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Peer reviewed

Freshwater and limno-terrestrial meiofauna of the Massane Forest Reserve in the Eastern French Pyrenees

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SUMMARY

We report the results of a faunistic survey focused on freshwater and limno-terrestrial meiofauna to improve biodiversity knowledge in a protected area in the Eastern part of the French Pyrénées: the Massane Forest Reserve (336 ha). The survey provided 1187 occurrence records from 315 taxa (most resolved at species level), uploaded as a shared online dataset and made freely available in the Global Biodiversity Information Facility (GBIF). The highest number of occurrences and distinguishable species belong to Nematoda (775 occurrences, 172 taxa), followed by Rotifera (219 occurrences, 67 taxa), Platyhelminthes (85 occurrences, 32 taxa), Tardigrada (69 occurrences, 25 taxa), and Gastrotricha (39 occurrences, 19 taxa). A diversity of meiofaunal organisms was found, in large numbers, in all the screened samples: from stream biofilms and sediments to forest floor soils, mosses, and litter, to a broad range of tree-related micro-habitats associated with beech epixylic mosses and lichens, tree cavities, woodpecker breeding holes, bark pockets and fruiting bodies of saproxylic fungi. This survey makes the Massane forest one of the few protected areas of the world with an all-taxa biodiversity inventory including meiofaunal groups, which could serve as a standard to further consider those cryptic groups of tiny animals in forest conservation efforts.

INTRODUCTION

Micro-invertebrates (aka. “Meiofauna”, or “Hygrophilous mesofauna” in soils) are an abundant and diverse component of soil and freshwater ecosystems worldwide, but often only known through the lens of nematodes (e.g. Zullini 2014, Majdi et al. 2020, van den Hoogen et al. 2020). However, even free-living nematodes remain fairly less studied than, for example, any other freshwater soil or benthic macro-invertebrate group (Majdi & Traunspurger 2021). Except for the nematode *Caenorhabditis elegans* (Maupas 1899), which has a long-standing record of being a model in biology, and perhaps more recently, tardigrades, whose popularity has soared thanks to their easy-going, cuddly appearance and their extraordinary resistance abilities (Goldstein 2022), the rest of the meiofauna have so far failed to gain widespread visibility and charm. As a result, outside of a limited number of specialized scientists, there is little awareness for meiofaunal groups such as rotifers, gastrotrichs and microturbellarians and thus a severe lack of support to investigate those groups (Giere 2008; Mammola et al. 2023).

Yet, there is mounting evidence that meiofauna play an important role in ecosystems. For example, meiofauna stimulate important

ecosystem functions like nutrient remineralization and photosynthesis through their grazing or facilitative interactions with microbial mats (e.g. Traunspurger et al. 1997, Mathieu et al. 2007, Liu et al. 2015, Yeates & Coleman 2021). Rotifers, for example, are major actors of pelagic-benthic couplings, comparable with bivalves, when it comes to the filtration of planktonic particles in large rivers (Kathol et al. 2011). Worm-shaped meiofauna such as nematodes and microturbellarians are usually extremely abundant and feed on an impressive range of prey items from microbial mats to other meiofauna, and even parasitize insects, vertebrates and plants (Kolasa 2000, Majdi et al. 2021, Kreuzinger-Janik et al. 2022). Although mostly falling behind groups such as Nematoda, Copepoda or Platyhelminthes in terms of density, biomass and diversity (e.g. Strayer 1986), Gastrotricha may also reach quite significant densities in freshwater biotopes (Nesteruk 1996). Due to their selective microbivorous trophic behaviour, Gastrotricha are considered having a significant influence on ecosystem dynamics, and are hypothesised as a link between the microbial (bacterial) loop and higher trophic levels (Balsamo & Todaro 2002). Concerning food web connectivity, meiofaunal organisms are a qualitatively and quantitatively valuable resource for young fish, snails and

aquatic insects (Weber & Traunspurger 2015; Ptatscheck et al. 2020). In a context of biodiversity erosion, alteration of ecosystem functions, and collapse of trophic networks, it is urgent to inventory the baseline conditions of diversity in nature reserve areas. This helps to get a glimpse of the role of species in unaltered ecosystems, and thus further develop proactive protection measures in non-protected areas.

These standard conditions may be found in the Massane Forest Reserve (Travé 2000). This old-growth forest situated near the Mediterranean in the piedmont of the Pyrénées (l'Albera massif) has been classified as a UNESCO World Heritage in 2021. It was one of the most northerly glacial refugia during the Holocene in western Europe for populations of European beech (*Fagus sylvatica* L.) (Magri 2008), and it stands now near the southernmost range of this species, near its drought-tolerance limit. Furthermore, the forest is considered in 'free-evolution' (i.e. very sporadic land-use at least for the last 150 years, strict protection status for 50 years), meaning more practically that the forest has remained mostly untouched by the recent burst of human activities, although the forest still suffers from indirect consequences (climate change, aerosol pollution and invasive species). The Massane forest has been extensively studied by ecologists as a biodiversity hotspot, offering the unique opportunity to monitor the "near-natural" dynamics of wood decomposition and the immense diversity of animals, fungi and bacteria associated with this process (Travé et al. 1954, Nicolau-Guillaumet 1959, Dajoz 1966, Skubała 2008, Skubała & Marzec 2013). Except for mites which have been extensively surveyed in the 50's – 196 species reported from 350 samples by Travé (1963) – the other microscopic invertebrates have been little studied in the reserve. In a first note, Travé et al. (1954) reported 5 species of tardigrades and 19 species of free-living nematodes in mosses and lichens of the reserve, but this was almost 70 years ago and since that time no further inventory has been done on those groups, and neither a single

gastrotrich, nor a rotifer, nor a microturbellarian specimen has been described in the reserve so far.

The aim of this study is to describe the assemblages of nematodes, gastrotrichs, rotifers, tardigrades and microturbellarians in limno-terrestrial and freshwater habitats commonly found in the Massane forest and in the small stream flowing through the reserve. With this baseline knowledge we expect to better understand (1) the biogeography of limno-terrestrial meiofauna communities and the environmental and/or biological filters that may shape those assemblages, and (2) the ecological role of these animals in this particular location. Finally, we also expect to shed light on a hidden but substantial component of biodiversity that would be worth including in current efforts in order to provide holistic scientific and educational knowledge about biodiversity in the form of "All Taxa Biodiversity Inventories" in protected areas or biodiversity hotspots in France and other regions of the world (e.g. Nichols & Langdon 2007, Villemant et al. 2015, Lacoëuilhe et al. 2023).

MATERIALS AND METHODS

Samples were collected from 2021 to 2022 for nematodes, in April 2023 for the other groups, covering as many microhabitats as possible in the Massane Forest Reserve (Figures 1 and 2) to obtain a large diversity of freshwater and limno-terrestrial meiofauna. Freshwater samples covered running and standing waters, focusing on submerged mosses, wet sediments, layers of fallen leaves on the bottom of scours in the stream bed, macrophytes, epilithic biofilms, and plankton samples. Samples for limno-terrestrial meiofauna included moss and lichen patches on different substrates (e.g. rock, tree trunk, soil), leaf litter, dry soils, fungi, tree holes, ivy rootlets, and other available tree-related microhabitats (TreMs) as defined in Larrieu et al. (2018).

Samples were collected in the field, stored in plastic containers, envelopes, or ziplock bags, brought to the lab of the reserve, and extracted within a few days or prepared for long-term storage. Dry samples for limno-terrestrial meiofauna were stored for longer periods in envelopes and studied in the following few months. We also used anesthetics, fixatives and stains for optimal microscopic observation of taxonomically relevant structures. Taxonomic identifications were performed to species-level whenever possible, or to the nearest reliable rank

by the expert taxonomists involved in the faunistic survey. Identifications were based on characteristic morphological features in each group. Some distinguishable but undescribed morphologies so far were marked with numbers and specimen were vouchered until more thorough morphological description and molecular data in further publications. The applied methods for meiofauna extraction from the samples and identification under the microscope varied depending on the selected group.

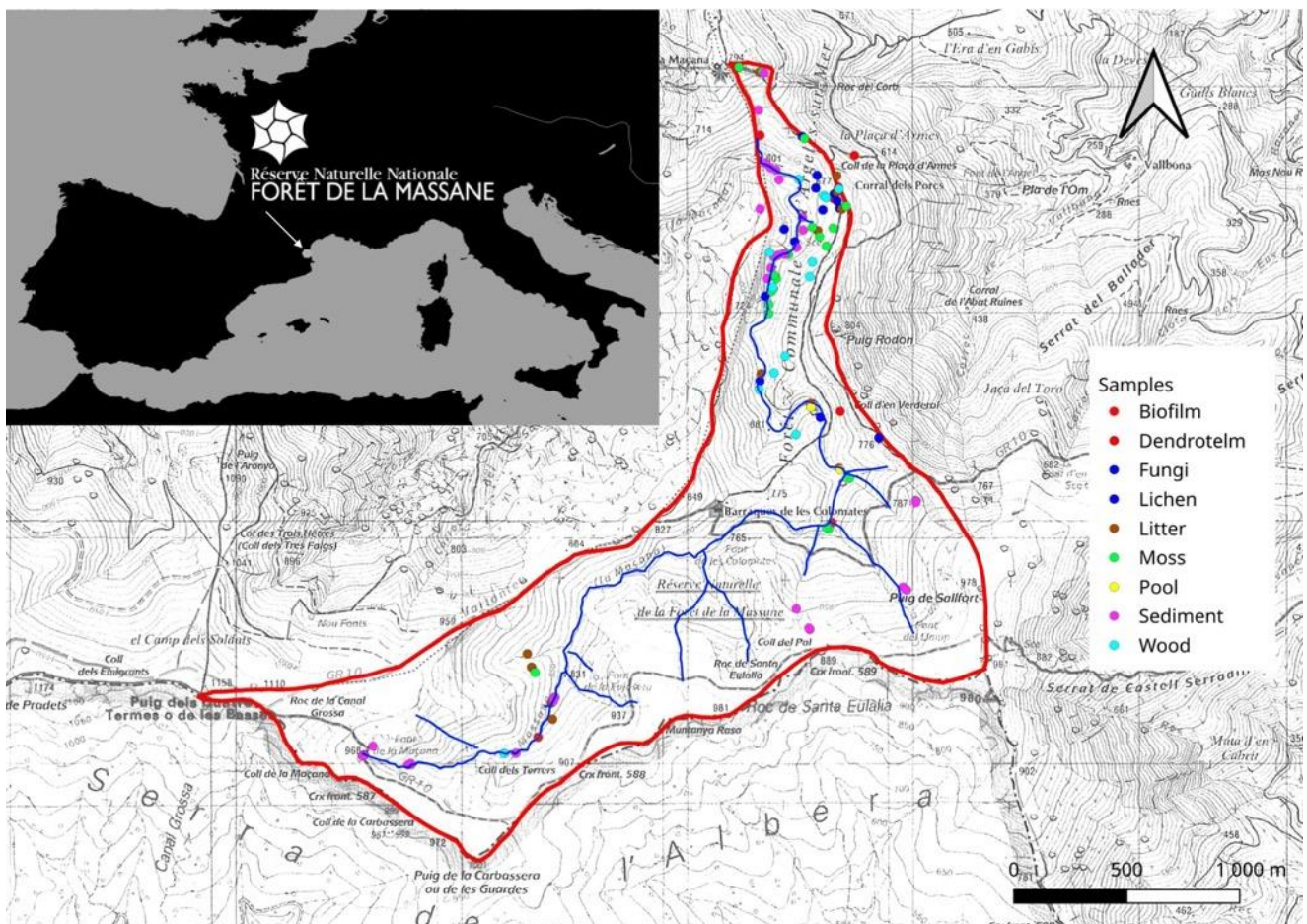


Figure 1. Localisation and type of samples collected. The red line represents the borders of the protected zone of the Massane Forest Reserve.



Figure 2. Illustration of field collection and laboratory extraction methods. A) Meiofauna collection using a plankton net in stream pools. B) Corer used to sample stream sediment. C) Material used to scrap stream epilithic biofilms. Sampling: D) dendrotelms bottom, E) epicormic shoots, F) collection of pyrenomycete-colonized bark. G) Extraction of TreM samples using "Baermann-funnels". H) sampling rotifers from aqueous phase of dendrotelms and I) from a stream pool. J) sampling litter and soils for flatworms at the Massane spring site. K) "Whitehead & Hemming's trays" used to extract flatworms from soil and litter samples.

For **Gastrotricha**, sedimentary substrata (sediment, organic debris, leaf litter etc.) were suspended in a bucket with ambient water in the field and initially pre-filtered through a coarse sieve in order to get rid of large particles and macrofauna. In a second step, this filtrate was sieved through a fine gauze (40 μm mesh size) and the captured meiofauna was rinsed into the

sampling jar using a squirt bottle filled with ambient water. Patches of aquatic mosses (*Fontinalis antipyretica* Hedw.), floating plants or submerged roots of ferns were hand-picked or sampled in stream pools with a plankton net (65 μm mesh size) mounted to a bar (Figure 2A). Qualitative sampling procedures mostly follow the methods described in Balsamo et al. (2014)

or Todaro et al. (2019). Back in the laboratory, subsamples of the filtrates were poured into petri dishes and screened under stereo microscopes using different illumination modes (see Rotifer section) and magnifications. Single gastrotrich specimens were picked from the petri dish using either a mouth pipette or a 2 μ L micropipette. For microscopic investigation and documentation, single specimens were placed on glass slides with a drop of ambient water and covered with a cover slip. Some specimens were anesthetized with few microliters of 0.25% Buccain (PUREN Pharma GmbH & Co. KG, München, Germany) prior to microscopic observation, alternatively the specimen was gently clamped between slide and coverslip by removing excess water carefully from the edge of the coverslip using a snippet of filter paper. Observation and live digital recording of specimens was carried out with a Olympus BX53 microscope equipped with high resolution objectives and differential interference contrast. An euromex HD-Ultra digital microscope camera VC.3036-HDS was adapted to the camera port of the microscope and every specimen was recorded with a series of still and video images. Taxonomic identification was mostly carried out after the expedition and was based on current monographs and taxonomic revisions (Balsamo 1983, Schwank 1990, Kisielewski 1991, Balsamo et al. 2014), original species descriptions and with the aid of the Gastrotricha World Portal (GWP, Todaro & Tongiorgi 2023).

For **Nematoda**, three categories of samples were distinguished: (1) The first 5-cm of stream sediments were collected using a PVC corer (diam. 9 cm, Figure 2B), and the sediment was fixed in a solution of 4% buffered formaldehyde. The nematodes were further extracted quantitatively from the sediment samples using a density-centrifugation procedure following Schenk & Traunspurger (2021). Briefly, the organic supernatant containing the nematodes was poured through 20 μ m meshes. After extraction, the organisms were stained with a few drops of Rose Bengal

and counted under a stereo-microscope (40x magnification). When available, the first 50 nematodes encountered while counting were removed from each sample, transferred to anhydrous glycerol and mounted on slides following the method of Seinhorst (1959). (2) Stream epilithic biofilms were collected by scraping with a toothbrush the superior face of three cobbles, washing off the detached biofilm over 20 μ m meshes (Figure 2C). Biofilm-dwelling organisms retained on meshes were preserved, stained, counted, and mounted on slides as in (1). (3) The nematodes colonizing TreMs associated with European beech were also sampled. After prior knowledge from field monitoring campaigns, we selected the most accessible and widespread TreM-types available in Massane forest after Larrieu et al.'s (2018) TreM typology. Approximately 100 g of TreM substrate was collected by hand, with a shovel, or with a spoon when they were easy to dislodge. In other cases, we used a knife, a small axe, or a handsaw to sample (Figure 2D, E, F). TreM samples were then slid in an airtight bag and transported to the laboratory within 24h. In the laboratory, we followed the protocol described by Travé et al. (1954): Briefly, the samples were placed in a fine gauze cloth (openings ca. 500 μ m), positioned on a steel grid placed in the upper part of a large funnel (largest diam. 40 cm, so-called “Baermann funnels”). The funnels were set with a water phase, expanding slightly over the steel grid, so the TreM samples in the gauze were slightly soaking at the interface with water. The funnels were positioned under a lamp (Figure 2G), so that small (usually lucifugous) hydrophilic organisms (such as nematodes, rotifers and tardigrades) were expected to quickly migrate in the water phase through the gauze's openings and steel grid, finally sinking into the bottom of the funnel. After 48 hours of migration – a period deemed long-enough to allow significant migration, and short enough to prevent substantial reproduction or predation within the funnel (Travé et al. 1954) – we poured the entire water phase through a 20 μ m sieve. The content of the sieve was preserved in 4% buffered formaldehyde and nematodes were

further stained with Rose Bengal, counted, and mounted on slides as in (1).

For **Rotifera**, lotic samples like *Fontinalis* moss patches growing in rocky riffles and cascades were directly collected into a screw-cap tube while submerged and further inspected in the laboratory. Water in dendrotelms and puddles was sampled by a hose coupled to a 50 mL syringe (Figure 2H). Water in stream pools was concentrated over 20 µm sieve (Figure 2I). Aquatic samples were kept in the dark at 5°C and processed within a week after their collection. Terrestrial samples (moss and lichen patches) were kept dry and inspected in the laboratory after rewetting within 2 months. The samples were screened at a stereomicroscope with magnification between 6x and 80x, using bright, oblique, and dark field to avoid biases in the description of species diversity, given differential abilities of different species to stick to the substrate particles when treated with various extraction techniques.

For **Platyhelminthes**, ca. 1 L of soil, river sediment, leaf litter, moss, woody detritus was scraped and handpicked in zip-lock bags (Figure 2J) and directly returned to the laboratory where they were stored at 5°C in the dark. For some waterlogged samples, we employed an oxygen-depletion method (overnight stagnation in a wide-mouthed glass jar) to drive larger microturbellaria to the surface where they could be handpicked and concentrated. For most samples however, microturbellarians were extracted following a modified version of the Whitehead & Hemming (1965) tray method (which coincidentally also proved an efficient extraction technique for all meiofaunal groups studied in this paper). Briefly, the sample was evenly spread onto a fine gauze tissue set onto a ~2 mm polypropylene sieve stacked within a seed sprouting tray filled with water to cover the surface of the substrate, and let to sit for up to 24 h (Figure 2K). After that, the water in the tray, containing minimal substrate, was poured on 20 and 62 µm meshes, and a squirt bottle was used to concentrate the contents into petri dishes, which were then

inspected for flatworms under a binocular. Specimens were further directly identified or wet-mounted on slides, semi-squeezing animals under a cover slip using tissue paper to wick away excess water. These were then microscopically observed in a Nikon Ni-U microscope equipped with DIC. Photos and/or videos of all specimens, emphasizing reproductive anatomy, were recorded as voucher data using a Nikon Digital Sight 10 microscope camera, and these were used to guide identification using primary literature and the Turbellarian Taxonomic Database Tyler et al (2023). Note that we use the term “microturbellaria” to refer to free-living Platyhelminthes (excluding Acoelomorpha) of microscopic size - a non-monophyletic group, which is nonetheless coherent from an ecological perspective, and distinct as well from their parasitic relatives in terms of the communities of researchers studying these animals.

For **Tardigrada**, samples of mosses and lichens were examined using standard methods as described in Stec et al. (2015). All specimens were mounted on microscope slides in a small drop of Hoyer’s medium and secured with a cover slip, following the protocol by Morek et al. (2016). Slides were then dried for five to seven days at 60 °C. Dried slides were sealed with a transparent nail polish and examined under an Leica DMLB light microscope with phase contrast, associated with digital camera. Taxonomic identification was carried out with the use of taxonomic keys and recent taxonomic revisions (Maucci (1986), Pilato & Binda (2010), Kaczmarek & Michalczyk (2017), Gąsiorek et al. (2019), Stec (2022)), and original species descriptions. All slides are deposited in the Tardigrada collection at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.

In cases when a definite species identification was not possible for now, we follow the suggestions of an ‘open nomenclature’ as compiled by Bengtson (1988).

This was either necessary if a determination was provisional, e. g. due to missing information or not optimally preserved (documented) specimens (indicated with a “cf.” between genus name and species name), or if a presumed new species was identified (indicated with a “aff.” between genus name and name of the suspected sister species). For many turbellarian in particular, morpho-species could be clearly recognized but not taxonomically assigned beyond subfamily level, owing to the problematic state of taxonomic classification of limno-terrestrial “Typhloplanidae” and “Protoplanellinae” Houben et al. (2022). They were thus assigned a provisional species “number” (based on the voucher videos) until substantial revision of the taxonomy would allow a more coherent classification of those putative species into valid genera and species.

RESULTS

Summary statistics

The dataset of freshwater and limno-terrestrial meiofauna from the Massane Reserve and surrounding areas was built starting from 150 samples collected during several surveys in the area between 2021 and 2023, covering diverse organismic groups (Figures 3-7) from different habitats.

The dataset includes 1187 occurrence records of 315 distinct meiofaunal species collected at the Massane Forest Reserve: 219 have been confidently diagnosed at species-level, 75 at genus-level and 21 at family-level. Of the species reported in the current dataset, 41 (13%) are Rotifera Bdelloidea (157 occurrences), 26 (8%) are Rotifera Monogononta (62 occurrences), 19 (6%) are Gastrotricha (39 occurrences), 25 (7.9%) are Tardigrada (69 occurrences), 172 (54%) are Nematoda (775 occurrences) and 32 (10%) are Platyhelminths-microtubellaria (85 occurrences).

The dataset is freely available as a supporting information file and from the Global Biodiversity Information Facility, GBIF (<https://doi.org/10.15468/96fy2a>).

Dataset description

The data were structured based on the Darwin Core standard (Wieczorek et al. 2012). The dataset is structured to report every record of a species from each sample from the Massane reserve. Information on species name, authorship, systematic hierarchy, coordinates, elevation, date of sampling, and habitat are recorded in the GBIF portal.

Object name: Massane meiofauna records.

Dataset citation: Massane meiofauna records.

Character encoding: UTF-8.

Format name: csv.

Format version: 1.0.

Distribution (permanent link): GBIF: <https://www.gbif.org/dataset/7b80c4e1-b2e1-486b-9403-cf4be12fd0ce>

Date of creation: 15 May 2023.

Date of last revision: 18 October 2023.

Date of publication: 19 January 2024

Update policy: The dataset at GBIF cannot be updated, and it represents the list of species that were identified during the workshop on meiofauna at the Massane.

Language: English.

Licence of use: both access and use are free to any user. The authors would appreciate users providing a link to the original dataset in GBIF (OSF: <https://doi.org/10.15468/96fy2a>) or when researchers use the data to cite the present paper, or Majdi et al. (2024). Stakeholders interested in additional information can contact authors via the contact information provided in the metadata.

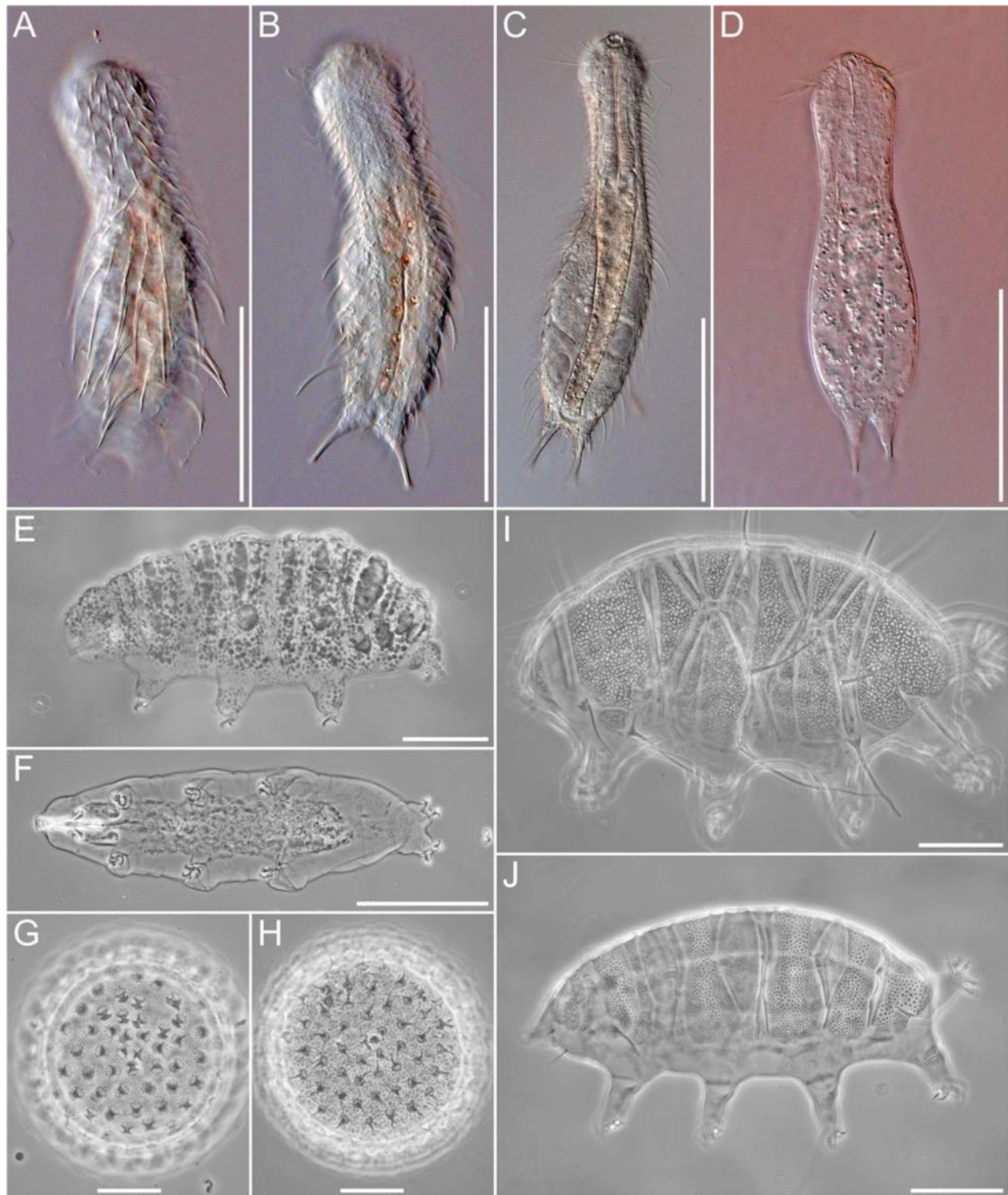


Figure 3. Examples of meiofaunal groups occurring at the Massane Forest Reserve: Gastrotricha (A-D) and Tardigrada (E-J). A-B: *Chaetonotus (Hystricochaetonotu) persetosus* Zelinka, 1889 in dorsal and ventral view. C: *Chaetonotus (Chaetonotus) maximus* Ehrenberg, 1838. D: *Ichthydium (Ichthydium) palustre* Kisielowski, 1981, both median views. E: *Fractonotus verrucosus* (Richters, 1900), lateral view F: *Macrobiotus macrocalix* Bertolani & Rebecchi, 1993, ventral view. G: sculptured egg of *Macrobiotus* cf. *terminalis*. H: sculptured egg of *Mesobiotus* cf. *binieki*. I: *Echiniscus quadrispinosus* Richters, 1902. J: *Pseudechiniscus* sp. 1, both lateral views. Scale bars: A-B, D: 50 μ m; C, F: 100 μ m; E, I-J: 40 μ m; G-H: 20 μ m.

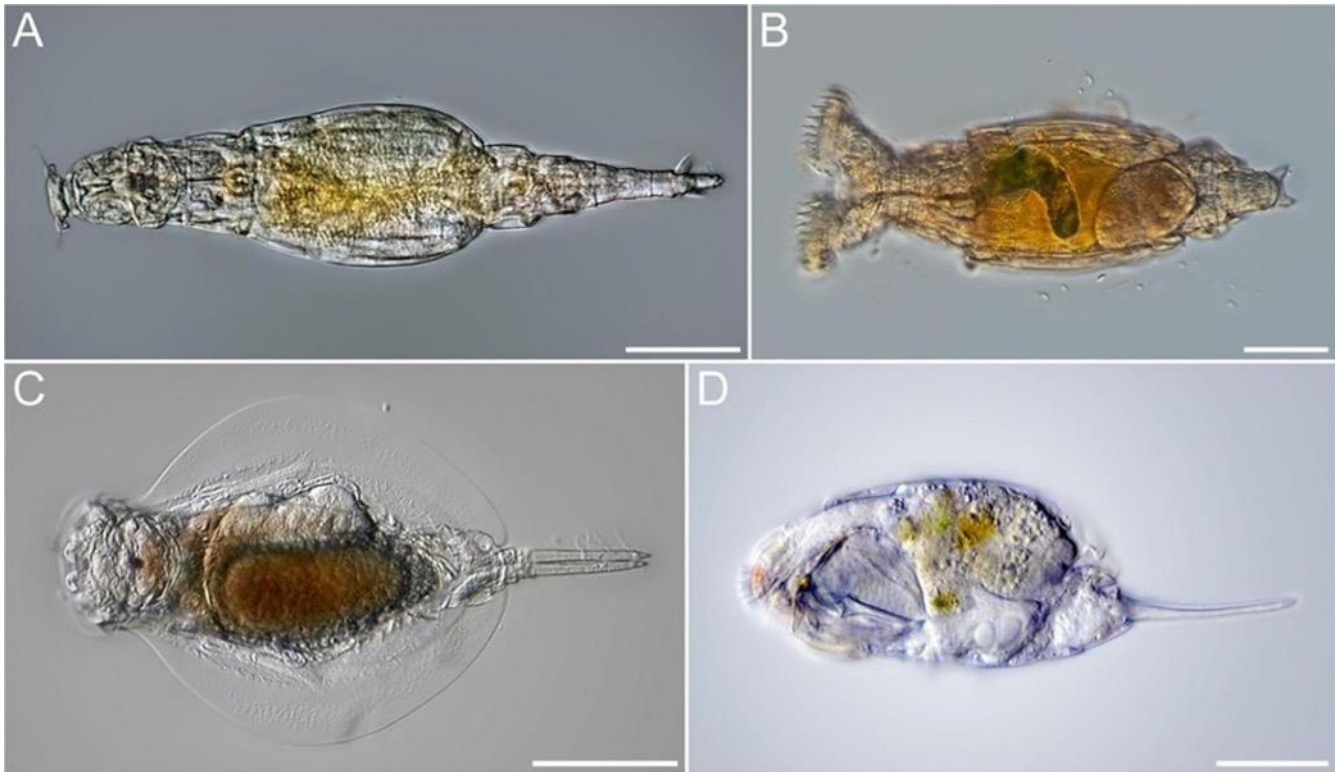
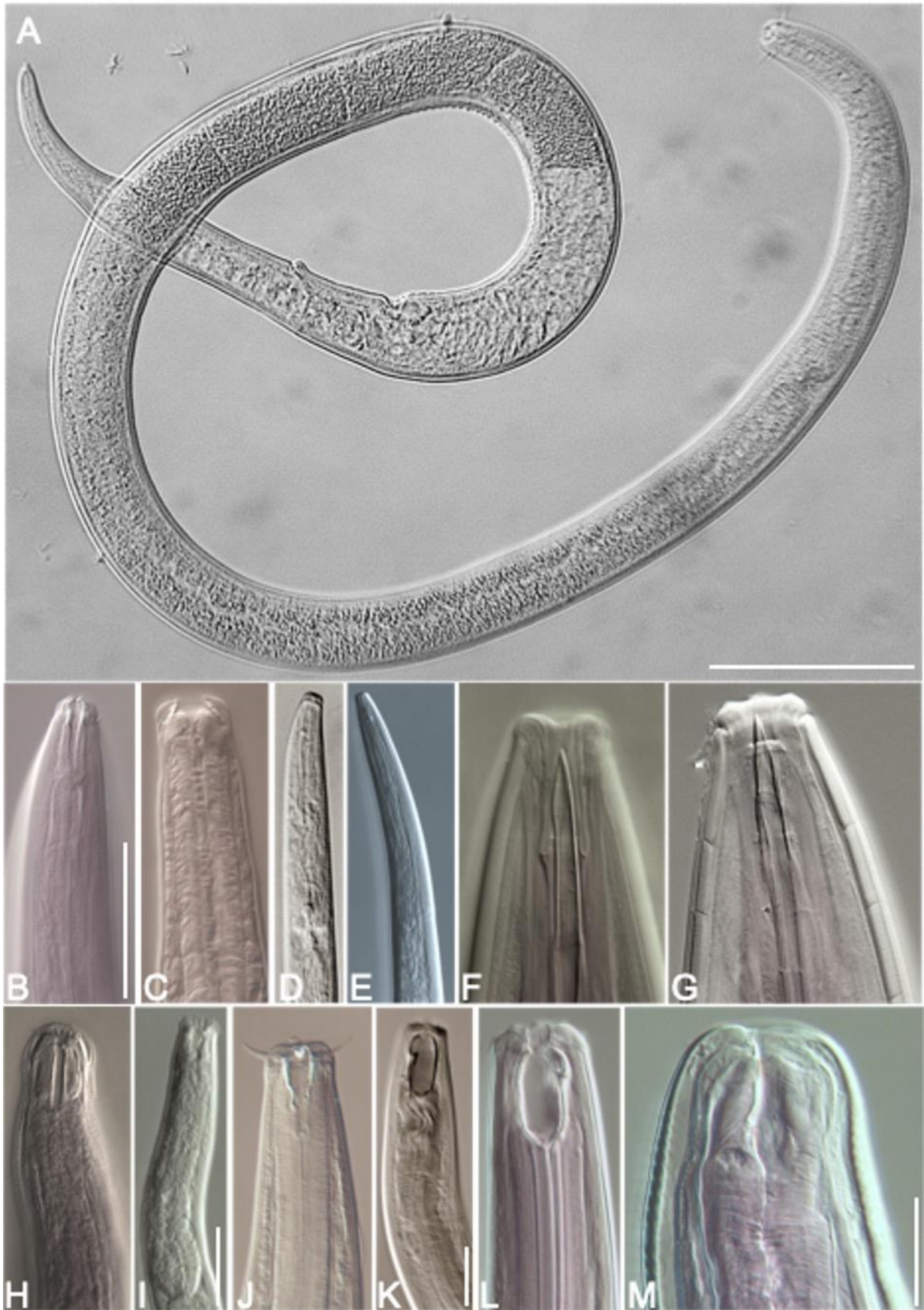


Figure 4. Rotifera Bdelloidea (A-B) and Rotifera Monogononta (C-D). A: *Adineta barbata* Janson 1893, dorsal view. B: *Mniobia cf. magna* Plate 1889, dorsal view. C: *Euchlanis triquetra* Ehrenberg 1838, dorsal view. D: *Cephalodella* sp., lateral view. Scale bars: A-B: 50 μm ; C: 100 μm ; D: 40 μm . images A-C by courtesy of M. Plewka.

Figure 5. Common Nematoda observed in the Massane Forest Reserve. Cephalic views illustrating the diversity of morphologies, feeding types and some notes on habitat and food preferences. A–C: *Geomonhystera* sp., common on tree bark, 'deposit-feeder' (mostly microbial diet: bacterial mats, tiny detritic particles, protozoa and algae). B: *Plectus* sp., in mosses and lichens, 'deposit-feeder'. C: *Eumonhystera* sp. in stream sediments/ biofilms, 'deposit-feeder'. D: *Aphelenchoides* sp. in soils and fungal fruiting bodies, 'suction-feeder' on fungal hyphae. E: *Tylenchus* sp. in soils, 'suction-feeder' (ectoparasitism) on plant rhizules. F: *Dorylaimus* sp., common in mosses and freshwater habitats, 'suction-feeder' omnivore (algae, large protozoans and other nematodes and meiofauna). G: *Aporcelaimellus* sp., freshwater, 'suction-feeder' omnivore. H: *Ethmolaimus* sp., algal biofilms and superficial stream sediment, 'epistrate-feeder' (mostly algal diet: diatoms, green algae; but also protozoans). I: *Achromadora* sp., sediment and biofilms, 'epistrate-feeder'. J: *Semitobrillus* sp., freshwater sediment, 'chewer' omnivore (bacterial mats, algae, protozoans and other nematodes and meiofauna). K: *Mononchus* sp., common in soils, 'chewer' predator (mostly carnivorous diet: other nematodes and small meiofauna). L: *Prionchulus* sp., mosses and lichens, 'chewer' predator. M: *Tripyla* sp., freshwater, 'chewer' predator. Scale bars: A: 100 μm ; B–M : 20 μm . B–H: Same scale. I–J: Same scale. K–L: Same scale.



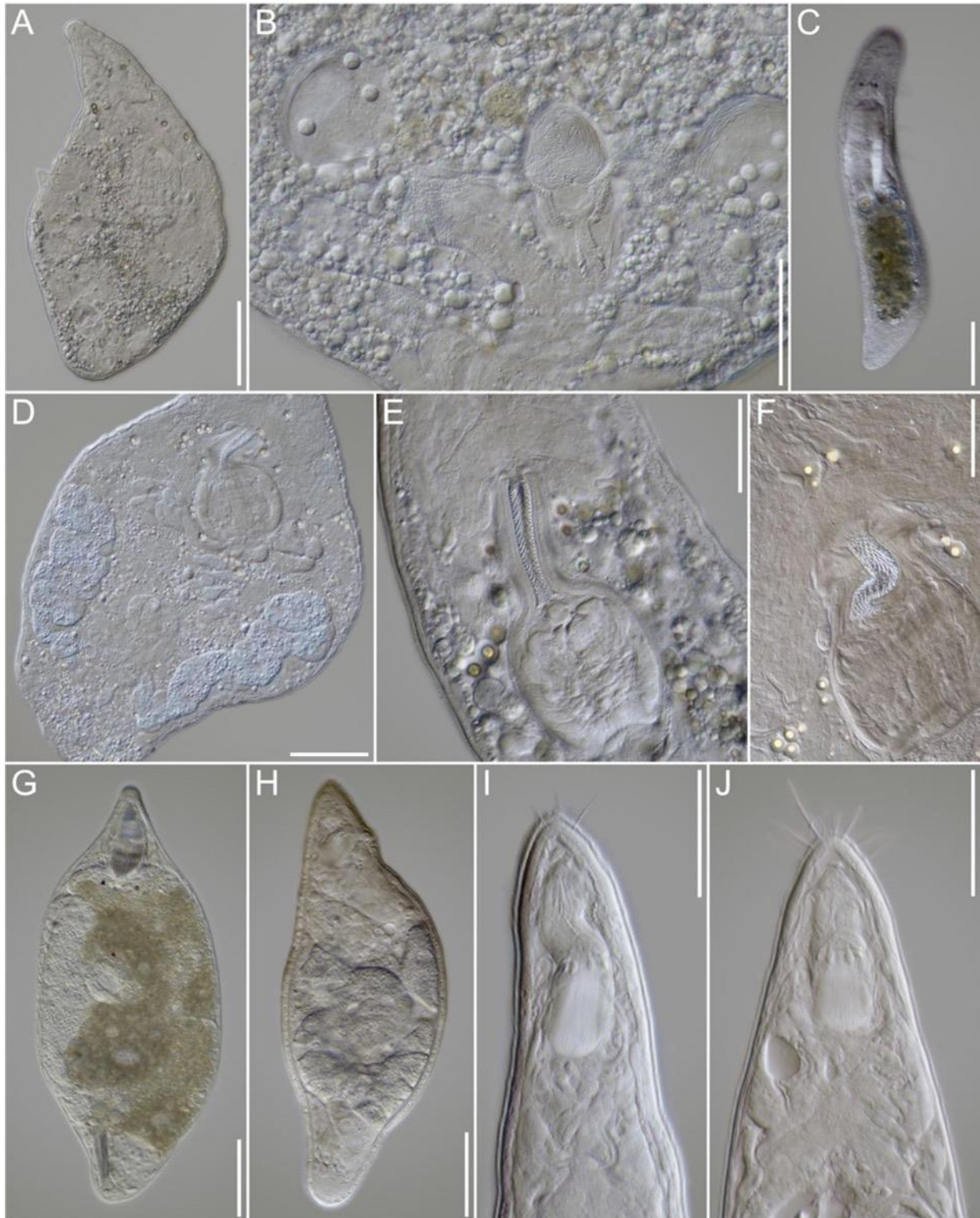


Figure 6. Platyhelminthes observed in the Massane Forest Reserve. A-B: *Acrochordonoposthia vandeputae* Houben, Proesmans & Artois, 2014, habitus and detail. C: A freshwater member of Neodallyelida, putatively *Kirgisella* sp., habitus, dorsal view. D-F: *Carcharodopharynx* cf. *arcanus* (Reisinger, 1924) Poche, 1926, habitus and details of the pharynx. G: Member of the *Gyratrix hermaphroditus* Ehrenberg, 1831. species cluster, habitus, dorsal view. H-J: *Ethmorhynchus youngi* Kolasa, 1977, habitus and details of the anterior end at different focal levels. Scale bars: A, D: 100 μ m; B, F, J: 50 μ m; C, I: 60 μ m; E: 40 μ m; G: 200 μ m; H: 120 μ m.

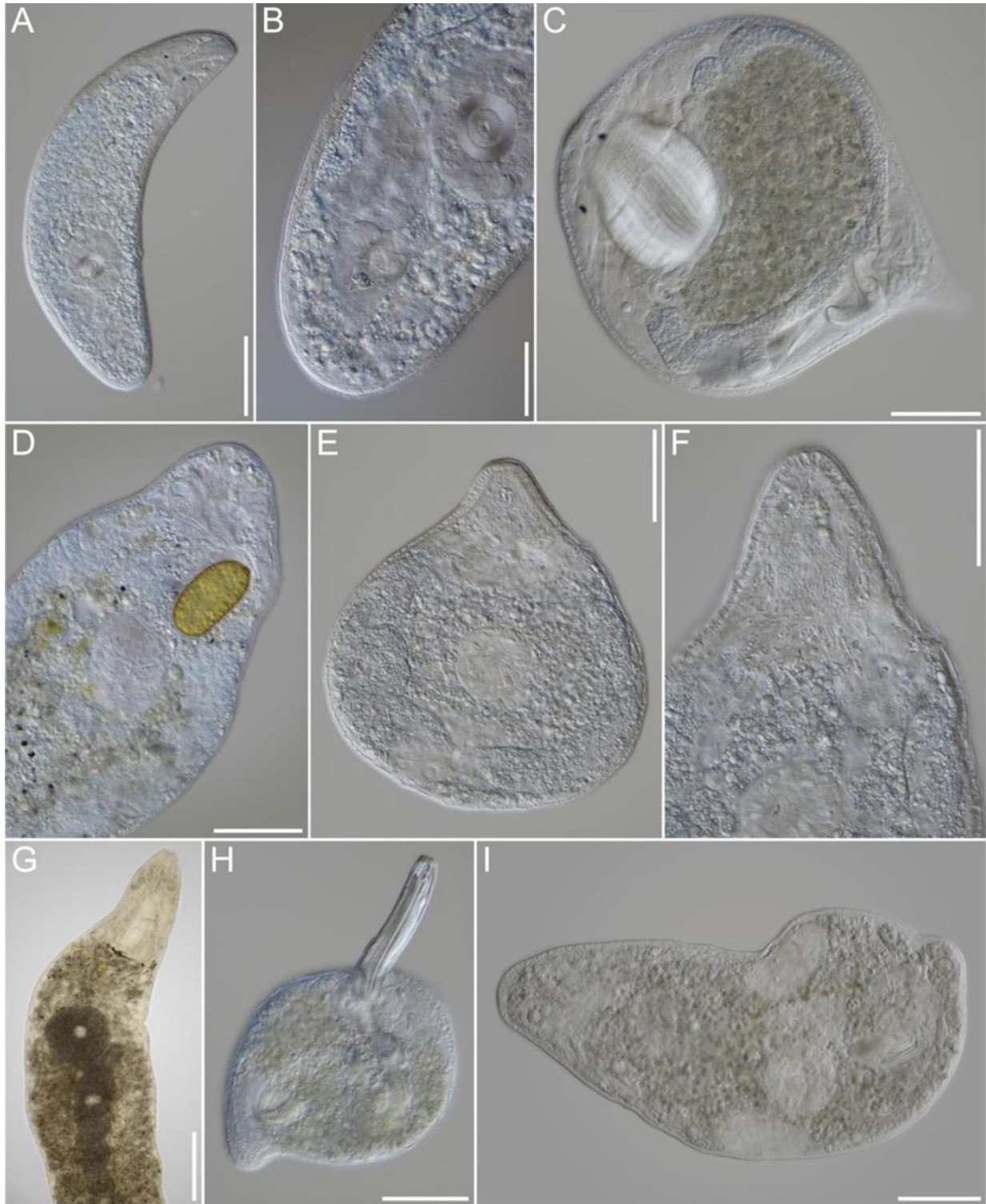


Figure 7. Platyhelminthes observed in the Massane Forest Reserve. A-B: *Limnoruanis romanae* Kolasa 1977, habitus, dorsal view, and detail of pharynx and reproductive tract. C: *Microdalyellia circulobursalis* (Ruebush, 1937) Ruebush & Hayes, 1939, habitus. D: *Opisthomum fuscum* Weise 1942, anterior body end. E-F: *Pseudobockia limicola* Kolasa 1981, habitus and detail of the anterior body end. G: *Prorhynchus stagnalis* Schultze 1851, anterior end. H: *Protoplanella macrorhabditophora* An der Lan, 1955, specimen ingesting a predatory nematode (mononchid) 'like a spaghetti'. I: unidentified species of terrestrial Protoplanellinae, habitus. Scale bars: A, C, E-F, H-I: 100 μ m; B: 60 μ m; D: 120 μ m; G: 240 μ m.

Metadata language: English.

Metadata managers: Nabil Majdi (nabil.majdi@espaces-naturels.fr), Diego Fontaneto (diego.fontaneto@cnr.it), Lyudmila Kamburska (lyudmila.kamburska@irsa.cnr.it).

Management details

Project title: A georeferenced dataset of meiofauna from the Massane Forest Reserve.

Database managers: Nabil Majdi, Lyudmila Kamburska.

Temporal coverage: the present dataset refers to all the records of meiofauna collected and identified in 2022 and 2023, but mostly during a workshop in April-May 2023, organised by the Massane Forest Reserve.

Record basis: Observation of individuals directly collected from the field.

IT specialists: Lyudmila Kamburska.

Funding: The research on tardigrades was supported by the National Science Centre, Poland (Grant no. 2022/44/C/NZ8/00050 to DS). The research on bdelloid rotifers was supported by the National Biodiversity Future Center of Italy. The research on nematodes was supported by the Hermès Foundation and PatriNat (OFB-MNHN-CNRS-IRD).

Geographic and ecological coverage

Study area: Sampling sites were distributed to cover the protected area of the Massane National Nature Reserve, in South France, and its surrounding areas. The reserve protects a large forest dominated by European beech, covering 336 hectares at the southern limit of distribution of the species (Garrigue et al. 2008). The forest has evolved naturally for over 150 years and the reserve is one of the best inventoried protected areas in Europe (Garrigue 2016).

Bounding box: min Longitude: 3.0310236 – min Latitude: 42.4917080 – max Longitude:

3.0366200 – max Latitude: 42.4981389. The data are georeferenced according to WGS 84.

Elevational gradient: Samples covered a gradient of elevation between 556 and 973 m asl, with a median of 725 m and average \pm standard deviation of 751 ± 116 m.

Sampling design: The general strategy was to try to obtain samples from all the types of habitat where freshwater and limno-terrestrial meiofauna can be found, covering habitats within the Massane reserve.

Habitat type: Substrates for freshwater habitats from where meiofauna was extracted included submerged mosses in running and standing waters, wet sediments, macrophytes, periphyton, biofilms, and plankton samples. Substrates for limno-terrestrial species included moss and lichen patches on different substrates, leaf litter, dry soils, fungi, phytotelmata, and other tree-related microhabitats associated with beech as defined in Larrieu et al. (2018).

Biogeographic region: Western Palearctic region, between Pyrenean and Mediterranean areas.

Country: France, Municipality of Argelès s/Mer.

Quality control for geographic data: Georeferenced data and elevation were obtained directly in the field using various GPS tools. Quality control was performed using Google maps identification of sites. Geographic coordinate format and absence of ASCII anomalous characters in the dataset were additionally controlled.

Taxonomic coverage

General description: The dataset covers only animals considered as meiofauna, defined as microscopic invertebrates, belonging to organism groups Gastrotricha, Nematoda, Platyhelminthes, Rotifera, and Tardigrada (Examples of specimen in Figures 3-7). Arthropoda (e.g. Acari and Copepoda) and meiofauna-sized single-celled eukaryotes were excluded owing to lack of available experts.

Taxonomic ranks: All identified organisms during the survey, with data from variety and subspecies levels to species, genus, and family rank were included in the dataset.

Taxonomic methods: All reported names are provided according to the currently (August 2023) most updated nomenclature of WoRMS (Horton et al. 2017; WoRMS 2023) and checked against the taxonomic backbone of GBIF (GBIF Secretariat 2023). For Rotifera, the rotifer List of Available Names, LAN (Segers et al. 2012), was used for all scientific names published before the year 2000. For Gastrotricha, validity of generic, sub-generic and species names was checked against Balsamo et al. (2009) and Todaro & Tongiorgi (2023).

Group specialists: for Gastrotricha, Thiago Quintao Araujo, Nicolas Bekkouche, Alexander Kieneke, Axell Kou Minowa; for Nematoda, Nabil Majdi, Walter Traunspurger; for Platyhelminthes, Christopher Laumer; for Rotifera Monogononta, Thiago Quintao Araujo; for Rotifera Bdelloidea, Diego Fontaneto; for Tardigrada, Daniel Stec.

Quality control for taxonomic data: Species identification was performed by taxonomic experts involved in the project. Nomenclature validation and cleaning were based on WoRMS (Horton et al. 2017; WoRMS 2023), on Tyler et al.'s database (2023), and on the GBIF taxonomic backbone (GBIF Backbone Taxonomy 2023).

Taxonomic remarks: All species of Gastrotricha found in the various habitats of the Massane belong to the sub-order Paucitubulatina. A comprehensive taxonomic discussion of every encountered gastrotrich species along with morphometric data will be provided in the course of a subsequent publication.

DISCUSSION

The main objective of this project was to inventory the species of gastrotrichs, nematodes, rotifers, tardigrades and microturbellarians found in limno-terrestrial habitats of the

Massane Forest Reserve (eastern Pyrenees). The meiofauna (315 species) collectively represents a small proportion (ca. 3%) of inventoried Massane's biodiversity (total of ca. 10,000 species, half of it being insects), but this minor contribution makes sense since this is the first inventory entirely dedicated to limno-terrestrial meiofauna, and we only considered a subset of the habitats available in the reserve. In contrast, insects have been consistently sampled for decades in a much larger range of habitats. Moreover, meiofauna are often composed of highly diverse clusters of cryptic species (Schenk & Fontaneto 2020), and our morphology-based inventory may therefore miss substantial real diversity present. Nevertheless, the results of this first meiofauna survey are encouraging, ending up with a substantial diversity of almost one species per Ha, some of which being new to science, and for the most part new records for continental France.

In terms of contribution to the French fauna, the present meiofauna inventory alone reported 75.9% of the number of bdelloids species previously reported in French inland waters, 36.6% of monogononts, 47.5% of gastrotrichs, 36.2% of the tardigrades, 35% of the nematodes, and 110.3% of limno-terrestrial microturbellarians (Table 1). Meaning that there are currently more microturbellarian species described from the Massane Forest Reserve than those known for the rest of continental France. These results further stress the existence of an enormous gap of knowledge concerning the biodiversity and distribution of meiofaunal groups (Giere & Schratzberger 2023), even in regions relatively well studied like the Mediterranean. It is not surprising to unravel remarkable assemblages and potentially new species when investigating poorly documented groups of organisms dwelling a diversity of forest micro-habitats. Indeed, the meiofauna living below- or aboveground, colonizing any tree crevice, or in any water body holds immense promises for discovery of new species, cognizance of biodiversity patterns and better understanding of ecosystem functioning, and

thus deserves more consideration in current efforts to develop “All Taxa Biodiversity Inventories” (Villemant et al. 2015).

No recent checklist of bdelloid rotifers exists for France. According to FaunaEuropaea (<https://fauna-eu.org/>, Jong et al. 2014), 54 species of bdelloids are found in continental France. Our survey confirmed 19 of them and added another 22 species, meaning that 28.9% of the known freshwater bdelloids from France are

so far only known from the Massane Forest Reserve. No recent checklist of monogonont rotifers has been done for France. To date, 71 species of monogononts were reported for France (<https://fauna-eu.org/>, Jong et al. 2014) and our survey confirmed three of them and potentially adds up to 20 species to France territory. If those 20 species are confirmed as new to France, Massane Forest reserve will house 22.2% of French freshwater monogononts.

Table 1. Comparison of the number of non-marine species reported per group in the World (according to WoRMS (2023) unless stated otherwise), in France (before this study, from multiple references/datasets) and in the Massane Forest Reserve (this study).

| Organism group | Number of species | | | % Number of species | |
|---------------------|-------------------|------------------|---------|---------------------|--------------------|
| | World | France | Massane | France vs. World | Massane vs. France |
| Nematoda | 7275 | 491 ^a | 172 | 6.7 | 35.0 |
| Bdelloid rotifers | 460 ^b | 54 ^c | 41 | 11.7 | 75.9 |
| Monogonont rotifers | 1570 ^b | 71 ^c | 26 | 4.5 | 36.6 |
| Gastrotricha | 361 | 40 ^d | 19 | 11.1 | 47.5 |
| Tardigrada | 819 | 69 | 25 | 8.4 | 36.2 |
| Microturbellarians | 1017 ^e | 29 ^f | 32 | 2.8 | 110.3 |

a: Forest nematofauna after TaxRef v13.0. b: Relatively little information about bdelloids in WoRMS database, global diversity data after Segers (2008). c: After FaunaEuropea database. d: Checklist of freshwater gastrotrichs in France and overseas territories (d'Hondt 2019). e: Balsamo et al. (2020). f: WoRMS database distribution tool results for continental France, filtering out exclusively marine species, and applying expert familiarity with these (WoRMS has all sorts of invalid records and errors in it for flatworms).

According to a recent checklist of species records of Gastrotricha from France including Corsica and the overseas territories, there are currently 40 species reported in French inland water biotopes, all belonging to the sub-order Paucitubulatina (d'Hondt 2019). Six species found in the Massane were already known from France, however, mostly from other regions: *Lepidodermella squamata* (Dujardin, 1841), *Heterolepidoderma ocellatum* (Metschnikoff, 1865), *Chaetonotus (Hystricochaetonotus) hystrix* Metschnikoff, 1865, and *Lepidochaetus zelinkai* (Grünspan, 1908). Two of these six species, i.e. *Chaetonotus maximus* Ehrenberg, 1838, and *Chaetonotus larius* (Müller, 1773), were additionally already reported from the

central Pyrenees (Lac de Lourdes) almost a century ago by Schodduyn (1925). Four species we collected in the Massane Reserve represent new species records for France: *Chaetonotus (Hystricochaetonotus) peretosus* Zelinka, 1889, *Chaetonotus (Primochaetus) fruticosus* Martin, 1981 (currently regarded as 'species inquirenda', however, validity of the species is highly likely and will be discussed in a subsequent publication), *Chaetonotus (Chaetonotus) laroides* Marcolongo, 1910, and *Ichthyidium (Ichthyidium) palustre* Kisielewski, 1981. The identification of the latter two species is highly likely although a little doubt remains due to individuals not being optimally preserved. However, in the case of *I. (I.) palustre*, we can

safely exclude all other similar species such as *I. (I.) podura* (much shorter adhesive tubes), or *I. (I.) maximum* (much larger with a body length of up to 258 µm). *C. (C.) laroides* can best be distinguished from its most similar congeneric species *C. (C.) maximus* by the different ventral scale pattern of the pharyngeal region (rounded keeled scales versus rectangular plates). Concerning all taxonomic decisions made for compiling the species list presented here, we need to refer to an upcoming publication that will include detailed taxonomic discussions for each species. A further three species from the Massane Reserve are both new species records for France and for science: *Lepidodermella* aff. *zelinkai* (Konsuloff, 1914), *Chaetonotus* (*Chaetonotus*) aff. *microchaetus* Preobrajenskaja, 1926, and *C. (C.)* aff. *multispinosus* Grünspan, 1908. While the single recorded specimen of the former species is not suitable for a proper taxonomic description, we documented several individuals of the latter two new species and the comprehensive descriptions will be provided in the course of a subsequent publication. We need to mention that the systematics especially of the family Chaetonotidae is a matter of current research (e.g. Kolicka et al. 2020) and hence there are currently changes and some controversy concerning the taxonomy and nomenclature of certain groups. This is also relevant to some species discovered in the Massane Forest area and reported in the checklist of Balsamo et al. (2009) regarding the subgenus *Hystricochaetonotus* as a synonym of the subgenus *Chaetonotus sensu lato* and furthermore claiming that the genus *Lepidochaetus* should be transposed to subgenus rank. However, according to the systematics presented on the GWP (Todaro & Tongiorgi 2023), *Hystricochaetonotus* is still considered a subgenus of genus *Chaetonotus* and *Lepidochaetus* still considered a genus rather than a subgenus. Furthermore, recent comprehensive molecular phylogenetic studies demonstrate the monophyly of *Lepidochaetus*, being quite separate from other species of *Chaetonotus* (Križanová & Vd'ačný 2021,

Križanová & Vd'ačný 2022) and a monophyletic *Hystricochaetonotus* (Križanová & Vd'ačný 2022) each time with a maximum statistic node support. In the present publication we therefore follow the current taxonomy according to the GWP with, a subgenus status for *Hystricochaetonotus* and a genus status for *Lepidochaetus*. Wrapping up, in the course of the current study we have been able to add seven species of freshwater Gastrotricha from the Paucitubulatina to the French fauna. In other words, almost 15% of the known freshwater gastrotrichs from France are so far only known from the Massane Reserve area. Until 2020, a total number of 253 freshwater species of Gastrotricha were known to occur in the whole Palearctic zoogeographic region (Balsamo et al. 2020). The version of FaunaEuropaea database (<https://fauna-eu.org/>, Jong et al. 2014) currently lists 215 species of freshwater dwelling Gastrotricha, while by the year 2015 already 224 species were known to occur in Europe (Balsamo et al. 2015). Recently, further new species were described from freshwater biotopes in central Europe (e.g. Križanová & Vd'ačný 2021, Križanová & Vd'ačný 2022) and therefore there may be around 260 species recorded from this continent. Compared to this number, 47 species records for the whole of France most likely do not reflect the true diversity present. The comparably limited effort for estimating the meiofauna diversity of the Massane already increased the number of species records considerably (this study). We expect that further surveys even again at the Massane reserve, but focussing on different seasons and further microhabitats, will yield further species that are currently not recorded yet.

The present Tardigrade survey based on 28 samples resulted in the detection of 25 tardigrade species (out of 604 specimen and 74 eggs). Five of them were confidently identified at the species level: *Echiniscus merokensis* Richters, 1904, *Echiniscus quadrispinosus* Richters, 1902, *Fractonotus verrucosus* (Richters, 1900), *Macrobotus hufelandi* C.A.S. Schultze, 1834, and *Macrobotus macrocalix*

Bertolani & Rebecchi, 1993. Due to vague descriptions of some nominal tardigrade species and lack of integrative analyses on the recovered specimens, the remaining specimens could not be precisely identified. Several samples contained specimens of the *Echiniscus blumi-canadensis* complex and they cannot be split into any potential species at this moment due to the high morphological variability and very blurry species boundaries. Furthermore, we discovered also an extremely rare tardigrade, namely *Fractonotus verrucosus*, which only recently was re-examined and redefined (Gąsiorek et al. 2019). Overall, our survey found five times more tardigrade species than the first survey conducted seventy years ago by Travé et al. (1954) in terrestrial and aquatic mosses of the Massane forest. Travé et al. (1954) observed five tardigrade species: *M. hufelandi*, *Macrobotus schultzei* Greeff, 1866 (which is now considered as synonym of *M. hufelandi* according to Marcus (1928)), *Macrobotus echinogenitus* Richters, 1903 (doubtful species according to Stec et al. (2020)), *Dianeia sattleri* (Richters, 1902) and *Adropion scoticum scoticum* (Murray, 1905). It should be noted that basically until its redescription (Bertolani and Rebecchi 1993), *M. hufelandi* was for many years an umbrella name for many macrobiotids whose eggs possess processes in shape of inverted goblets. Therefore, it is not surprising that compared to Travé et al. (1954) our study discovered at least five distinct *Macrobotus* species based on the specific morphology of egg ornamentation. Finally, *Mesobiotus* cf. *binieki* Kaczmarek, Goldyn, Prokop & Michalczyk, 2011, *Minibiotus* cf. *diversus* Ciobanu, Roszkowska & Kaczmarek, 2015, and *Macrobotus* cf. *persimilis* Binda & Pilato, 1972 may constitute new species, but differentiating them from other similar species would require comprehensive integrative analyses due to incomplete and general descriptions of these similar species.

Nematodes are probably the most extensively studied group of organisms among the meiofauna, presumably because they are extremely abundant in benthic habitats and

because nematodes' cuticle preserves well under common fixatives like Ethanol or Formaldehyde, which is not the case for more delicate, soft-bodied meiofauna such as microturbellarians, gastrotrichs and rotifers (Balsamo et al. 2020), which need to be identified alive. For nematodes no new species candidate seemed to emerge from the Massane survey, and typical freshwater (e.g. Monhysterids) and typical limno-terrestrial species (e.g. Plectids) were found in the habitats where they were expected to be found. However, the Massane dataset still represents an addition of 104 new records to the French forest nematofauna record (Tax Ref13, "nématodes forestiers" in Gargominy et al. 2019) meaning that 21% of the known limno-terrestrial nematodes from France are so far only known from the Massane Reserve. This might encourage nematologists to look aboveground: TreMs should be more considered to get a more comprehensive panorama of nematodes' biotopes (any epiphytic structure hanging on tree bark, young and old fungal fruiting bodies, as well as birds' nests and woodpecker lodges with copious amounts of guano were heavily inhabited by nematodes). This will be the topic of a subsequent publication since, to our knowledge, very few studies have focused on TreM-dwelling meiofauna besides dendrotelmata (Ptatscheck & Traunspurger 2015; Petermann & Gössner 2022). Interestingly, freshwater nematode communities were also unusually diverse for an oligotrophic stream system, perhaps as a beneficial effect of the protection status of the forest. A total of 89 nematode species was encountered in Massane stream biofilms alone, which represents a considerable species richness in comparison with much poorer biofilm-associated nematofaunas which have been reported in other lotic systems nearby: e.g. 28 species were found in epilithic biofilms of the Garonne River surveyed weekly over 18 months (Majdi et al. 2011), only 6 to 8 species were found in a snapshot sampling of epixylic biofilms in headwater streams of "La Montagne Noire" in areas affected by forestry (Majdi et al. 2015),

again only 6 species were described from cyanobacterial biofilms of the Llobregat River surveyed over 5 months (Gaudes et al. 2006). On the other hand, our results are more in line with the study by Brüchner-Hütteman and Traunspurger (2020), reporting 43, 46 and 66 species of nematodes over 12 months in biofilms growing on macrophytes, dead wood and litter, respectively. These nematode-rich biofilms were found in the "Furlbach", a headwater stream also flowing through a protected beech forest in a vestigial moraine of North-Rhine Westphalia, Germany. Together, our results and those of Brüchner-Hütteman and Traunspurger (2020) suggest that the diversity of biofilm nematodes may be related to a strict protection status for the surrounding riparian forest.

Microturbellarians remain acutely taxonomically under-investigated throughout the globe, owing to the lack of modern English monographic treatments and the need to observe reproductive characters in detail from live specimens. An additional factor, evident from our results, is the habitat sampling bias of the few turbellarian taxonomists active today - most focussing on marine habitats, with fewer studies of freshwater environments and fewer still of terrestrial habitats, though these harbour their own distinct faunas. Indeed, the 32 species we encountered during a two-week survey of the Massane reserve exceed the total diversity (29) of freshwater and terrestrial microturbellaria reported across all of France. Noteworthy individual results of our survey include the recovery of freshwater interstitial species seldom reported since Kolasa's original descriptions (Kolasa 1977, 1980), for instance *Ethmorhynchus youngi* Kolasa, 1977, and *Uncinorhynchus karlingi* Kolasa, 1977 (freshwater cicerinid and gnathorhynchid kalyptorhynchs, respectively), *Pseudobockia limicola* Kolasa, 1981, and *Limnoruanis romanae* Kolasa, 1977. The presence of two distinct (however, extremely delicate and rare) neodalyellid species is also remarkable, as this is a group seldom reported from freshwater and terrestrial habitats (though one specimen, again,

may conform to a Kolasa species, *Balgetia papii* Kolasa, 1976). Many of the 32 recognizable species we encountered were "Protoplanellinae" and "Typhloplanidae", some of which conformed to established descriptions, but many of which could not be assigned to genus and species, owing to either immaturity (unique non-reproductive characters, however, being visible), opacity, or failure to conform to available descriptions. Several of these likely represent new records, and in general especially the terrestrial members of "Protoplanellinae" and "Typhloplaninae" (i.e. Adenoplea) - likely non-monophyletic subfamilies (Houben et al. 2022) - are badly in need of deeper systematic treatment, turbellarians lacking sclerotized stylets (as is the norm outside marine settings) being notoriously difficult to properly identify and describe. Three fragile and strongly photophobic specimens of a prorhynchid species conforming to the habitus of the rare *Prorhynchus fontinalis* (Vejdovski, 1895) (which is likely a cryptic species cluster nested within the diversity of *Geocentrophora*; Laumer, unpublished) were encountered in a cold and coarse-sand bottomed segment of the Massane stream. Several species were restricted to small springs feeding into the Massane stream, highlighting the conservation importance of such habitats as microbiogeographic units for freshwater meiofauna. Greater clarity on taxonomic identity and the total diversity represented among the 127 vouchered specimens from the Massane reserve is anticipated following a planned DNA taxonomic treatment, and many of the unique species recorded in this survey are anticipated to bring high value to a per se phylogenetic investigation of limno-terrestrial Rhabdocoela.

CONCLUSION

Through a scientific expedition in an old-growth beech forest situated in southern France near the Mediterranean, followed by an intensive meiofauna identification workshop, we were able to detect and identify 315 species of nematodes, rotifers, gastrotrichs, tardigrades and

microturbellarians living in a variety of limno-terrestrial habitats. Amongst the numerous specimens collected, several are for sure representatives of species new to science. However, this survey can only be regarded as a first baseline study, though a quite comprehensive one. Future expeditions in this unique protected biotope may consider further microhabitats, different seasons, and quantitative sampling to unravel the ecological preferences and the population dynamics of those intriguing species.

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AUTHOR CONTRIBUTIONS

NM: Experiment design, project/ workshop organization, sampling. TQA: Sampling, identification. NB: Sampling, identification. DF: Sampling, identification. JG: Experiment design, sampling. LL: Experiment design, sampling. LK: Data analysis. AK: Sampling, identification. AKM: Sampling, identification. CL: Sampling, identification. RS: Data analysis. DSo: Sampling. DSt: Identification. WT: Identification. All authors contributed equally to dataset curation and manuscript preparation.

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