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Nematodes as bioindicators of polluted sediments using metabarcoding and microscopic taxonomy

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ABSTRACT

The use of bioindicator species is a widely applied approach to evaluate ecological conditions, and several indices have been designed for this purpose. To assess the impact of pollution, especially in sediments, a pollution-sensitive index based on nematodes, one of the most abundant and species-rich groups of metazoa, was developed. The NemaSPEAR[%] index in its original form relies on the morphological inspection of nematode species. The application of a morphologically based NemaSPEAR[%] at the genus-level was previously validated. The present study evaluated a NemaSPEAR[%] index based on metabarcoding of nematode communities and tested the potential of fragments from the 28S rDNA, 18S rDNA and cytochrome *c* oxidase subunit I (COI) genes. In general, molecular-based results tended to show a poorer condition than morphology-based results for the investigated sites. At the genus level, NemaSPEAR[%] values based on morphological data strongly correlated with those based on molecular data for both the 28S rDNA and the 18S rDNA gene fragments (R^2 = 0.86 and R^2 = 0.74, respectively). Within the dominant genera (> 3%) identified by morphology, 68% were detected by at least one of the two ribosomal markers. At the species level, however, concordance was less pronounced, as there were several deviations of the molecular from the morphological data. These differences could mostly be attributed to shortcomings in the reference database used in the molecular-based assignments. Our pilot study shows that a molecularly based, genus-level NemaSPEAR[%] can be successfully applied to evaluate polluted sediment. Future studies need to validate this approach further, e.g. with bulk extractions of whole meiofaunal communities in order to circumvent time-consuming nematode isolation. Further database curation with abundant NemaSPEAR[%] species will also increase the applicability of this approach.

1. Introduction

In the 21st century, the routine measurement of water quality has become particularly important, whether in the context of decreasing water quality (e.g. pesticides and heavy metals), climate change or the decline in species diversity (Young et al., 2016; Cowart et al., 2015; Bucklin et al., 2016). In Europe, the aim of the Water Framework Directive (WFD) (Directive 2000) is to achieve the "good chemical and ecological status" of all ground and surface waters. The WFD has been accompanied by the development of several supporting initiatives, such as the EU COST Action "DNAqua-Net," which seeks to identify and apply genomic tools to promote bioindication in the assessment of a "good ecological status"(Leese et al., 2016).

Water quality is commonly evaluated using biological indices that allow standardized evaluations in different labs and across borders. Commonly applied indices include those based on diatoms (Kelly 2013) and macro-invertebrates (Birk and Hering 2006; Vivien et al., 2016). However, environmental evaluations of aquatic sediment are complicated by a lack of bioindicator tools, because sediment macroinvertebrates are often scarce, especially in fine sediments. Furthermore, several macroinvertebrates, such as insect larvae, are present only during larval stages (Wolfram et al., 2010; Wagenhoff et al., 2012). Nonetheless, as a highly biodiverse habitat important for nutrient cycling, healthy infaunal communities are essential to the functioning of

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Table 1
Overview of the species number and individuals (Ind) analyzed in a morphological analysis, as well as the number of OTUs and individuals (Ind) analyzed by metabarcoding of the 18S rDNA, 28S rDNA and COI genes for the seven investigated locations. (−) = no data. The rarefied species number for the morphological analysis is given in parentheses and is based on the number of individuals analyzed in the molecular approach.

River/Location	Coordinates	Morphology		Molecular			
		Species [rarefied]	Ind	OTUs 28S	OTUs 18S	OTUs COI	Ind
Furlbach (FB)	N51°53.724 E008°42.931	23 ([500]: 23.3)	485	13	–	–	500
Veerse (VE)	N53°08.483 E009°30.060	59 ([130]: 32.4)	516	16	7	14	130
Örtze (ÖR)	N53°00.944 E010°04.974	20 ([500]: 20)	499	34	21	72	500
Saale-Rischmühle (RM)	N51°21.038 E012°00.213	35 ([700]: 39.4)	498	38	31	97	700
Elbe-Hitzacker (HI)	N53°09.643 E011°02.787	60 ([1000]: 76.5)	504	44	38	49	1000
Elbe-Cumlosen (CUM)	N53°02.432 E011°38.592	51 ([800]: 57)	503	53	31	53	800
Luppe (LU)	N51°23.116 E012°00.526	36 ([250]: 29.5)	491	13	8	36	250

aquatic ecosystems (Traunspurger et al., 1997). Moreover, because sediments act as both sink and source for toxic chemicals (den Besten et al., 2003; Wetzel et al., 2013), their quality contributes to the overall quality of the overlying water column (SedNet, 2017).

As an alternative to macroinvertebrates, nematodes are a good proxy in sediment quality assessment, including fine sediments (Heininger et al., 2007; Höss et al., 2011). Nematodes form one of the most species-rich groups on Earth and account for ~90% of the organismal abundance in soils (Ferris et al., 2001; Porazinska et al., 2010; van den Hoogen et al., 2019) as well as in rivers and lakes (Traunspurger 2000; Traunspurger et al., 2012). Furthermore, the use of nematodes as bioindicators was previously described (Wilson and Kakouli-Duarte 2009). To standardize the use of nematodes as a bioindicator, the Nematode SPEcies At Risk (NemaSPEAR[%]) index was developed and subsequently shown to correlate well with sediment quality (Höss et al., 2011; Höss et al., 2017). However, a drawback of NemaSPEAR[%] is that, due to the minute body size and uniform structure of nematodes, their identification requires considerable taxonomic expertise (Bhadury et al., 2008; Nielsen 1998). As a result, the application of nematodes as a biomonitoring tool has been limited.

An alternative species identification method, based on molecular information, has emerged over the last few decades. In this so-called barcoding approach, species delimitation is achieved using short specific gene fragments. Barcoding has been rapidly adopted to assess the diversity of organisms ranging from bacteria to mammals (Hebert et al., 2003). The most prominent genetic markers used are ribosomal and mitochondrial gene fragments. The 18S rDNA gene evolves conservatively, allowing a broad amplification range but a relatively low resolution efficiency between closely related species (Abad et al., 2016; Derycke et al., 2010; Sahraean et al., 2017). Nonetheless, it is the most frequently amplified gene in studies of metazoans (Bucklin et al., 2016; Zimmermann et al., 2011). The 28S rDNA gene evolves less conservatively than the 18S rDNA gene and its resolution efficiency is accordingly higher (Pereira et al., 2010). Its ability to reliably distinguish between species of aquatic nematodes has been demonstrated (Ristau et al., 2013; Schenk et al., 2017). The mitochondrial cytochrome *c* oxidase subunit I (COI) is maternally inherited and has a high intraspecific variability, allowing its use in the differentiation of species and even populations (Carugati et al., 2015; Deagle et al., 2014; Ristau et al., 2013). Although the COI gene was initially the barcoding marker of choice, its use as a biomarker is hindered by mutations in primer binding regions and inadequate reference databases (Lejzerowicz et al., 2015). The latter also affecting ribosomal reference sequences (Abad et al., 2016; Holovachov 2016).

Metabarcoding, developed over the last decade for molecular species analysis, couples amplicon barcoding with high-throughput sequencing and allows the simultaneous processing of an extremely large number of samples (Cristescu 2014; Shokralla et al., 2015). Consequently, whole communities can be analyzed without the need to first extract indicator species (Hajibabaei et al., 2011). However, despite the enormous potential of metabarcoding, standardized protocols and routine applications are currently not available because of problems associated with biased results due to primer mismatches, differences in gene copy numbers and PCR bias, all of which may lead to misamplification and the under- or overrepresentation of taxa (Bik et al., 2013; Elbrecht et al., 2017; Pawlowski et al., 2018; Piñol et al., 2019).

Nonetheless, molecularly based species identification is a promising approach and several metabarcoding-based indices have been developed. Examples include the IOBS, used to assess oligochaetes (Vivien et al., 2015), and the macroinvertebrate-based marine biotic index AMBI (Aylagas et al., 2016). In studies of diatoms, metabarcoding has been used to recover even rare species (Rimet et al., 2018). Yet, despite their apparent feasibility, indices based on molecular data have yet to be widely implemented (Hering et al., 2018).

Nematodes have been well studied at the molecular level (Darby et al., 2013; Derycke et al., 2013; Holovachov 2016; van Megen et al., 2009) but their potential as a bioindicator based on a molecular approach has been evaluated rarely before (Griffiths et al., 2018). Therefore, in this pilot study we used nematode metabarcoding to evaluate the performance of the NemaSPEAR[%] based on one mitochondrial and two ribosomal DNA markers. Thus, the nematode communities in sediment samples covering a pollution gradient were assessed based on metabarcoding and morphological inspection and the performance of the two approaches at the species and genus levels was compared. Nonetheless, due to database deficiencies, problems with species assignment and differences in species proportions arising from over- or under-representation were anticipated. We predicted that, in the classification of sediment quality, the performance of the molecular-based NemaSPEAR[%]would be comparable to that achieved using morphological data.

2. Material & methods

2.1. Sampling

Samples of river sediments were collected in May 2017 (Furlbach) and 2018 (all other sites) at seven locations in Germany (Table 1). A sediment corer (diameter: 2.6 cm; 24.5 ml) was used to extract sediment from the upper 10 cm of sediment in a 1.5 m^2 radius, following the method of (Higgins and Thiel, 1988). Each replicate consisted of five pooled corer loads. Ten replicates were taken at each site, from which five were fixed with formaldehyde (4%) for morphological analysis and five with 80% ethanol for molecular analysis. Also, at each site, sediment (approx. 1 kg) collected from the same area and depth using a grab sampler was used to analyze the physico-chemical properties of the sediment and the concentrations of sediment pollutants.

2.2. Chemical analysis and toxic potential of sediment

Chemical analyses were conducted for 33 compounds including arsenic (As), 7 metals (Cd, Cu, Pb, Cr, Hg, Ni, Zn), 16 polycyclic aromatic hydrocarbons (PAHs according to the US EPA), 7 polychlorinated

biphenols (PCB 28, 52, 101, 118, 138, 153, 180), *p,p'*-DDD and *p,p'*-DDE (Schenk et al., 2020, Table 1). Sediment quality guidelines were applied to the chemicals measured in the sediment samples. For each chemical, the measured sediment concentration was divided by the consensus-based probable effect concentration (PEC) according to de Deckere et al. (2011) , resulting in a PEC-quotient (PEC-Q) for each chemical. As a measure of the toxic potential, the mean of all PEC-Q values was calculated for each sample (mean PEC-Q). According to MacDonald et al. (2000), a mean PEC-Q < 0.5 indicates a very low probability of toxicity and a PEC-Q > 0.5 a proportionally higher probability of toxicity and thus a proportionally higher toxic potential. Thus, the toxic potential of the sampling sites was ranked based on the calculated mean PEC-Q values.

2.3. Morphological inspection and metabarcoding

Aqueous samples containing the nematodes present in the different sediment samples were obtained by density centrifugation of the collected sediments with Ludox following the method of Higgins and Thiel (1988). Formalin-preserved nematodes were manually sorted and the first 100 individuals were prepared in glycerin following the method of Seinhorst (1962) for subsequent microscopy-based (1250× magnification; Leitz, Dialux) identification to the species-level and according to life stage. Additionally, the biomass of each identified species was calculated based on the measured specimen in this study. For the juvenile stages 1–4 the measurements showed a mean of 25% of the adult biomass, while juvenile sage 4 showed a mean of 90% of the adult's biomass. Therefore, juveniles were corrected with the factors 0.90 and 0.25 applied to juvenile stages 4 and stages 1–3, respectively (Schenk et al., 2020, Table 9). The same approach was previously used in (Ristau and Traunspurger, 2011).

Ethanol-preserved nematodes were manually sorted and transferred to Eppendorf tubes containing 80 µl of lysis buffer from the NucleoSpin tissue XS kit (Macherey & Nagel, Hilden, Germany). If possible, for each sample 200 nematodes were used in the molecular analysis, as fewer individuals could result in insufficient DNA yields. This was the case at two sites (LU and VE), where due to the low nematode yields replicates had to be combined to obtain enough DNA. A detailed overview of the number of nematodes in each replicate is given in Schenk et al. (2020, Table 2). DNA was extracted using the above-mentioned kit following the manufacturer's protocol, with an 8-h lysis time. A ~540-bp fragment of the V4 region of the 18S rDNA gene (3NDf/C_1132f: 5′–GGC AAGTCTGGTGCCAG– 3′/5′–TCCGTCAATTYCTTTAAGT–3′, (Geisen et al., 2018), a 520-bp fragment of the D3–D5 region of the 28S rDNA gene (1274/706: 5′–GACCCGTCTTGAAACACGGA-–3′/5′–GCCAGTTCT GCTTACC–3‘) introduced by Markmann and Tautz (2005) and a 313-bp fragment of the COI gene (mlCOIintF/HCO2198: 5′–GGWACWGGWT-GAACWGTWTAYCCYCC/5′–TAAACTTCAGGGTGACCAAARAAYCA–3′, (Leray et al., 2013) were amplified for all samples. PCR amplification was conducted in 30 cycles as follows: 1 min 96 °C pre-denaturation; 96 °C for 15 s, 58 °C for 30 s, and 70 °C for 90 s. In a second PCR with 10 cycles and the same conditions, an individual index-tag was ligated. For each sample, 20 ng of DNA were pooled and then sequenced at LGC Genomics using Illumina MiSeq using V3 chemistry (2 × 300 bp; 2.5 M read pairs).

2.4. Bioinformatic analysis

Raw reads from Illumina MiSeq, delivered demultiplexed, were merged in Mothur (Kozich et al., 2013; Schloss et al., 2009) using the make.contigs function (default settings). Cutadapt (Martin, 2011) with an error rate of 0.1 was used to remove primer sequences from the combined reads. Reads that did not contain both primer sequences before cutting were removed from the dataset. Additionally, screen_seqs with default parameters was used to remove reads with ambiguous bases, homopolymers > 10 bases and unexpectedly short or long reads (allowed range: 503–562 bases for 18S, 483–506 for 28S and 313 for COI). The sequences were aligned using the SILVA reference (release 132) alignment (Martin, 2011) to determine the spanned 18S and 28S rDNA gene region within the alignment and then remove sequences outside this region as well as overhangs. The sequences were then clustered into operational taxonomic units (OTUs) at 99% identity for the 18S and 28S rDNA fragment and at 97% identity for the COI gene. Chimeras were removed using UCHIME (Edgar et al., 2011). OTUs were taxonomically classified based on the best BLAST hits in the NCBI nucleotide (nt) database. The identity cutoff was 95%. OTUs represented by < 10 reads were discarded. In addition to the OTU-based downstream analysis, a cluster independent approach was tested (Callahan et al., 2016). However, as this resulted in many unassigned Amplicon Sequence Variants (ASVs), this approach was not used in further analysis. An overview about ASV results is given in Table 3 of Schenk et al. (2020).

2.5. Species number and composition

As molecular and morphological samples often yielded different individuals numbers, rarefaction analysis with the R (R Core Team, 2013) package "iNEXT" (Hsieh et al., 2016) was used. Additionally to the number of morphologically identified species, the rarefied number based on the number of molecularly analyzed individuals is also reported, allowing direct comparisons between both approaches. Rarefaction curves for the morphological data of up to 1000 individuals are given in Schenk et al. (2020, Fig. 1).

In order to evaluate the variability of species and genus composition between the replicates of the various sites, non-metric multidimensional scaling (nMDS) was carried out separately for the three

Table 2
Toxic potentials (mean PEC-Q), NemaSPEAR[%] and NemaSPEAR[%]$_{genus}$ for the seven investigated sites, (ranked by increasing mean PEC-Q). NemaSPEAR[%] was calculated based on morphological and molecular (18S and 28S) taxonomic data (mean ± standard deviation if n > 1). NemaSPEAR[%] values are color coded according to the ecological status of the respective site as defined for nematode communities (Höss et al, 0.2017); blue = high; green = good; yellow = moderate; orange = poor; red = bad.

Site		NemaSPEAR[%]			NemaSPEAR[%]$_{genus}$		
	mean PEC-Q	Morph	Mol (28S)	Mol (18S)	Morph	Mol (28S)	Mol (18S)
FB	0.01	51.4 ± 6.8	55.4	-	51.1 ± 5.8	54.8	-
VE	0.01	42.8 ± 9.2	34.4	54.0	59.1 ± 7.6	78.1	43.9
ÖR	0.02	70.9 ± 6.5	32.8 ± 7.5	40.7 ± 8.1	75.6 ± 10.2	76.1 ± 10.7	36.4 ± 8.4
RM	0.34	31.8 ± 5.4	14.0 ± 9.7	10.2 ± 4.7	36.6 ± 7.9	16.7 ± 11.7	15.1 ± 9.0
CUM	0.71	31.4 ± 4.3	33.5 ± 12.8	5.6 ± 5.8	48.1 ± 4.8	37.4 ± 11.7	19.1 ± 6.4
HI	1.2	26.7 ± 3.6	23.5 ± 7.0	3.7 ± 3.8	39.6 ± 3.1	25.3 ± 7.9	23.2 ± 3.8
LU	7.71	16.5 ± 7.1	0.6 ± 0.8	21.5 ± 0.6	25.7 ± 5.3	13.7 ± 7.1	6.8 ± 9.7

taxonomic methods, with not-transformed relative abundance data of the species and genera in the respective replicates using PRIMER_v6 software (Clarke and Gorley, 2006). To evaluate the difference of the three different taxonomic approaches in assessing nematode taxa composition, nMDS was performed for pooled data combining all three taxonomic approaches with relative genus composition based on abundance and biomass data (using Bray-Curtis similarities). In order to keep quantitative information for the various taxa, data was not transformed to presence/absence. Schenk et al. (2019) could show that relative abundances and biomasses were consistent with molecular abundance data.

To visualize statistically different groupings of data points in the nMDS-plot, hierarchical cluster analysis with similarity profile analysis (SIMPROF) were performed with the Bray-Curtis similarity matrix. Significantly different clusters (at a 20% similarity level) were laid over the nMDS plots. To test for effects of the taxonomic method on the outcome of the community structure analysis, a Permutational Multivariate Analysis of Variance (PERMANOVA) was performed in PRIMER_v6 with a two-way design and the factors “method” and “location” with 9999 permutations.

2.6. NemaSPEAR[%]

The NemaSPEAR[%] was calculated from the species and genus data of all replicates used to generate the molecular and morphological data. Abundance data were log (x + 1)-transformed as described in Höss et al. (2017). For the two above-mentioned sites (VE and LU), where the samples had to be combined due to the low specimen number, only one replicate was available for the molecular approach. We also determined whether all nematode species and genera classified as species at risk (NemaSPEAR; Höss et al., 2017) were listed in the molecular database (NCBI) for the chosen genetic marker (Schenk et al., 2020, Table 8).

3. Results

3.1. Toxic potential of sediments

Chemical analysis of the seven locations revealed a clear gradient of chemical pollution, with mean PEC-Q values ranging from 0.01 to 7.71 (Table 2). A very low toxic potential was determined for the samples from the sites FB, VE (0.01) and ÖR (0.02), and a low toxic potential for those from RM (0.34). Samples from locations where toxic effects were likely to occur had a high toxic potential, including those from CUM (0.71), HI (1.2) and LU (7.71).

3.2. OTU and species number

From the 939932 raw reads for the 28S gene region, 526519 reads for the 18S gene region and 219693 reads for the COI gene, the bioinformatics pipeline reduced the dataset to 461690, 56835 and 152549 reads, respectively (Schenk et al., 2020, Table 7). The COI marker revealed no “nematode” or other “unknown” classifications and thus could not be used to describe the species composition of the studied sediments, although the ASV approach could identify single-find nematode matches (3 from 308 ASVs in total; Schenk et al., 2020, Table 3, Figure 5). However, as the COI yielded uninformative results, the OTU numbers determined from the COI analysis were compared to those obtained with the other gene regions, but the COI was not regarded in further analyses.

The samples from the seven locations yielded different numbers of OTUs for the three genetic markers used in the molecular analysis and different species numbers for the morphological analysis (Table 1). The OTU number obtained with the COI gene was higher than that obtained with either of the other markers at all locations and was also higher than the species number determined by morphological inspection of the corresponding samples. The site with the highest number of OTUs according to the 18S rDNA ribosomal marker (38 OTUs) and with the highest species number according to the morphological analysis (60 species, rarefied [1000]: 76.5 species) was HI, while for the 28S rDNA marker the site CUM had the highest OUT number (44 OTUs) and the COI marker showed the highest number of OTUs at site RM (97 OTUs). Site VE had the lowest number of OTUs according to both the 18S rDNA and the COI gene region (18S: 7 OTUs, COI: 14 OTUs) and site LU according to the 28S gene region (13 OTUs). Based on morphology, the lowest species number was recovered at site ÖR (20 species).

3.3. Taxonomic assignment

Taxonomic assignments based on morphological and molecular data differed, especially at the species level. Moreover, the molecular species assignment was strongly restricted by 13 “unknown” assignments for the 28S gene region and 9 “unknown” assignments for the 18S gene region, i.e., no database entry matched these OTUs with the designated threshold. Other matches, such as “uncultured eukaryote,” prevented an assignment at the genus or species level and were therefore excluded from further analysis. Therefore, for both markers several OTUs could not be assigned at an informative level (Schenk et al., 2020, Table 4, 5, 6).

Overall, 123 species were detected morphologically, with 36 (29.3%) species detected using at least one molecular marker. From the 25 most common species detected by each method, 9 (36%) morphologically identified species were also molecularly detected. Thus, 7 species (28% of the most common species) were identified by both the morphological and the 28S rDNA gene analyses, 2 species (8% of the most common species) both morphologically and by the 18S and 28S rDNA markers and four species (16% of the most common species) morphologically and by the 18S rDNA marker (Fig. 1a). Among the 12 dominant species (> 3%) identified by morphology, six were also detected using the molecular approach but the other six were not. A better match was achieved at the genus level. Overall, 80 genera were identified by morphology, including 39 (48.8%) by at least one molecular marker. Among the 25 most abundant genera identified morphologically, 17 (68%) were also detected using metabarcoding and 5 (20%) were found by all three (morphological, 28S-based and 18S-based) approaches. Twelve genera (48% of the most abundant genera) were identified by the morphological analysis and using the 28S gene region, 10 genera (40% of the most abundant genera) by the morphological analysis and using the 18S gene region, and 7 genera (28% of the most abundant genera) by both molecular markers (Fig. 1b). All of the most common genera (> 3% of the total abundance) revealed by morphology could be detected molecularly, except the genus *Filenchus*.

3.4. NemaSPEAR[%]

The different locations showed a gradient of ecological quality as defined by Höss et al. (2017) and indicated by the morphological NemaSPEAR[%]. Thus, ecological quality ranged from high (70.9%) to a poor (16.5%) and corresponded well to the toxic potential of the sites (Table 2). All values for the NemaSPEAR[%]$_{genus}$ were higher than the species-based values (NemaSPEAR[%]), so that only the location with the highest toxic potential didn’t reach good ecological quality. For all sites with a low toxic potential (FB, VE, ÖR), the NemaSPEAR[%] and NemaSPEAR[%]$_{genus}$ values based on morphological and molecular data pointed to a good or high an ecological quality, as defined for nematode communities according to Höss et al. (2017). The NemaSPEAR[%] and NemaSPEAR[%]$_{genus}$, ranging between 33 and 71% and 36–78%, respectively (Table 2), showed significant correlations with the toxic potential of the sediments, whereas the index on species-level was slightly better correlated (Fig. 3a and b; Schenk et al., 2020).

The morphologically and molecularly based NemaSPEAR[%] values for the two study sites with the highest toxic potentials, HI and LU, were

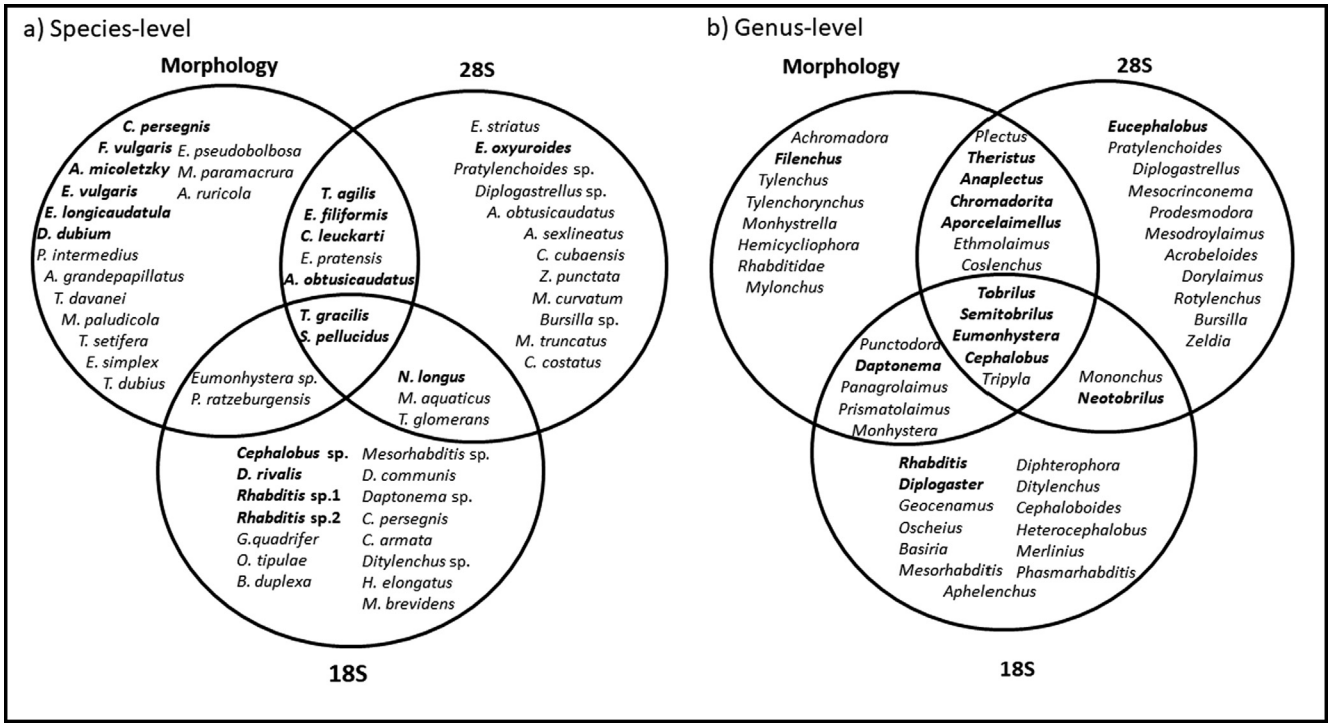

Fig. 1. Venn diagram showing (a) the 25 most abundant species identified by microscopy, the28S gene region and 18S gene region and (b) the 25 most abundant genera identified by microscopy, the 28S gene region and the 18S gene region. Dominant species and genera (relative abundance > 3%) are marked in bold.

in good agreement, with values ranging from 1 to 27%, indicative of the moderate to bad quality of the sites. Only the NemaSPEAR[%]$_{genus}$ calculated for the morphologically derived data of HI indicated a good quality of this site despite its high toxic potential. For the sites RM and CUM, whose toxic potential was close to the mean PEC-Q threshold of 0.5 defined by MacDonald et al. (2000), there was no agreement between the NemaSPEAR[%] determined based on morphology and that determined molecularly and the quality of these sites ranged from good to bad.

Although, the classifications obtained with the molecular and morphological approaches did not always agree, especially for moderately contaminated sites, the site rankings were similar. Plotting the 28S- and 18S rDNA NemaSPEAR[%]-values against the mean PEC-Q values, also revealed significant correlations ($p < 0.05$; with the exception of 18S NemaSPEAR[%]: $p = 0.07$; Schenk et al., 2020, Fig. 3 c to f). For the molecular data, however, the NemaSPEAR[%]$_{genus}$ correlated better than the NemaSPEAR[%] with the toxic potential. A plot of the 28S-rDNA-based NemaSPEAR[%] and the morphological NemaSPEAR[%] revealed a positive, albeit not significant correlation ($R^2 = 0.44$, $p = 0.104$; Fig. 2a). The correlation based on the 18S-derived taxonomy was not significant (Fig. 2b). By contrast, the NemaSPEAR[%]$_{genus}$ calculated from the molecular data correlated strongly with the morphologically derived NemaSPEAR[%]$_{genus}$. The correlation with the morphological data was stronger for the 28S marker ($R^2 = 0.859$, $p = 0.003$; Fig. 2c), than for the 18S marker ($R^2 = 0.741$, $p = 0.028$; Fig. 2d).

3.5. Community structure

The nematode species and genus compositions were quite similar between the replicates of the various sites, regardless of the taxonomic method used (Fig. 2, Schenk et al., 2020). Morphological and molecular taxonomic methods revealed similar results regarding the nematode community structure at the seven sites. In Fig. 3, nMDS plots show that distinct nematode communities could be assigned to the site location, to sites belonging to one river system and to various levels of contamination. This could be shown for both, abundance-based (Fig. 3a) and biomass-based (Fig. 3b) data. Regardless of the taxonomic method, the reference sites with low-level contamination (ÖR, VE, FB) could be found on the left side of the nMDS plot, whereas FB could still be distinguished from VE and ÖR (cluster analysis: $p < 0.05$; Simprof; 20% similarity level). The sites with elevated chemical contamination (HI, CUM, RM and LU) could be found on the right side of the plot, whereas here, the sites of the river Elbe (HI and CUM; right, upper side) could be differentiated from RM and Lu, closely clustering together at the right bottom side of the plot (Fig. 3).

Although, the three taxonomic methods generally clustered the different sites in a similar way, there were still significant differences between regarding the different methods. For sites HI and CUM, the three taxonomic methods revealed significantly distinct nematode communities, in terms of both species (data not shown) and genus composition (cluster analysis: $p < 0.05$, Simprof; Fig. 3). For the biomass-based data of HI and CUM, morphological and 28S data clustered together at a 20% similarity level (Fig. 3b), however, could be distinguished at a 40%-similarity level (Fig. 3c and d; $p < 0.05$; SIMPROF). Also, for ÖR and VE, all three methods clustered together at the 20%-similarity level (Fig. 3a and b), however, were significantly distinguishable at a higher similarity level (Fig. 3c and d; $p < 0.05$; SIMPROF). For sites LU and RM, the three methods were not distinguishable in the cluster analysis ($p > 0.05$, Simprof; Fig. 3). The results of the nMDS and cluster analyses were supported by PERMANOVA results, showing a significant effect of both factors, “location” and “method” ($p < 0.001$), while the pseudo-F value for the factor “location” (pseudo-F: 5.56) was higher than the value for the factor “method” (pseudo-F: 3.53).

4. Discussion

4.1. Species diversity and species assignment

Our analysis of nematode communities at seven sites showed a higher diversity estimated by morphology than by 28S- and 18S-based

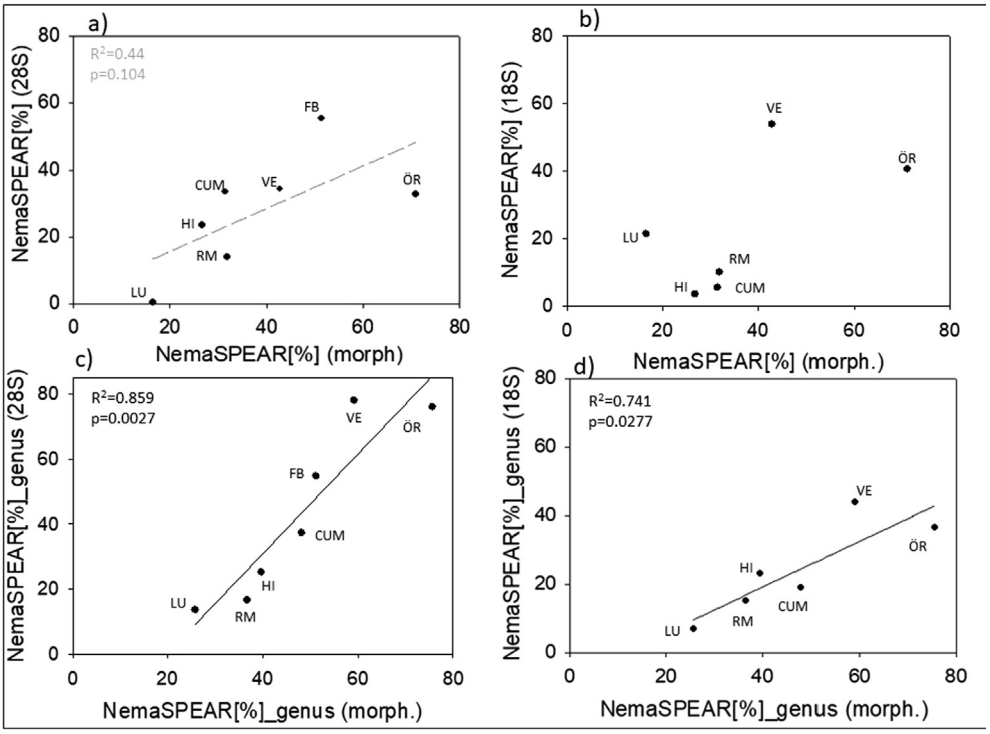

Fig. 2. Correlations between NemaSPEAR [%] and NemaSPEAR[%]$_{genus}$ calculated based on morphological (morph) and molecular (28S, 18S) taxonomic data. (a) NemaSPEAR[%] 28S vs. NemaSPEAR[%] morph; (b) NemaSPEAR[%] 18S vs. NemaSPEAR[%] morph; (c) NemaSPEAR [%]$_{genus}$ 28S vs. NemaSPEAR[%]$_{genus}$ morph; (d) NemaSPEAR[%]$_{genus}$ 18S vs. NemaSPEAR[%]$_{genus}$ morph. The dotted line shows a rather low correlation, which is therefore only partial.

molecular approaches. The difference can be attributed to the known tendency of metabarcoding to miss species, due either to insufficient DNA or to possible primer mismatches that lead to misamplification (Elbrecht et al., 2017; Piñol et al., 2019). Different taxonomic resolutions of the chosen gene regions will also alter the outcome of diversity estimates. This is particularly the case with ribosomal genes, which underestimate diversity due to low evolutionary rates, resulting in the inseparability of closely related species (Machida et al., 2017; Capra et al., 2016). Although the molecular and morphological diversity determined in this study were comparable, metabarcoding may lead to flawed estimates of diversity and therefore should not be regarded as a standalone method (Tang et al., 2015). Furthermore, the use of one larger sample, which is homogenized and subsequently subsampled for the different taxonomic approaches, might have been more accurate for direct comparisons. Moreover, subsamples would have been more evenly distributed, e.g. lower discrepancies between individuals sequenced and analyzed (Table 1).

In this study, the taxonomic assignments at the genus level showed a reliable congruence between the molecular and morphological approaches, as about 70% of the 25 most common genera identified by the latter were found by molecular approaches and included almost all dominant genera (> 3%). Compared to the morphological analysis, a larger number of common genera was found using the 28S rDNA gene (48%) than the 18S rDNA gene (40%), thus demonstrating the potential of 28S rDNA nematode metabarcoding in environmental assessments. However, at the species level, the taxonomic assignments based on 28S rDNA were not congruent with those of the morphological analysis, as only 36% (9 species) of the 25 most abundant species were identified by both. This was most likely due to the shortcomings of the reference database regarding this gene, especially for nematodes (Ahmed et al., 2015). While the 18S rRNA gene is the most thoroughly investigated gene region for meiofauna and nematodes, there are fewer sequences available for the 28S rDNA gene (Creer et al., 2010; Schenk and Fontaneto 2019). This was exemplified by the ubiquitous and dominant genus *Eumonhystera*, for which up to eight species were found by microscopy but only one by metabarcoding of the 28S rDNA using the NCBI database (Benson et al., 2013, accession: Nov. 2019; *E. filiformis;* Schenk et al., 2020, Table 8). Similar species-level underestimations are likely to occur for other taxa as well, although the 28S rDNA gene region might be well suited to distinguishing between closely related nematode species (Carugati et al., 2015). For the 18S rDNA gene, up to seven species of *Eumonhystera* have been deposited at NCBI (accession: Nov. 2019), but only five of them were also discovered by morphology (Schenk et al., 2020, Table 8), such that the underrepresentation was likely due to the lower species-level resolution of the 18S rDNA gene, as previously reported (Derycke et al., 2010; Tang et al., 2012). Nonetheless, both molecular markers and especially the 28S rDNA marker performed well, as the numbers and proportions of OTUs obtained with either one were comparable to the number of species identified morphologically.

Ribosomal genes have a lower evolutionary rate than the COI gene and therefore a lower resolution efficiency (Hirai et al., 2015). However, the COI gene, while used in molecular studies of many organisms, is unable to amplify nematode gene regions (Weigand and Macher 2018). In this study, while we were able to amplify a large number of OTUs for the chosen COI gene region, they could not be assigned to nematode species. Phylogenetic trees (Schenk et al., 2020, Figure 4 and 5) did not increase the resolution of this genetic marker. Therefore, ribosomal genes are a better choice for metazoan metabarcoding.

4.2. The NemaSPEAR[%] index as an indicator of chemical pollution

The NemaSPEAR[%] calculated using the morphologically derived genus data was negatively related to the toxic potential of the seven study sites (Table 2; Schenk et al., 2020, Figure 3). This result demonstrated the power of the NemaSPEAR[%] index in assessments of the risk posed by pollutants to benthic invertebrates in rivers (Höss et al., 2011; Wolfram et al., 2012). Generally, NemaSPEAR[%]$_{genus}$ was higher than the species-level NemaSPEAR[%] and led to an underestimation of the risk at some of the study sites. Höss et al. (2017) reported similar findings. The difference in the two indexes can partly be attributed to the exclusion of the diverse but ambiguous genus *Eumonhystera* in the calculation of NemaSPEAR[%]$_{genus}$, including the indexes for species at risk (NemaSPEAR) and for those not at risk (NemaSPnotAR). Our study showed a good correlation between the NemaSPEAR[%] derived from molecular taxonomy and that based on

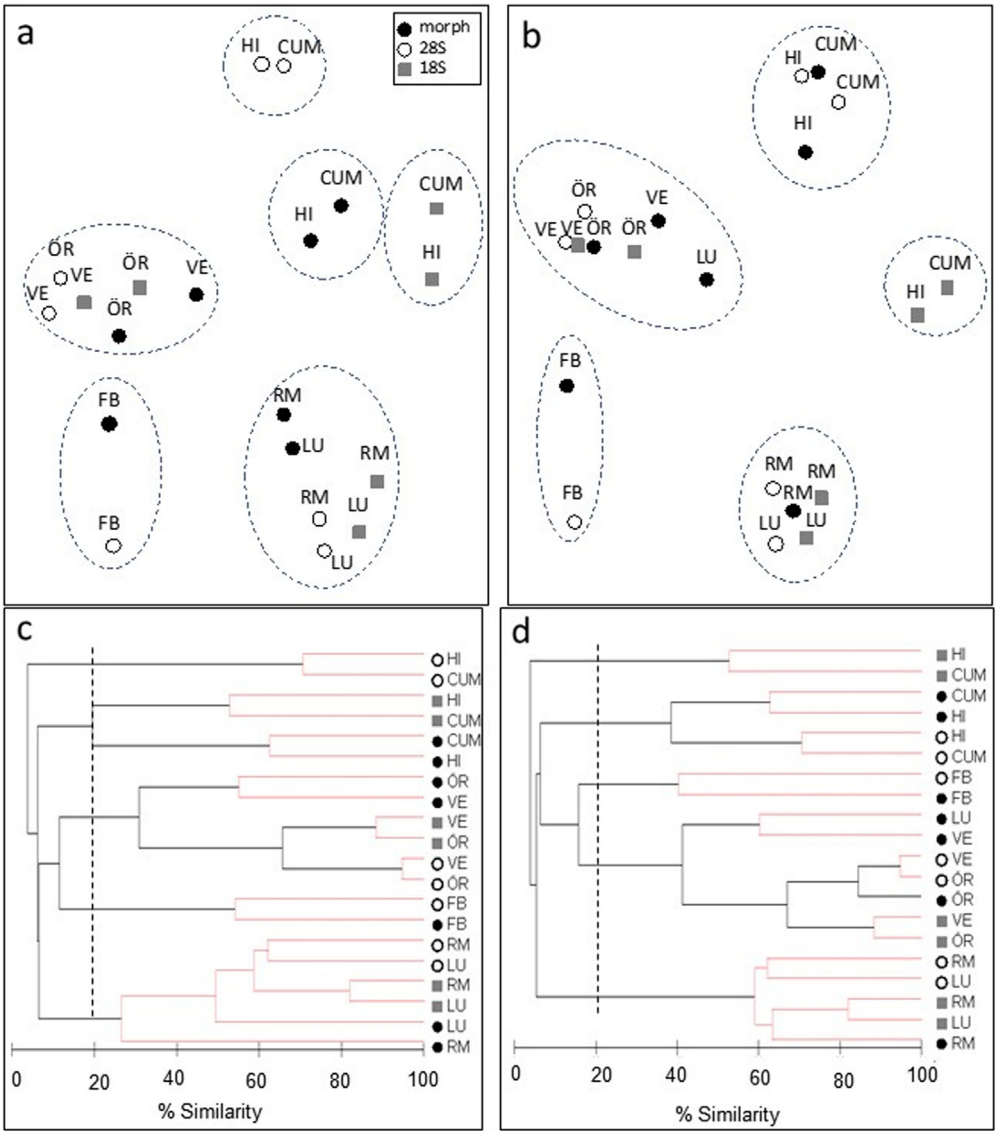

Fig. 3. nMDS (a and b) and hierarchical cluster analysis (c and d) comparing the nematode genus composition in river sediments sampled from 7 sites (CUM = Cumlosen; Hi = Hitzacker, RM = Mischmühle, LU = Luppe, ÖR = Örtze, VE = Veerse, FB = Furlbach); analyses used Bray-Curtis similarities of untransformed data based on abundance (a, c) and biomass (b, d); for the cluster analysis (c, d), non-significantly different data sets were identified by Similarity Profile Analysis (SIMPROF; $p < 0.05$) and are connected with red lines; the 20%-similarity level was used to define clusters laid over the respective nMDS plots (a, b); the analyses compared three different taxonomic methods: morphological taxonomy (morph) and the molecular taxonomy (28S, 18S); stress of nMDS plots: (a) = 0.20; (b) = 0.16.

the morphological data. However, whereas at the species level only the NemaSPEAR[%] based on metabarcoding of the 28S rDNA gene yielded acceptable results, on the genus level both the 28S- and the 18S-based indices correlated well with the morphologically derived NemaSPEAR [%]. These results show that molecular data can be used to establish nematodes as bioindicators, as already demonstrated for freshwater diatoms (Cordier et al., 2017) and marine macroinvertebrates (Aylagas et al., 2014). The lower congruence at the species than the genus level, attributable to incorrect taxonomic assignments mostly caused by missing reference sequences, also had consequences for the NemaSPEAR[%] calculations. For example, the discrepancy at the ÖR site between the NemaSPEAR[%] based on morphology (70.9%) vs. on the 28S data (32.8%) was mainly caused by the absence of *Eumonhystera* species in reference databases for the 28S rDNA gene. However, two dominant species represented up to 36.7% of the overall community and were therefore responsible for the high NemaSPEAR[%] classification. As explained above, the genus *Eumonhystera* is not included in the NemaSPEAR[%]$_{genus}$-calculation, which in the case of ÖR explains the better match of this index for the different taxonomic approaches. Similarly, the higher NemaSPEAR[%] value of the RM site based on morphological data (31.8%) was mainly influenced by the species *Punctodora ratzeburgensis* and *Monhystrella paramacrura*, for which 28S rDNA gene sequences are missing and the NemaSPEAR[%] value for this location based on molecular data was accordingly lower (14%).

Among the 58 nematode species listed as being at risk, 38% (22 species) are currently represented in the reference database for the 28S rDNA gene and 55% (32 species) for the 18S rDNA gene. Sequences for the 28S rDNA are missing completely for 62% (36 species), and 18S rDNA sequences are missing for 45% (26 species), such that species-level detections of these nematodes are not possible (Schenk et al., 2020, Table 8). At the genus level, 18S rDNA sequences have been deposited for 95% of the at-risk nematodes (36 out of 38 genera) and 28S rDNA sequences for 74% (28 genera;Schenk et al., 2020, Table 8), explaining the higher coverage at the genus level. Therefore, an integrated approach that couples nematode identification at the species level with single-specimen barcoding will improve reference databases and should especially aim for the missing NemaSPEAR[%] species and the most abundant nematode species as determined by Gansfort and Traunspurger (2019) and Heininger et al. (2007). From the 10 most abundant nematode species (> 20,000 analyzed), the extremely abundant *E. pseudobulbosa*, as well as *Daptonema dubium* are missing completely in the reference database, whereas for the also very abundant *E. longicaudatula* and *E. vulgaris* only one 18S rDNA reference sequence is available (NCBI accession: February 2020). These species should be preferential sequencing candidates for the future, while reliable primer pairs for the abundant genus *Eumonhystera* are also

needed. Further sequencing of species from Höss et al. (2017), particularly nematode species at risk, is also needed for more accurate sediment quality assessments, especially at the species level.

4.3. Community structure

Species abundance determinations are needed for the calculation of many indices but those obtained by metabarcoding approaches are potentially flawed (Darby et al., 2013; Lamb et al., 2019; Piñol et al., 2015). Recent studies indicate that species proportions rather than abundances are influenced by biomass, as already shown for ciliates (Pitsch et al., 2010) and macroinvertebrates (Elbrecht et al., 2017). Although our biomass-based analysis did not substantially improve the agreement between the molecular and morphological approaches, the congruence between the biomass-based morphological and 28S-based data for the most dominant species was higher, suggesting that the 28SrRNA gene can represent biomass proportions to a satisfying degree, as recently proposed by Schenk et al. (2019). Each of the three methods showed that all unpolluted sites with a low toxic potential clustered together (VE, FB, ÖR), regardless of the depiction method, as well as the locations HI and RM and the most polluted sites with the highest toxic potential in this study (LU, CUM; Fig. 3a, b). This underlines the strong potential of metabarcoding for the in-situ monitoring of nematode communities in freshwater sediments.

5. Conclusion

This study evaluated the application of the NemaSPEAR[%] based on molecular data and showed that the results achieved with this index were similar to those of the NemaSPEAR[%] based on conventional morphological inspection. While there were several shortcomings of the NemaSPEAR[%] at the species-level, NemaSPEAR[%]$_{genus}$ determined with the 28S and the18S rDNA markers was a reliable indicator of sediment quality. As NemaSPEAR[%]$_{genus}$ has already been validated (Höss et al., 2017), NemaSPEAR[%] derived from molecular data will enable molecular-based biomonitoring approaches, as successfully demonstrated by this study. Nematodes have several advantages with respect to biomonitoring, including their ubiquitous occurrence and high sensitivity towards pollutants. Thus, a molecular NemaSPEAR[%] will facilitate assessments of sediment quality. Given the declining number of taxonomic experts but also the new possibilities offered by high-throughput sequencing, our study contributes to the development of a method using nematode data in molecularly based bioindicator surveys, as suggested by other studies (Weigand et al., 2019). Further sequencing approaches with the priority of completing databases with the most abundant NemaSPEAR[%] species will help to achieve reliable results, while further validation with a larger dataset, especially with bulk extractions of whole nematode communities in order to circumvent time-consuming nematode isolation, will lead to the more rapid implementation of this approach.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Accessibility

Raw reads of Illumina sequencing are deposited at NCBI under the accession number PRJNA513975 and PRJNA608650. The data that support the findings of this study are available in Schenk et al., 2020 (submitted).

Authors Contributions

J.S., S.H., H.B-H. and W.T. planned and discussed the experimental design. H. B.-H. carried out the sampling and J.S. performed isolation of nematodes and laboratory work. J.S., S.H. and M.B calculated the NemaSPEAR[%] and analyzed the data, while N.K. performed bioinformatic analyses. All authors wrote and discussed the manuscript.

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