopic species of the genera *Chaetonotus*, *Lepidodermella*, *Heterolepidoderma*, and *Ichthydium*, whereas epiphytic assemblages also include semiplanktonic species of Dasydytidae and Neogosseidae (Nesteruk, 2000; Minowa & Garraffoni, 2017). Sandy sediments of lentic and running waters host all four freshwater species of Macrotrichida, but few species of Chaetonotida (see Balsamo et al., 2014). Trophic levels and zonality of water bodies also influence the diversity and density of gastrotrich populations. Water bodies with a clear zonality provide a higher habitat diversity and consequently have a richer and more abundant fauna, especially in the littoral zone (Kisielewski, 1981; Nesteruk, 2004b, 2005). Compositional differences also exist between the sublittoral and the deep zone (Nesteruk, 1996b, 2004b). Alpha-mesotrophic waters are 26–45% richer in species than waters with a lower trophic level (Nesteruk, 1996b, 2004a). The few semiplanktonic or planktonic species preferentially live in eutrophic ponds, *Sphagnum* bogs, and transitional peat bogs, which appear to have the highest species richness, independent from altitude, vegetation, and trophic level (Kisielewski, 1981, 1986, 1991; Balsamo, 1982; Balsamo & Todaro, 1995). In lotic habitats, gastrotrichs are mostly present where the water current is slower, such as vegetated river banks, bends of the water course, and in small streams (Kisielewski & Kisielewska, 1986; Kisielewski, 1991). A few interstitial species have been reported from sediments of springs, rivers, and streams (Ricci & Balsamo, 2000; Nesteruk, 2008; Garraffoni et al., 2017). Most gastrotrich species are able to colonize more than a single habitat and can migrate between the epibenthos, periphyton, and interstitial.

Very few studies specifically focus on the influence of abiotic variables on the occurrence and abundance

of freshwater microturbellarians. Kolasa (2002) provides a brief overview on general preferences and tolerance ranges of several abiotic parameters, but only for few species tolerance ranges for temperature, oxygen, water level, oxygen, pH, and calcium are known (Heitkamp, 1982). Most species have an optimal temperature range for reproduction and population growth to occur and temperature can have a significant influence on hatching and on the generation time (Heitkamp, 1988; Sayre & Wergin, 1994; Dumont et al., 2014). Some species are stenotherm, while others are eurytherm. Microturbellarians require oxygenated layers of water and sediment. Species that live in substrates of well-oxygenated, fast-running streams are particularly sensitive to low oxygen concentrations (Kolasa, 1983). A small number of freshwater rhabdocoels are euryhaline and can also be found in brackish water habitats (Ax, 2008). However, most microturbellarians that occur in brackish water are euryhaline marine species or genuine brackish water species that do not occur in freshwater habitats. Granulometry of freshwater sediments also influences species composition and occurrence. Kolasa et al. (1987) found higher species richness and abundance in stream sediments with a grain size of 0.4–0.7 mm, compared to a low species richness and abundance for small stones or large gravel. Young (1973) found that calcium-rich and calcium-poor lakes each have their specific species of microturbellarians, but also share a number of species.

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



2000). In large lakes and reservoirs, species richness and abundance are significantly higher in sediments and aquatic vegetation in the littoral zone, but some species have also been found in the limnetic zone as part of the pelagic (Dumont et al., 2014). Permanent bodies of water are usually dominated by catenulids, macrostomids, prorhynchids, and rhabdocoels associated with aquatic vegetation, plant roots, and sediment, while temporary pools typically harbor species with drought resistant resting eggs, such as typhloplanid and dalyelliid rhabdocoels (Artois et al., 2004; Eitam et al., 2004). Species compositions in lotic systems are highly variable. Mountain springs and fast flowing streams or rivers have unique hyporheic and psammophilic species or species associated with mosses and other vegetation along its course (Schwank, 1982a, b). The lower courses of rivers systems are inhabited by eurytopic species also found in lentic habitats. Assemblages of species are further enriched by species from habitats at the interface of lotic and lentic habitats, including limnoterrestrial, brackish water, and groundwater elements (Kolasa 1983, 2000). A very detailed review on the distribution and abundance of microturbellarians in different aquatic habitats is given by Young (2001).

Spatial and temporal dynamics of rousphozoans' populations

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

The vertical distribution of gastrotrichs is highly related to grain size, oxygen concentration, presence and velocity of water flow, quantity of organic matter present in the interstitial water, predation pressure, as well as the tolerating abilities of different species (Palmer, 1990; Danielopol et al., 1997). The few 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 availability. Periods of drought and freezing in temperate zones strongly influence both the abundance and the structure 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 individuals 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 *Mesostoma* 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 community compositions throughout the year (Young, 2001; Braccini & Leal-Zanchet, 2013). Seasonal occurrence and abundance of microturbellarians also

seem to vary according to geographic location and are most likely linked to the influence of temperature, food availability, droughts and floods, and several other abiotic and biotic variables. The scarcity of studies available on these temporal dynamics highlights the need for more research in different parts of the world.

Trophic and biotic interactions

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

careful examination of pharynx structure by transmission electron microscopy and confocal laser scanning microscopy. We predict that species with monosarcomeral pharynges will be biofilm feeders, whereas species with multisarcomeral pharynges will feed primarily on eukaryotes. These studies should determine 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, polychaetes, and larvae of Diptera have been reported as natural predators of Gastrotricha (Strayer & Hummon, 1991; d'Hondt, pers. comm.). The heliozoon *Actinophrys 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; Kisieleska 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 Rhabdocoela) and carnivory on other meiofauna and the larvae of macroinvertebrates (see Watzin, 1983, 1986; Giere, 2009). Catenulids have a distensible 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 smithrii</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)

opening. A few species in the catenulid genus *Paracatenula* are mouthless and maintain symbiotic bacteria in the gut (Dirks et al., 2011, 2012). Other microturbellarians use their muscular pharynx for the capture and uptake of prey items. The pharynx can be distended to capture and ingest prey as a whole (Stenostomidae, Dalyelliidae) or protruded to breach the body wall of larger prey and suck up prey fluids and tissues (Typhloplanidae, Proseriata). Kalypatorhynchs use their anterior proboscis to capture and possibly envenomate prey and immobilize it while positioning their pharynx. Some flatworms, such as prorhynchids and *Gyratrix hermaphroditus* Ehrenberg, 1831, use their stylet to stab prey.

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

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

Taxon	Pharynx type ¹	Ex. FW Genera	Ex. Prey ^{2,3}	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 ^{4,5}
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>Gieysztor</i>	Diatoms, green algae, rotifers, nematodes, oligochaetes, other flatworms	Suction, holozoic

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

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

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

Microturbellarians are also eaten by other invertebrates, small vertebrates, and even protists. Defensive behavior such as the release of mucous to escape from predatory ciliates has been observed (Buonanno,

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

Freshwater microturbellarians, and then predominantly rhabdocoels, can be both ectosymbionts on other freshwater animals and hosts for other organisms. Temnocephalids are small freshwater epibionts on macroinvertebrates and turtles. They prey on other co-symbiotic organisms and feed opportunistically on particles of the host's food. The dalyelliid *Varsoviella kozminskii* Gięsztor & Wiszniewski, 1947 lives on the gills of freshwater gammarids (Gięsztor & Wiszniewski, 1947). A number of freshwater species in the genera *Castrada*, *Dalyellia*, *Gięsztor*, *Phaenocora*, and *Typhloplana* harbor endosymbiotic chlorophytes. Little is known about this symbiosis, but studies on *Phaenocora typhlops* (Vejdovsky, 1880), *Dalyellia viridis* (Shaw, 1791), and *Typhloplana viridata* (Abildgaard, 1789) suggest that worms could benefit from the photosynthate and oxygen produced by the algae (Young, 2001 and references therein). Kleptoplasty, a form of endosymbiosis where only the algal plastids are sequestered and retained, has recently been observed in marine and brackish water rhabdocoels (Van Steenkiste et al., 2019). Species of the genus *Microstomum* often retain nematocysts from digested *Hydra* tentacles as kleptocnids (Fig. 3f, arrows). Parasites of freshwater microturbellarians have occasionally been recorded in older taxonomic literature, but very few studies characterize the observed parasites in detail. Most of these parasites are protists, including apicomplexans, microsporidians, ciliates, and euglenozoans. Only a few records mention metazoan parasites such as nematodes or neodermatan flatworms (for details, see Young, 2001 and references therein). It is noteworthy that the last comprehensive review of microturbellarians as parasites and hosts was published over 100 years ago (von Graff, 1903), and less comprehensive modern summaries are available (Jennings, 1971, 1974a, 1977).

Life strategies

Gastrotrichs have various reproductive modalities. While marine Macrodasyda are hermaphrodite with cross-fertilization, freshwater Chaetonotida generally reproduce by thelytokous parthenogenesis. As a consequence, freshwater populations can start from any single individual. Many freshwater species can also produce resting eggs that can withstand environmental adverse conditions and act as dispersal propagules. The factors triggering the production and the hatching of the resting eggs are not yet known. Parthenogenesis, resting eggs, and short life cycles allow gastrotrichs to survive extreme variations in environmental conditions (e.g., droughts, floods) and colonize challenging habitats such as lotic sediments (Ricci & Balsamo, 2000), caves (Vandel, 1964; Renaud-Mornant, 1986; Kolicka et al., 2017), high mountain biotopes (Baumann, 1910; Tonolli & Tonolli, 1951; Gadea, 1988), hot springs (De Guerne, 1888), and deep crater lakes (Barrois, 1896; R. Schabetsberger, unpublished data). In addition, individuals might also be able to survive critical conditions by migrating deeper into the sediment (Nesteruk, 2007c).

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

Microturbellarians are hermaphrodites and display both sexual (cross- and self-fertilization) and asexual (paratomy) modes of reproduction (Kolasa, 2000). Catenulids and some macrostomids (e.g., *Microstomum*) reproduce asexually, although sexual reproduction can also occur. Most other freshwater microturbellarians reproduce by internal cross-fertilization, either by mutual copulation or sometimes by hypodermic impregnation. Self-fertilization is rare and has only been observed in a few species (Young, 2001).

Life histories of freshwater microturbellarians are 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; Kanneby & 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; Kanneby 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

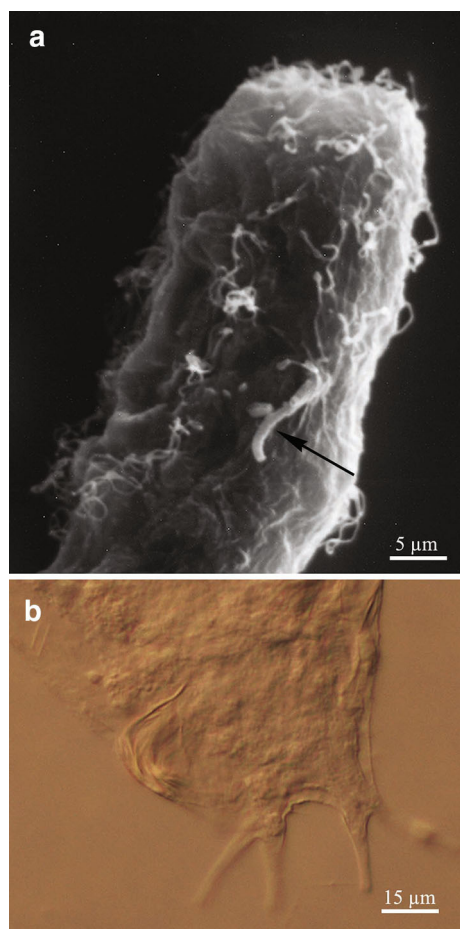


Fig. 6 A freshwater genus of Macrotrichidae, *Redudasys*. **a** Lateral view of the head showing the diagnostic single adhesive tube (SEM); **b** detail of the caudal body end with four adhesive tubes (DIC microscopy)

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), Rhabdocoela (739 species), Prolecithophora (20 species), and Bothrioplanida (2 species) (Table 6b). The majority of freshwater species belong to three groups within the rhabdocoel clade Limnotyphloplanida: 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), rhabdocoels (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 *Microstomum* (Atherton & Jondelius, 2018). Species numbers and records in the Nearctic have increased slightly due to recent surveys of rhabdocoels 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 rhabdocoels and

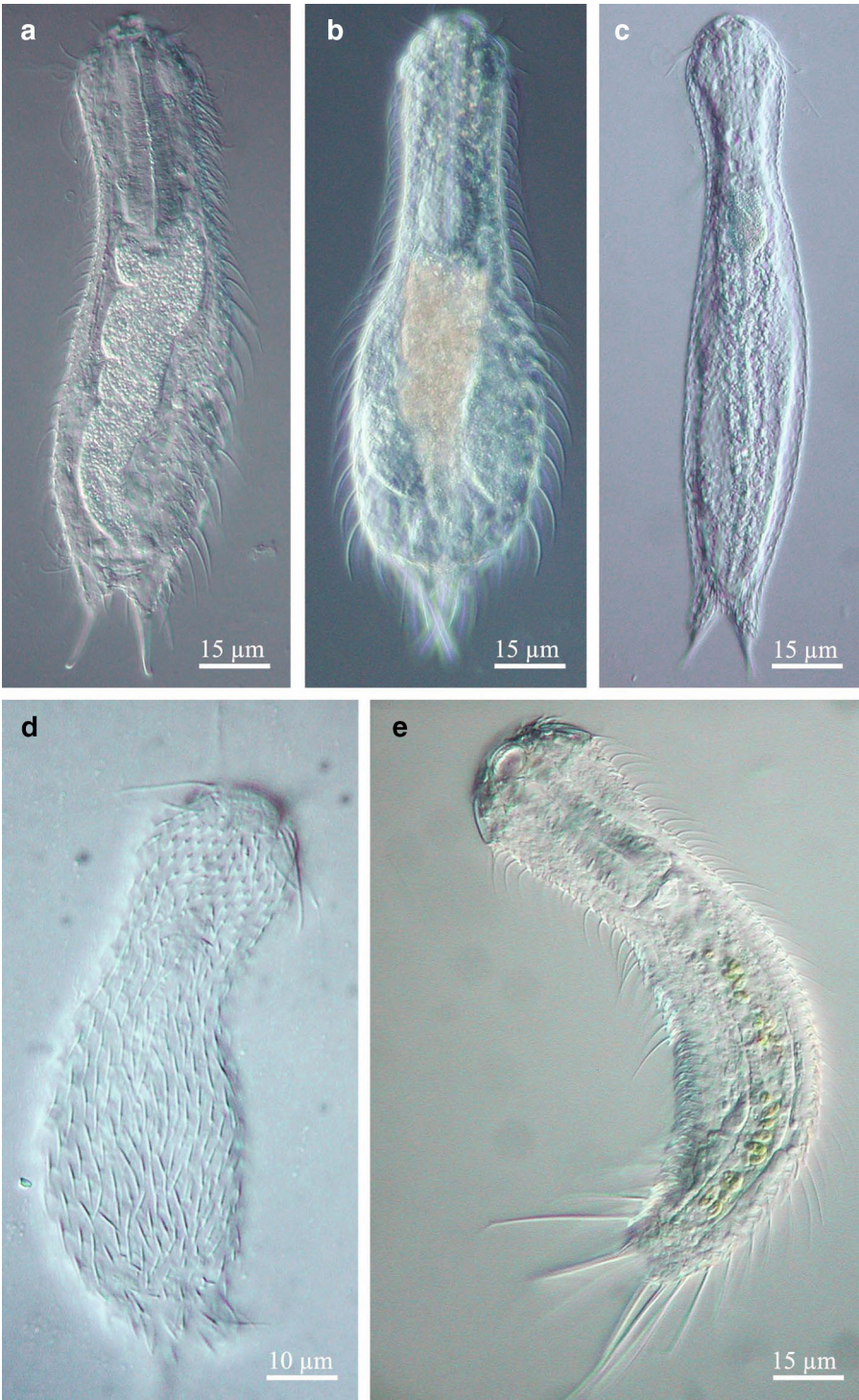


Fig. 7 Representatives of freshwater genera of Chaetonotida: **a, b** *Chaetonotus*; **c, d** *Heterolepidoderma*; **e** *Lepidochaetus* (DIC microscopy)

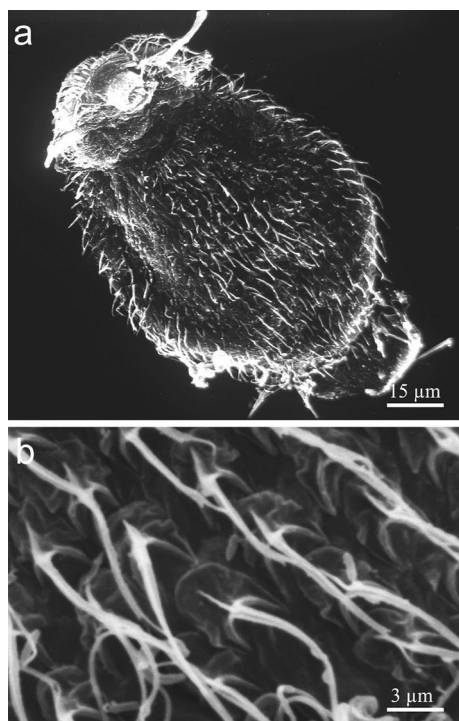


Fig. 8 A semiplanktonic genus of Chaetonotida, *Neogosseia*. **a** ventral view; **b** detail of cuticular scales and spines (SEM)

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), *Gieysztoria 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
(a) Gastrotricha										
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
Macrodasysida	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) Microturbellaria										
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
Macrostrompha	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
Prolethopora	Current census	14	2	1	1	1	1	0	0	20
	2008 census	12	2	0	0	1	1	0	0	12
	increase	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
	increase	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

estimation of microturbellarian and gastrotrich species diversity, but are at risk because of rapid habitat destruction and climate change. Wide-ranging European research programs on the freshwater animal biodiversity have been carried out in the past years (2000–2008) leading to the compilation of European and global databases of the known biodiversity at the time (Fauna.Europaea Project, see de Jong, 2014; FADA Freshwater Animal Diversity Assessment Project, see Balsamo et al., 2008). Increased species discovery should be a concerted effort with expanding and updating databases that consolidate existing and new taxonomic and biogeographic data. An important first step would be the development of regularly updated identification keys for freshwater rouphozoans. This could be part of a broader effort on freshwater meiofauna analogous to current efforts for marine meiofauna (Schmidt-Rhaesa, 2020). To accelerate biodiversity surveys of rouphozoans, protocols for animal collection, voucherizing, DNA extraction, DNA barcode marker selection, amplification, and sequencing should be adjusted to the upcoming and promising third-generation sequencing techniques (e.g., Nanopore).

A large impediment for future research on taxonomy, biogeography, and phylogeny of Rouphozoa (and all other Metazoa for that matter) is the implementation of the Nagoya Protocol (NP). Since October 2014, NP regulates all access to, and benefit sharing of, genetic resources worldwide. The protocol was designed to ensure fair use of countries' genetic resources, including the use of traditional knowledge. However, as logical and fair such legislation might

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

Dispersal abilities of freshwater gastrotrichs and microturbellarians and the relationship between dispersal and distribution have not been specifically investigated so far. The small size of gastrotrichs and microturbellarians and the absence of planktonic stages limit active dispersal of live individuals to short distances. Wind, running water, and more mobile

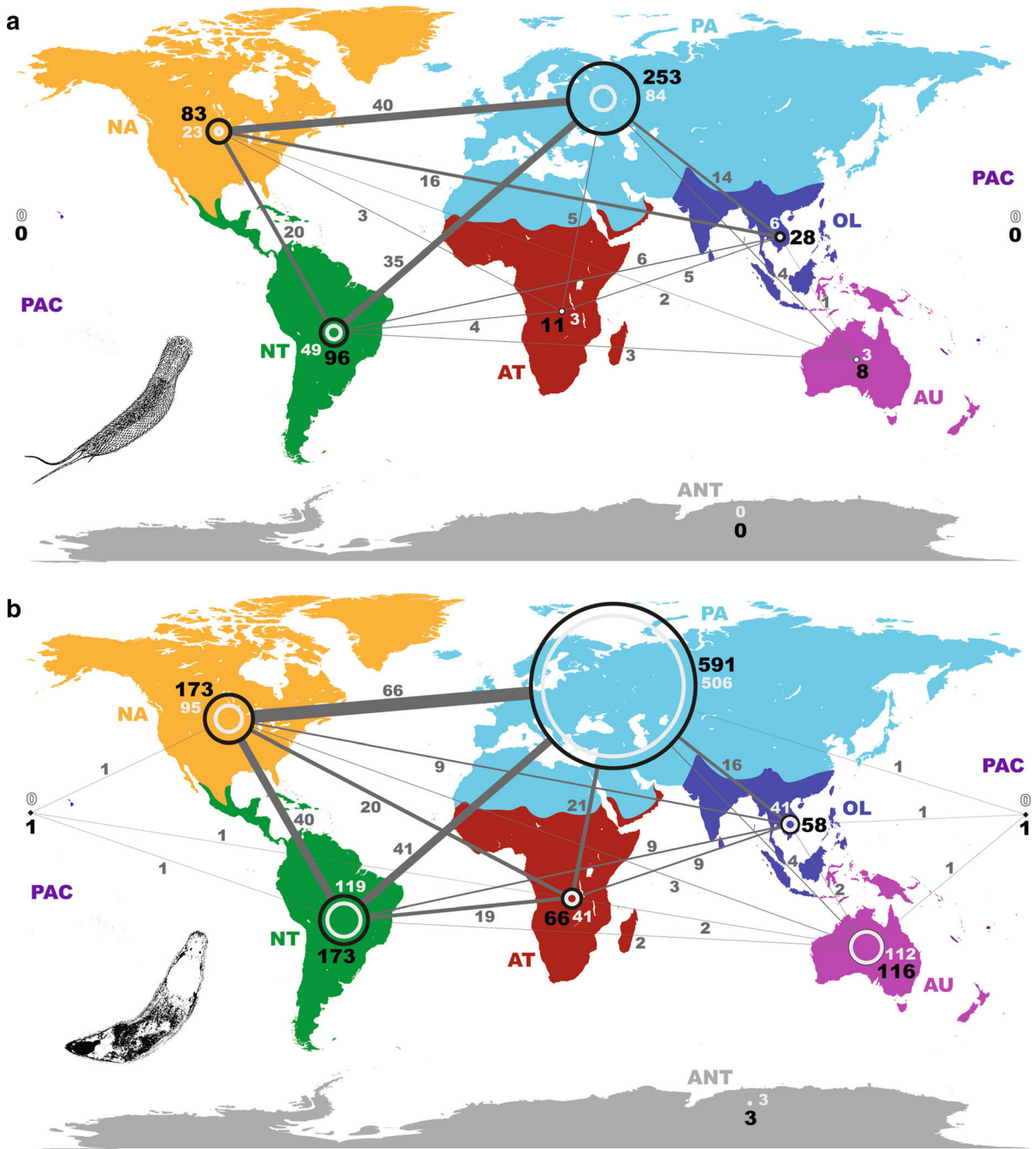


Fig. 9 Current species numbers for **a.** Gastrotricha and **b.** microturbellarians in different biogeographical regions of the world (black numbers and circles), including numbers of endemic species per region (light gray numbers and circles)

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

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

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

ballast waters, etc.) of gastrotrichs, microturbellarians, and many other aquatic micro-invertebrates is likely, but has not yet been the subject of specific studies (Artois et al., 2011). Future studies focusing on the spatial connectivity and gene flow of freshwater gastrotrich and microturbellarian populations are highly needed to support these assumptions. Cerca et al. (2018) have stressed the importance of including ecological and life-history traits, evolutionary history and cryptic speciation, metapopulation dynamics, as well as considering vicariant events and (ancient) dispersal routes on different geographic and temporal scales to explain current-day distribution of marine meiofauna. These are all important considerations to also elucidate recent distribution patterns of freshwater gastrotrichs and microturbellarians.

The task of untangling hidden diversity, spatial connectivity, and trophic networks in Rousphozoa will certainly fall to molecular methods. Metagenetic, genomic, and transcriptomic data—when combined and integrated with morphological and ecological data—can also provide new insights into additional properties and patterns such as niche differentiation, differential gene expression, genome duplication or reduction, character evolution, reproduction modes and traits related to sexual selection, origins of symbiotic interactions, co-evolution and host specificity, nutritional strategies, and life cycle modifications. The current lack of such integrated studies impedes our understanding on evolutionary processes within rousphozoans. Many closely related species of freshwater gastrotrichs and microturbellarians occur in sympatry suggesting some kind of ecological differentiation. However, non-ecological speciation in allopatry at some point in the past has been proposed for present-day sympatric organisms (Czekanski-Moir & Rundell, 2019). Species flocks of rhabdocoels in Lake Baikal are the product of spectacular speciation events, but the mechanisms behind these radiations are not known. Revealing these underlying processes remains challenging and will require holistic multi-evidence approaches employing new techniques in high-resolution microscopy and high-throughput sequencing.

Finally, there is still a valuable role for functional morphology, specifically, studies with a biomechanical approach. From the original analysis of the role of connective tissue in soft-bodied worms (Clark & Cowey, 1958), and subsequent refinements applied to

soft-tissue extensible structures more generally (Kier, 2010), biomechanical studies in Rousphozoa are rare: proboscis function in *Cheliplana* (Uyeno & Kier, 2010) and in Schizorhynchia more generally (Smith et al., 2015), and dynamics of duo-gland adhesion in marine microturbellaria (Wunderer et al., 2019). Accordingly, additional studies directed at a better understanding of rousphozoan biomechanics would provide a much richer context for the evolutionary and ecological work proposed above.

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