Assessment of abundance and community composition of benthic macroinvertebrates:

recommendations for improved sampling, fixation and extraction of oligochaetes

Evaluation de l'abondance et de la composition des communautés de macroinvertébrés benthiques :

recommandations pour améliorer les méthodes d'échantillonnage, de fixation et d'extraction des oligochètes

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Abstract

For several decades, the abundance and community structure of benthic macroinvertebrates have been studied to assess the biological quality of aquatic ecosystems. Procedures aiming at studying the whole of macroinvertebrate communities were mainly designed for the evaluation of the diversity of insects at a site and are not adapted for assessing the effect of environmental factors on the community structure and abundance of specimen from other taxonomic groups, including oligochaetes. However, oligochaete abundance and community composition resulting from the implementation of such procedures have sometimes been used for establishing/complementing ecological diagnoses. Here, we propose a number of procedure adaptations, from the choice of the studied habitat, mesh sizes for the net and sieve, fixation of samples, to the sorting of specimens. Following these recommendations will allow to properly assess the effect of environmental factors on the abundance and community composition of oligochaetes at each of the sampled sites as well as make comparisons between sites possible. If procedures described herein are not adapted for the study of the whole macroinvertebrate fauna, oligochaetes and the other macroinvertebrates should be analyzed separately.

Keywords: *Biomonitoring; Benthic macroinvertebrates; Oligochaetes; Sampling; Sample treatment*

Résumé

Depuis plusieurs décennies, les abondances et la structure des communautés de macroinvertébrés benthiques ont été étudiées pour évaluer la qualité biologique des écosystèmes aquatiques. Les procédures visant à étudier l'ensemble des communautés de macroinvertébrés ont été principalement conçues pour l'évaluation de la diversité des insectes sur l'ensemble d'un site et

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ne sont pas adaptées pour évaluer l'effet de facteurs environnementaux sur la structure des communautés et l'abondance de spécimens d'autres groupes taxonomiques tels que les oligochètes. Cependant, l'abondance et la composition des communautés des oligochètes résultant de l'application de telles procédures ont parfois été utilisées pour établir/compléter des diagnostics écologiques. Dans le présent travail, nous suggérons des adaptations des procédures, allant du choix de l'habitat étudié au tri des spécimens, en incluant la fixation des échantillons et les tailles du vide de maille du filet et du tamis. Le suivi de ces recommandations permettra d'évaluer correctement l'effet des facteurs environnementaux sur les peuplements d'oligochètes (abondance, composition) sur un site et de comparer les résultats obtenus entre les sites. Les oligochètes et les autres macroinvertébrés devraient être analysés séparément si les procédures décrites ici n'étaient pas adaptées pour l'étude de l'ensemble des macroinvertébrés.
Mots-clés : *Biosurveillance ; Macroinvertébrés benthiques ; Oligochètes ; Échantillonnage ; Traitements des échantillons*

1. Introduction

For several decades, the abundance and community structure of benthic macroinvertebrates have been used as biological markers for the quality of freshwater ecosystems. Procedures aiming at cataloguing all of the macroinvertebrates found in a stream or a lake, or specific groups such as oligochaetes, mollusks and nematodes, have been developed. For example, different standardized methods exist for specifically studying communities and the abundance of aquatic oligochaetes in fine/sandy sediments in rivers and lakes (AFNOR 2016, Vivien *et al.* 2020) as well as in surficial coarse sediments and in the hyporheic zone (Vivier 2006, Vivien *et al.* 2019a). Pictures of a community of aquatic oligochaetes extracted from one stream site and of specimens belonging to different aquatic oligochaete taxa are provided in Figure 1.

Procedures aiming at studying all macroinvertebrates were mostly conceived to analyze the effect of environmental factors on the diversity of insects, that were generally identified at the family or genus levels. Several standardized methods to investigate macroinvertebrate communities have been applied in routine analyses and for research (e.g. Woodiwiss 1964, Verneaux & Tufféry 1967, AFNOR 2004, OFEV 2020). These methods are not suitable for specifically assessing the effect of environmental factors on the community structures and abundance of specimen from other taxonomic groups, and of oligochaetes in particular. Consequently, oligochaete communities/abundance are given either no or only very little consideration in most macroinvertebrate studies (e.g. Pardo *et al.* 2014, Turley *et al.* 2016, Serrana *et al.* 2019). However, oligochaete abundance and community composition resulting from the implementation of macroinvertebrate methodologies have sometimes been used to establish or complement ecological diagnoses (e.g. Burdon *et al.* 2016; 2019, Carew *et al.* 2018, Aylagas *et al.* 2018).

Here, we propose a number of procedure adaptations that should be applied in order to correctly assess the effect of environmental factors on the abundance and community structure of oligochaetes and allow to compare oligochaete results from different sites. These adaptations range from the choice of the studied habitat to the sorting of specimens.

2. Recommendations

Sampling: choice of the studied habitat

Macroinvertebrates are generally collected at a site in several habitats and in various grain sizes, using the "kick-sampling" technique. The aim of this method is to sample a majority of taxa, in particular of insects, present at a site (e.g. Burdon *et al.* 2016, OFEV 2020). Such a sampling procedure is not adequate for the study of oligochaete for two reasons. First, the density and community composition of these organisms largely depend upon the habitat structure. For example, some species are more abundant in coarse than in fine/sandy sediments (e.g. *Chaetogaster* spp., *Cernosvitoviella* spp.) and inversely (e.g. tubificids), while oligochaete populations are often larger in fine/sandy rather than in coarse sediments. Given the existing variations in structure and composition of studied habitats, the type of sampling procedure may significantly influence the results of oligochaete community composition and abundance. Secondly, the degree of sensitivity of many oligochaete species is differently classified in fine/sandy sediments and in coarse sediments. For example, *Pristina* spp., *Nais elinguis* and *Lumbricillus* spp. are considered as resistant to pollution in coarse sediments (Vivier 2006, Vivien et al. 2019a) and moderately tolerant to pollution in fine/sandy sediments. The interpretation of oligochaete community results is possible only when oligochaetes are sampled either in fine/sandy sediments or in coarse sediments. In addition, the "kick-sampling" technique, which consists to capture with a net the benthic fauna raised by scratching the river bed with the foot on a plot equivalent to an area of one square foot (OFEV, 2020), is not adequate (or optimal) to determine with precision densities of organisms and thus to compare abundance of specimen between sites.

In order to properly assess the effects of environmental factors (e.g. chemical pollution) on the abundance and community composition of oligochaetes and compare results between sites, it is therefore essential at each site (Vivier 2006, AFNOR 2016) i) to select only one type of habitat (coarse or sandy or fine sediments). It is recommended to sample this habitat at three different places of a site, and ii) to collect sediments on a specific surface using a net, a grab sampler or a core, in order to measure precise

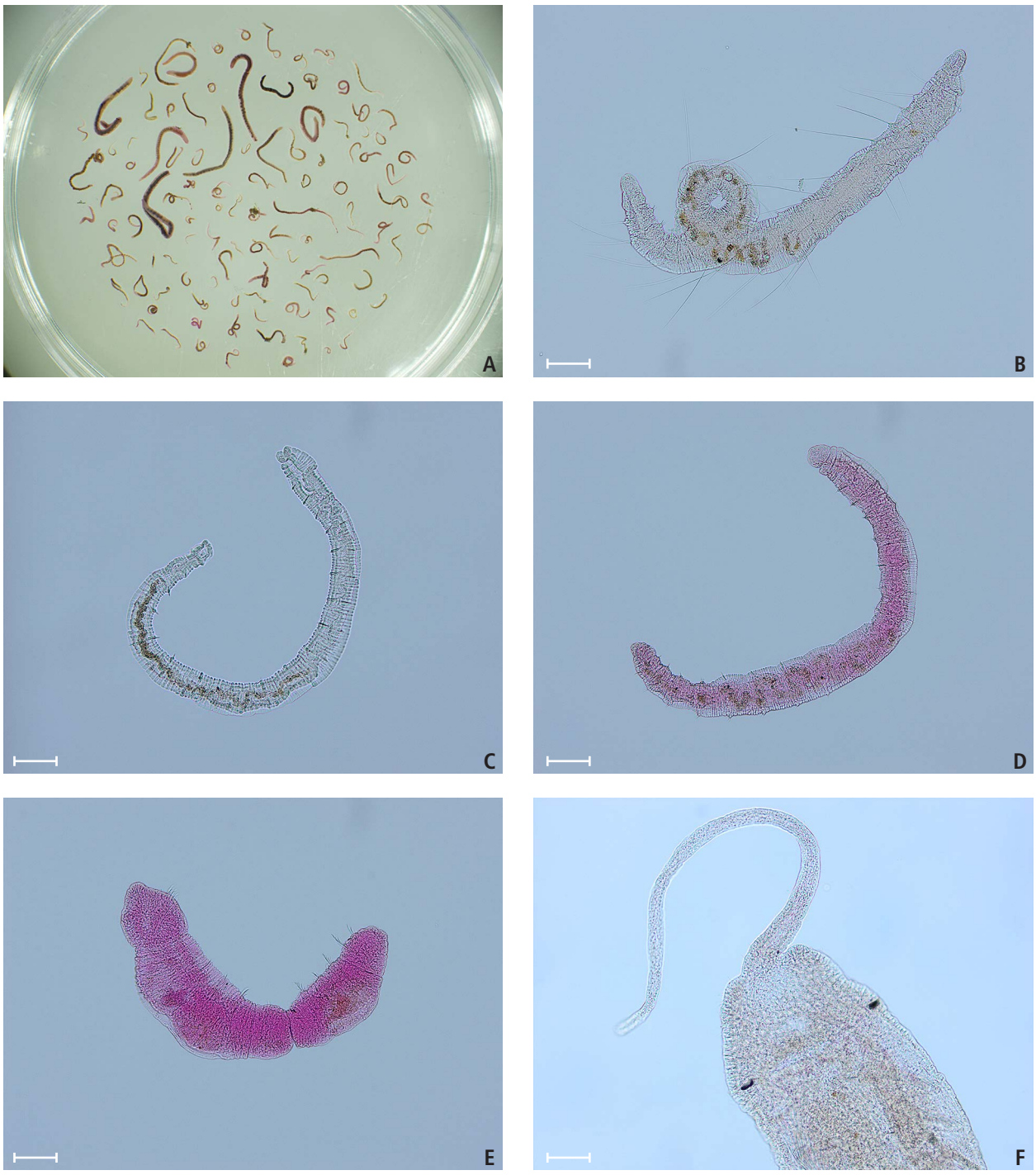

Figure 1. **A:** Photo of an aquatic oligochaete community of about 100 specimens isolated from one stream site. The specimens are slightly stained with eosin. The diameter of the Petri dish is 5.1 cm. **B-F:** Photos of aquatic oligochaete specimens belonging to the taxa Tubificinae sp. (B), *Marionina argentea* (Enchytraeidae) (C), *Cernosvitoviell*a sp. (Enchytraeidae) (D). *Chaetogaster diastrophus* (Naidinae) (E) and *Stylaria lacustris* (Naidinae) (F). The scale bars (all photos) correspond to a length of 100 µm. Specimens shown in D and E are stained with eosin, while the other specimens are slightly or not stained with eosin. Author: Régis Vivien

densities of organisms. To obtain a sufficient number of oligochaete specimens, it is necessary to sample at least 3 litres of coarse or fine/sandy sediments per site (1 litre per subsample).

Choice of fixative

Until about fifteen years ago, formalin was commonly used for fixing macroinvertebrates (e.g. AFNOR 2004), and ethanol for preserving the formalin-fixed organisms. In most studies dealing with the use of macroinvertebrates for biomonitoring, ethanol was then favored over formalin (e.g. OFEV 2020) because of formalin toxicity to humans and for better preservation of samples for later genetic studies. Formalin, which induces cross linking, irreversible denaturation, modification and fragmentation of nucleic acids (Chaw *et al.* 1980, Tang 2006), was found to be inadequate for molecular barcoding studies.

However, while ethanol was probably optimal for fixing insect larvae, insufficient ethanol concentrations were reported to induce disintegration and fragmentation of oligochaete specimens and therefore to bias density and diversity estimates (Rodriguez & Reynoldson 2011, Vivien *et al.* 2018). In addition, insufficient concentrations of ethanol during fixation make oligochaetes soft and fragile, and thus vulnerable to sieving. We emphasize that low ethanol concentrations may also damage other soft bodied organisms such as leeches or flatworms. Macroinvertebrates should therefore always be fixed and preserved in ≥ 70% ethanol (Timm & Martin 2015). When macroinvertebrates are collected for genetic studies, organisms should be kept at 4 to 8°C during transport and preserved in concentrations of 80 to 96% ethanol and at -20°C when in laboratory (Timm & Martin 2015) to preserve DNA from degradation. A direct fixation with 100% ethanol should be avoided as it can damage oligochaete specimens by osmotic shock. It is also important to emphasize that, unlike specimens treated with formalin, the oligochaetes that are fixed and preserved in ethanol often become soft and fragile when in contact with water. Therefore, damage and loss of oligochaete specimens fixed and preserved in ethanol can occur during the sieving of sediments and sorting of specimens once immersed in water. Finally, morphological identification of oligochaetes is generally easier when organisms were fixed and preserved in formalin rather than in ethanol. This was observed during a study in the Swiss Val Roseg River (Malard *et al.* 2001), when the presence of *Cernosvitoviella carpatica* was only confirmed when field samples were fixed and preserved in formalin (Michel Lafont, pers. com.).

For collecting macroinvertebrates, several protocols with (e.g. OFEV 2020) or without sieving in the field (e.g. AFNOR 2004, Pardo *et al.* 2014) have been proposed.

When no sieving in the field is performed, the sampled material (either fine or coarse sediments) should be transferred into a container before being fixed/preserved preferably with formalin (Table 1). The risk of using ethanol when large volumes of samples are collected is the destruction of a number of oligochaete specimens (in contact with low concentrations of ethanol, see above) normally found in the site, since it is difficult to rapidly homogenize ethanol concentrations throughout sediment samples. The use of formalin also presents the advantage to require low volumes of fixative in the field: 4% formalin suffice for fixation and preservation of organisms, whereas 70 to 100% ethanol concentrations are needed.

By contrast, when samples are elutriated and sieved in the field (live specimens), it is possible to fix the smaller volumes of retained material with ethanol (Table 1). We recommend sorting ethanol-fixed/preserved oligochaete specimens immersed in ethanol and not in water and suggest transferring them into 4% formalin just before mounting them on slides to facilitate their morphological identification (see above). To minimize contaminations, we recommend using tap instead of river water for field sieving, and de-chlorinated water should be favored.

While it is possible to sieve on site, we recommend laboratory sieving. First, sieving in the field can cause maiming of oligochaete specimens (Timm & Martin 2015). Secondly, given the rather large volumes we recommend to sample (at least 3 litres per site), sieving the sediments once back in the laboratory is much more convenient, but only when biological material has been fixed when still on site. Frequent sieve clogging and abundant oligochaetes attached to vegetal debris, are other reasons to favor laboratory sieving. We suggest using 5 litres containers and 1 container per site, for transport of sediment samples to the laboratory.

Formalin fixation does not necessarily inhibit subsequent genetic analyses since success of DNA amplifications largely depends upon the duration of storage and pH of formalin (Schander & Halanych 2003, Bucklin & Allen 2004, Baird *et al.* 2011). For example, it has been shown that a cytochrome c oxidase (COI) gene fragment from oligochaete tissues fixed in 4% neutral buffered formalin and stored in this medium for up to one month could be successfully amplified and sequenced (Vivien *et al.* 2018). In this respect, a protocol for preserving oligochaetes using neutral buffered formalin and describing all the steps

from collection of sediments to preservation of biological materials in absolute ethanol, was proposed in Vivien *et al.* (2018).

Because of toxicity, a number of measures must be taken to protect operators from formalin vapors. In the field, wearing a mask, protective glasses and gloves is recommended. In the laboratory, sieving the samples in a fume hood limits human contact with formalin vapors. An infrastructure for sieving the samples, comprising a basin and a showerhead, can be installed in most fume hoods. A substitute to formalin is being actively searched for, including in the medical field (e.g. Bussolati *et al.* 2017, Sarot *et al.* 2017), and testing of these proposed formalin-substitutes for fixing macroinvertebrates should be regularly carried out. Since ethanol vapors are most probably not harmless either, sorting macroinvertebrates fixed and preserved in ethanol should also be carried out in well ventilated premises. With the development of bioindication methods based on DNA analyses of environmental samples (Cordier *et al.* 2017, Pawlowski *et al.* 2018), the use of harmful fixatives might be considerably reduced in the future. Such methods, still under development for oligochaete communities, are promising (Vivien *et al.* 2017; 2019b).

Mesh-sizes of the net and sieve

The standard mesh size of 0.5 to 1 mm for nets commonly used for sampling macroinvertebrates (AFNOR 2004, Turley *et al.* 2016, Aylagas *et al.* 2018, OFEV 2020) is not adequate for sampling oligochaetes, since many specimens have diameters lower than 0.5 mm. This important issue that was already raised by Nalepa & Robertson (1981), should call for a mesh size of nets of maximum 0.25 mm for sampling oligochaetes (AFNOR 2016) (Table 1). The maximum mesh size for sieving sediments containing live specimens is also 0.25 mm. However, during the sieving procedure in the laboratory (fixed material), a sieve mesh size equal or superior to 0.25 mm but not larger than 0.5 mm is appropriate as oligochaetes fixed in formalin or ≥70% ethanol are rigid and coiled. In fact, the optimal mesh size of the sieve depends upon the type of sample that is being analyzed. For example, while a mesh size of 0.5 mm is acceptable for fine/sandy sediments since a large majority of sampled specimens is retained (Rosso *et al.* 1994, AFNOR 2016), a mesh size no greater than 0.25 mm is recommended for samples from coarse sediments (Vivier 2006). In these samples an important part of very small specimens belonging to taxa such as *Cernosvitoviella* spp., *Nais* spp. and *Chaetogaster* spp. are generally present and their abundance could be underestimated when using a mesh size greater than 0.25 mm. Furthermore, as the field sieving of fine/sandy sediments using the mesh size of 0.25 mm will generally retain large quantities of non-biological material, it is necessary, once the sample has been fixed with formalin or ≥70% ethanol, to sieve one more time in the laboratory using a mesh size of 0.5 mm.

Sorting of specimens

The sorting of specimens should be performed in the laboratory using a stereomicroscope and once organisms have been fixed. Sorting of live specimens in the field, as was suggested in the Rapid Bioassessment Protocols developed by EPA Victoria (Carew *et al.* 2018, EPA Victoria 2003), is unsuitable for oligochaetes since they are only visible under a stereomicroscope, except for big specimens of a few species. In addition, fixed specimens are faster and easier to sort than live specimens.

3. Discussion, Conclusion

Here we have reviewed some of the problems encountered during the studies of oligochaete communities and abundance when using procedures designed for whole macroinvertebrate communities. Although these procedures are well suited to evaluate the diversity of insects at a site, they are not adapted for assessing the effects of environmental factors on the abundance and community structure of oligochaetes. To address this issue, we proposed to implement a number of adaptations to the sampling, fixation and extraction of oligochaetes.

We are aware these procedure adaptations may not necessarily be compatible for studying the diversity of certain macroinvertebrates, including that of insect larvae. For example, the collection of all macroinvertebrates in only one type of habitat per site could lead to underestimating their biodiversity, which may have significant consequences on the final biological quality diagnostics for the sampled sites. However, other recommendations such as the use of lower net and sieve mesh sizes and of neutral buffered formalin instead of ethanol for genetic identifications could be suitable for the study of all macroinvertebrates (Vivien et al. 2018). Oligochaetes and whole macroinvertebrates should be analyzed separately when the procedures we recommend implementing are not fully adapted for studying the targeted macroinvertebrates.

The study of oligochaete communities/abundance allows the ecological diagnoses established based on the analysis of whole macroinvertebrates (in particular, insects) to be efficiently complemented by providing, for example, information on the causes

Table 1. Summary of the choice of mesh sizes (net and sieve) and of fixative according to the grain size of samples and mode of sieving (in the field or in laboratory) and to the subsequent mode of identification (morphological or genetic). Remarks and advantages/disadvantages of sieving sediments in laboratory (1) and in the field (2) are included

	Mesh size of the net	Mesh size of the sieve	Fixative	Remarks	Advantages	Disadvantages
(1) Samples not sieved in the field - oligochaetes fixed in the field	0.20-0.25 mm (max 0.25 mm)	0.20-0.25 mm (max 0.25 mm) for coarse sediments / hyporheic zone samples 0.5 mm for fine/sandy sediments	Low-pH formalin 37% (4% formaldehyde in the container) in the case of identification by **morphological analysis** **or** neutral buffered formalin 10% (4% formaldehyde in the container) in the case of identification by **genetic analysis** (material kept in 4% formaldehyde at 4°C for maximum 4 weeks)	Recommended when large quantities of sediments (>1-2 litres) and/or fine/sandy sediments are collected	Globally very convenient compared to (2), facilitates the field work No destruction of specimens induced Ideal for the morphological identification	Necessity to use formalin
(2) Samples sieved in the field (live specimens) - oligochaetes fixed after sieving (in the field)	0.20-0.25 mm (max 0.25 mm)	Sieving in the field (live specimens): 0.20-0.25 mm (max 0.25 mm) for fine/sandy sediments and coarse sediments / hyporheic zone samples Second sieving for fine/sandy sediment samples (in laboratory, fixed specimens): 0.5 mm	Low-pH formalin 37% (4% formaldehyde in the container) in the case of identification by **morphological analysis** **or** neutral buffered formalin 10% (4% formaldehyde in the container) in the case of identification by **genetic analysis** (material kept in 4% formaldehyde at 4°C for maximum 4 weeks) **or** absolute ethanol: at least 70% ethanol in the container **(morphological analysis)**; 80-96% ethanol in the container and preservation of samples in a cool box in the field and at -20°C as soon as possible **(genetic analysis)**	Not recommended when large quantities of sediments (>1-2 litres) and/or fine/sandy sediments are collected Use river water or de-chlorinated tap water for sieving in the field In the case of fixation/preservation in ethanol: - better to transfer oligochaete specimens into 4% formalin just before morphological identification - necessary to sort oligochaete specimens immersed in ethanol	No necessity to use formalin	Long and hard field work Sieving in the field (live specimens) can cause maiming of oligochaete specimens Fixation and preservation of oligochaete specimens in ethanol not ideal for morphological identification

of environmental disturbances or by revealing the presence of chemical pollutions (Lafont *et al.* 2001, Vivien *et al.* 2015; 2019a; 2020). Therefore, we strongly encourage researchers to carefully consider the importance of oligochaete populations during macroinvertebrate studies. By following the various recommendations outlined above, analyses of oligochaete abundance and community composition for one site will be robust and reliable, and thus appropriate when several sites need to be compared. This will guarantee a sound evaluation of the impact of environmental factors on this particular taxonomic group.

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