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Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study

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Summary

The influence of bacterivorous nematodes (*Diplolaimelloides meyli, Diplolaimelloides oschei, Diplolaimella dievengatensis, Panagrolaimus paetzoldi*) on the development of a bacterial community growing on decaying cordgrass detritus was studied in laboratory microcosm experiments. Cordgrass leaves were incubated on a sediment surface with a natural bacterial mixture containing bacteria from sediment, cordgrass detritus and habitat water. The four nematode species were applied separately to the microcosms; controls remained without nematodes. Samples were taken seven times over a 65-day period. The bacterial community structure was analysed by means of DGGE of the 16S rRNA genes. Multi Dimensional Scaling showed grouping of the samples per treatment. Analysis of Similarities indicated that the differences between treatments were significantly larger than differences within treatments. Our results suggest that nematodes can have a significant structuring top-down influence on the 'pool' of bacteria growing on the detritus, even at low densities. Dissimilarities were similar between all treatments. Differences in bacterial community composition within the treatments with monhysterids (*D. meyli, D. oschei and D. dievengatensis*) can be explained by species-specific food preferences. *Panagrolaimus paetzoldi* on the other hand feeds unselectively, and thus affects the bacterial community differently. A top-down effect of the nematodes on the diversity of the bacterial community was only evident under high grazing pressure, i.e. in the presence of *P. paetzoldi*.

Introduction

Heterotrophic bacteria and fungi are an important link in the functioning of many ecosystems, particularly for the mineralization of nutrients (Valiela, 1995). An increase in nutrient mineralization has been reported in the presence of grazers of bacteria, mainly nematodes and protists (Johannes, 1965; Ferris *et al*., 1997; Bonkowski *et al*., 2000). Grazers can affect bacterial communities at different levels. They are able to influence bacterial activity, either stimulatory (Findlay and Tenore, 1982; Alkemade *et al*., 1992a,b) or inhibitory (De Mesel *et al*., 2003). This can be a direct effect of grazing (Ingham *et al*., 1985), but bioturbation (Abrams and Mitchell, 1980; Alkemade *et al*., 1992b) and secretion of mucus trails (Riemann and Schrage, 1978; Jensen, 1996) by nematodes can also be important. Grazing impacts on the bacterial community structure have also been reported. Protists can cause a size shift by grazing the medium-sized part of the community (Hahn and Höfle, 2001). Van Hannen *et al*. (1999) were the first to demonstrate changes in the bacterial species composition under protist grazing pressure, whereas Rønn *et al*. (2002) found that these changes were protist-species specific. The structuring influence of nematodes, however, has not yet been studied well. Only Griffiths *et al*. (1999) reported bacterial shifts under nematode grazing using phospholipid fatty acid analysis, community level physiological profiling (using BIOLOG plates) and DNA fingerprinting (Denaturing Gradient Gel Electrophoresis). The shifts in the bacterial community composition would presumably be the result of selective grazing. For example, large and filamentous bacterial cells can escape uptake by some nematodes as a result of their small buccal cavity (Tietjen, 1980). Additionally, experimental data suggest that some nematode species can distinguish between and preferentially consume different strains of bacteria, the preference differing between even closely related nematode species (Tietjen and Lee, 1977; Moens *et al*., 1999).

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The aims of the present paper were: (i) to investigate the potential effect of bacterivorous nematodes on the bacterial community composition and diversity and (ii) to assess whether any such effects are nematode-species specific. Cordgrass (*Spartina alterniflora*) detritus was used as a substrate for the bacterial community. Cordgrass is often the dominant vegetation in the lower parts of salt marshes and decomposes mainly *in situ*, on the sediment surface (Groenendijk, 1984; Newell *et al*., 1985; Buth and Voesenek, 1988). High densities of bacterivorous nematodes occur on the detritus (Montagna and Ruber, 1980; Reice and Stiven, 1983; Buth and de Wolf, 1985; Hemminga and Buth, 1991).

Four bacterivore nematode species commonly found on decaying cordgrass leaves (*Diplolaimelloides meyli, Diplolaimelloides oschei, Diplolaimella dievengatensis* and *Panagrolaimus paetzoldi*) were inoculated separately in microcosms containing cordgrass detritus and a natural bacterial inoculum; controls without nematodes were incubated in parallel. *Panagrolaimus paetzoldi* (Rhabditida) is an extreme colonizer, appearing promptly at sites with high organic input where they can quickly reach high densities. The three other species belong to the Monhysteridae and are also characteristic for organically enriched sites. Rhabditida and Monhysteridae generally dominate the nematode community on decaying phytodetritus in salt marshes (Warwick, 1987). The choice of the three species within a single family (Monhysteridae) allows testing for different effects on the bacterial community between closely related grazer species, with a similar life strategy.

At seven sampling occasions over a period of 65 days, the bacterial communities were analysed by means of Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S rRNA gene. Denaturing gradient gel electrophoresis allows a fast screening of complex bacterial communities, without going into detail on community assemblage or functional aspects of the bacterial community, which was beyond the scope of this study. The use of DGGE was first introduced in ecological studies by Muyzer *et al*. (1993), and has since proven to be very useful in analysing bacterial communities originating from a large number of habitats (Muyzer, 1999). Recently, this technique has been applied successfully in a number of experimental studies (Degans *et al*., 2002; Massana and Jürgens, 2003; Zöllner *et al*., 2003)

Results and discussion

Effects on bacterial community composition

The bacterial community composition was analysed by means of Multi Dimensional Scaling (MDS) and Analysis of Similarities (ANOSIM), both non-parametric multivariate tests for changes in community structure (Clarke, 1993; Clarke and Warwick, 2001). The use of either relative band intensities, or of a reduced data matrix based on presence/absence of bands, to analyse community structure has been discussed and questioned before (Schauer *et al*., 2000). Both are commonly used, depending on the type of dataset studied. We believe that the use of relative band intensities in this study was justified because bands could clearly be seen fading away or appearing and intensifying through the succession. Using a binary dataset would strongly influence our analysis by overestimating very weak, sometimes uncertain bands. Moreover, Muylaert *et al*. (2002) showed that the use of relative band intensities is better suited for ordination analysis than a binary data matrix. Ordinations have been found very useful in ecological research in general and for processing community analyses by means of DGGE in specific (Fromin *et al*., 2002).

The MDS analysis on all samples from each sampling event showed a clustering per treatment (Fig. 1). A pronounced time aspect was observed in the presence of nematodes, but was less clear in the control. Although the stress value, indicating how faithful the high-dimensional relationship among the samples is represented in the 2D plot, was rather high, ANOSIM confirmed that the variation in DGGE profiles was higher between samples from different treatments than between samples within one treatment (Table 1). Similar observations have been made under protist grazing pressure in terrestrial soil systems (Rønn *et al*., 2002). The pairwise comparisons further showed that dissimilarities between all treatments were similar.

A first explanation for the differences between treatments may be related to differences in feeding habits between the nematode species used here. Panagrolaimidae are typical enrichment opportunists (Bongers *et al*., 1995). Direct observations indicate they non-selectively consume food particles at high rates as they show a constant pumping activity of the oesophagus (Moens, unpublished). As the passage through the gut is very fast, it is plausible that not all ingested food particles are digested, implying that some viable cells are again released. The subset of bacterial cells that maintains viability after passage through the gut is assumed to be protected mainly by their cell wall. Within the Monhysteridae however, bacterial selection would rather occur at the level of food ingestion. Moens *et al*. (1999) carried out a number of experiments with the monhysterid species used in our study, showing they can select between bacterial strains. Chemotaxic responses in which ‘taste’ and ‘smell’ of the prey would be of importance may determine this selection (Moens *et al*., 1999 and references therein). This implies that chemical cues emanating from the bacteria rather than bacterial cell wall structure influence their palatability and uptake by monhysterid nematodes. The

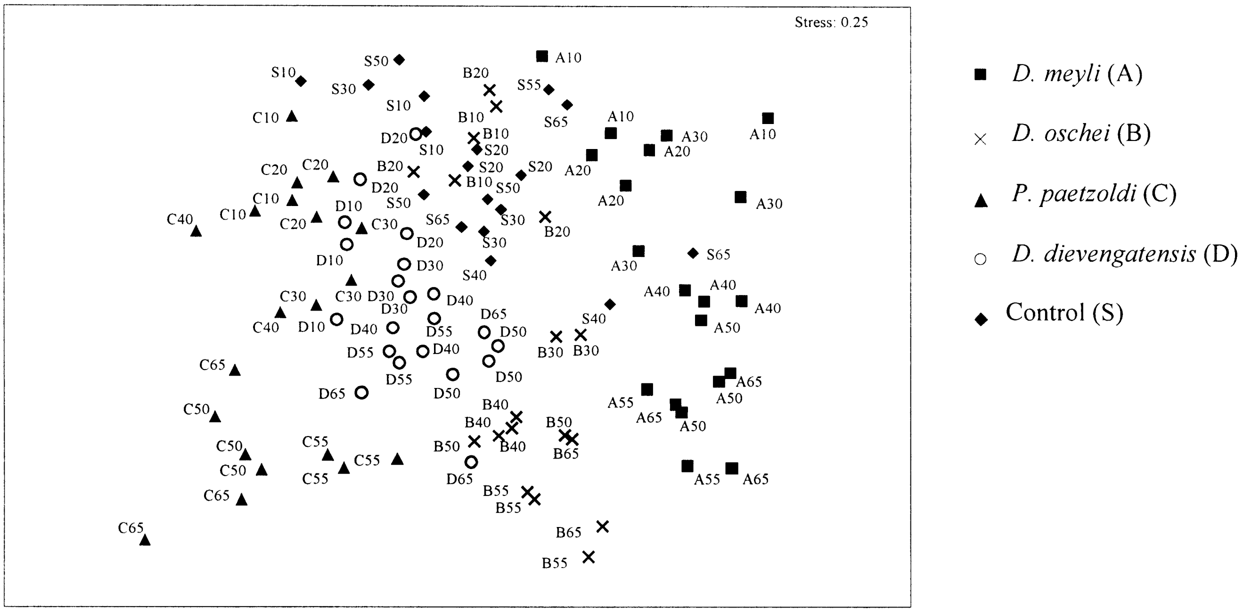


Fig. 1. MDS ordination plot based on the relative band intensities of the DGGE profiles, with the letter referring to the treatments and the number to the sampling day.

nematode-species-specific preference for different bacterial strains, observed by Moens *et al.* (1999), may have caused the development of different bacterial communities in the presence of each monhysterid nematode species.

The structure of the bacterial community may also have been affected by some other nematode traits that have been found important in stimulating bacterial activity. Bioturbation by the nematodes enhances diffusion of nutrients and oxygen through sediments (Abrams and Mitchell, 1980; Alkemade *et al.*, 1992b; Aller and Aller, 1992) and could thus affect the bacterial community structure. However, we assume that bioturbation is mainly of importance when organic matter is mixed in the sediment, and not, as in our experiment, when larger organic fragments decompose on the sediment surface (De Mesel *et al.*, 2003). Nematodes can secrete mucus trails which are characterized by a dense growth of bacteria (Riemann and Schrage, 1978; Jensen, 1996). These bacteria could be nematode-species-specific, but research on their identity is, to our knowledge, non-existent. However, this kind of 'cultivation' of bacteria by the nematodes is presumably of minor importance, as most bands can be found in two or more treatments, but with other relative intensities and/or different frequencies.

Sequencing of DGGE-bands provided some taxonomic information on the bacteria. The DNA fragments we cut out of the DGGE gel were 200 bp at most; therefore information at the species level could not be obtained. The identification is restricted to the genus or higher taxonomic level (see Table 2).

Most of the sequences showed high similarities (>95%) with species commonly found in marine and brackish environments. Members of the genus *Clostridium* are often found in marine sediments and on decaying vegetation and are characteristic for anaerobic environments. They

Table 1. Results of the ANOSIM routine to analyse the differences between treatments.

	Comparison		R Statistic	Significance level (%)
D. meyli	versus	*D. oschei*	0.727	0.1
D. meyli	versus	*P. paetzoldi*	0.838	0.1
D. meyli	versus	*D. dievengatensis*	0.839	0.1
D. meyli	versus	control	0.769	0.1
D. oschei	versus	*P. paetzoldi*	0.689	0.1
D. oschei	versus	*D. dievengatensis*	0.637	0.1
D. oschei	versus	control	0.774	0.1
P. paetzoldi	versus	*D. dievengatensis*	0.448	0.1
P. paetzoldi	versus	control	0.602	0.1
D. dievengatensis	versus	control	0.652	0.1

Table 2. Sequence similarities of the excised DGGE bands, for the position on the DGGE fingerprints, see Fig. 2. Unidentified species 7–9 only showed very low similarity with sequences of the database.

Band no.	Band name	Closest relative	Percentage similarity	Accession no.
1	*Clostridium* sp.1	*Clostridium algidixylanolyticum*	99%	AY510428
2	*Clostridium* sp.2	*Clostridium lentocellum*	95%	AY510429
3	*Clostridium* sp.3	*Clostridium* sp.	97%	AY510430
4	Unidentified 1	Uncultured CFB-group bacterium from ocean floor	96%	AY510431
5	Unidentified 2	Uncultured bacterium clone s3 form marine sediment	97%	AY510432
6	Unidentified 3	Uncultured eubacterium clone F10.2	96%	AY510433
7	Unidentified 4	Marine bacterium SCRIPPS_739	100%	AY510434
8	*Sulfurospirillum* sp.	*Sulfurospirillum* sp.	96%	AY510435
9	*Cytophaga* sp.	*Cytophaga* sp.	100%	AY510436
10	Unidentified 5	Uncultured gamma proteobacterium clone CD3B12	97%	AY510437
11	*Arcobacter* sp.	*Arcobacter* sp.	98%	AY510438
12	*Clostridium* related	Uncultured bacterium RSb40	100%	AY510439
13	*Oceanospirillum* sp.	*Oceanospirillum pusillum*	99%	AY510440
14	Unidentified 6	Uncultured bacterium clone ZB131	98%	AY510441
15	*Bdellovibrio* sp.	*Bdellovibrio* sp.	98%	AY510442
16	*Neptunomonas* sp.	*Neptunomonas naphthovorans*	96%	AY510443
17	*Anaerofilum* sp.	*Anaerofilum pentosovorans*	99%	AY510444
18	Unidentified 7	no match		AY510445
19	Unidentified 8	no match		AY510446
20	Unidentified 9	no match		AY510447

have the ability to break down a large number of organic compounds (Hippe *et al*., 1992). *Cytophaga* species are marine cellulose degraders (Reichenbach, 1992), whereas *Neptunomonas* species are marine bacteria breaking down aromatic hydrocarbons (Hedlund *et al*., 1999). *Oceanospirillum* species are often associated with particulate plant matter in marine sites (Pot *et al*., 1992). One *Arcobacter* species (*A. nitrofigilis*) has been described associated with *Spartina* roots in salt marshes (McClung *et al*., 1983). *Sulfurospirillum* and *Bdellovibrio* species occur in sediments of marine and freshwater systems (Ruby, 1992; Kersters *et al*., 2003). Unidentified species 1 belongs to the CFB-group bacteria. Unidentified species 2–6 showed high similarities with other partial sequences in the database of which no taxonomic information was available; no match with available sequences (< < 95%) was obtained for Unidentified species 7–9 (Table 2).

Similarity percentages (SIMPER) analysis was used to look for those bacterial species which contributed most to the average similarity within treatments and average dissimilarity between treatments (Clarke and Gorley, 2001). (Tables 3 and 4; see Table 2 for sequence information of the excised bands). *Clostridium* sp.1 and sp.2 and *Arcobacter* sp. were prominent members of the bacterial community in each treatment. Density differences in *Arcobacter* sp. generally explained a substantial part of the dissimilarity between treatments. One unknown species occurred only in the presence of *D. oschei* and explained most of the dissimilarity with other treatments. For the control, the presence of *Anaerofilum* sp. accounted for much of the dissimilarity with the nematode treatments.

In all treatments a clear shift of bands with time could be observed (Fig. 2), which, for the nematode treatments, was also reflected in the MDS plot (Fig. 1). Simper analysis (results not shown) of the effect of time within each treatment was performed to determine those bands which were most important in determining the succession. The dynamics of *Arcobacter* sp. were important in all treatments. In the presence of *D. meyli*, the appearance of *Sulfurospirillum* sp. from day 50 onwards and the dynamics of four unknown species determined the major differences in time. In the treatment with *D. oschei* the decreasing intensity of *Clostridium* sp.2, the disappearance of Unidentified sp. 6 and the appearance of two unknown species were of major importance. In the presence of *P. paetzoldi* the succession was primarily driven by dynamics in *Clostridium* sp.1, Unidentified sp.2 and three unknown species. The succession in the *D. dievengatensis* treatment was mainly determined by the appearance of *Oceanospirillum* sp. after 20 days and the variations of Unidentified sp.5 and two unknown species. In the control treatment the decreasing intensity of *Clostridium* sp.2 and the variation of Unidentified sp. 4 and two unknown species through time were the most important dynamics in the succession.

Effects on bacterial diversity

It was assumed that each band on the DGGE gel represents one 'Operational Taxonomic Unit' (OTU). However, some shortcomings of the DGGE technique should be considered when interpreting the results. Species that make up less than 1% of the total bacterial community will

Table 3. Results of the Simper analysis giving the similarities within treatments. Five species which contribute most to the similarity are listed. $\overline{A}$: average abundance of the i th species over all samples of the treatment; $\overline{S}_i$: contribution of the i th species to the total similartity; $\overline{S}_i/SD$: the value of the i the species as a discriminating species; $\overline{S}_i\%$: percentage contribution of the i th species to the total similarity; $\sum\overline{S}_i\%$: cumulative contribution to the total similarity.

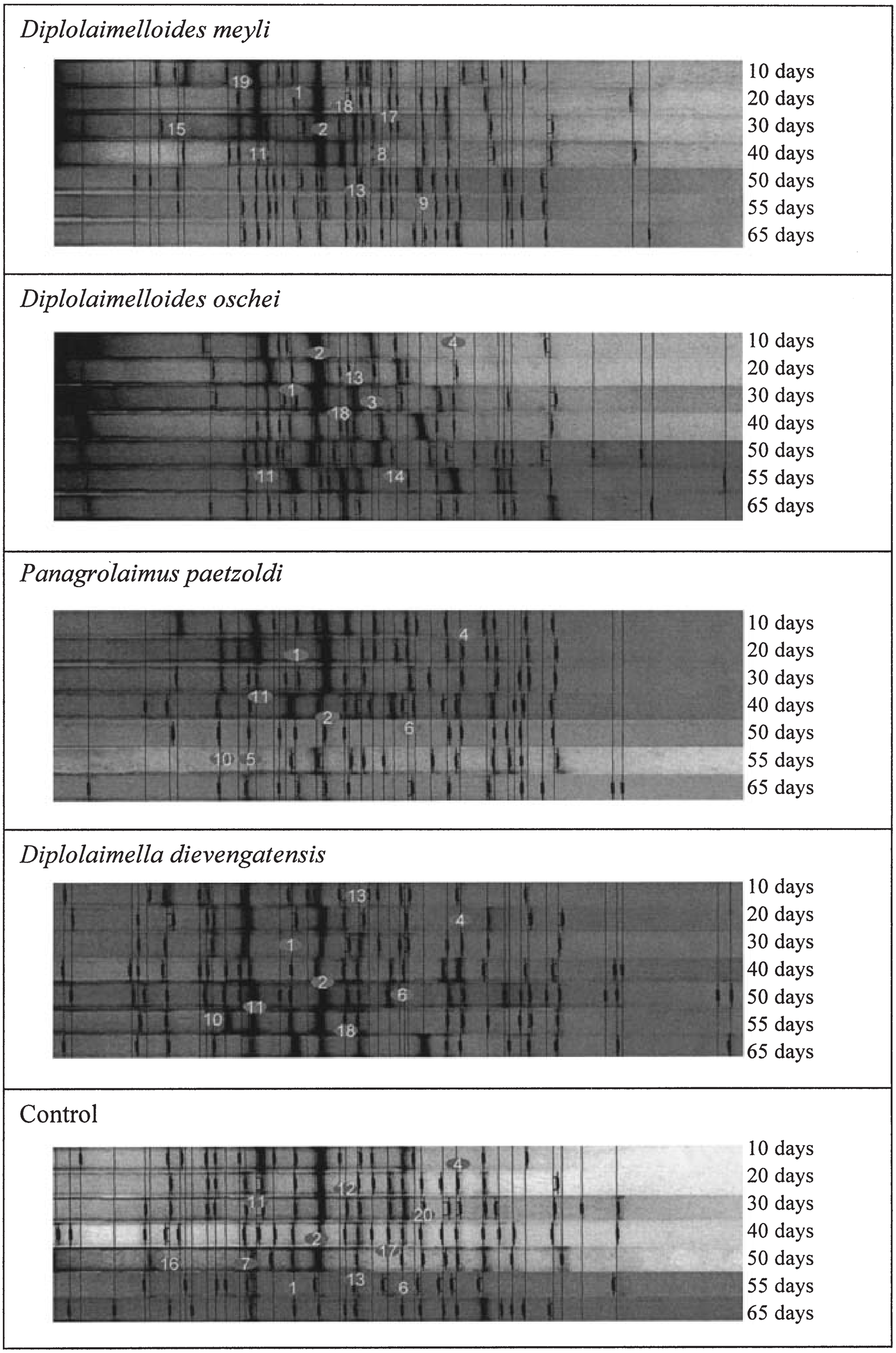
	$\overline{A}$	$\overline{S}_i$	$\overline{S}_i/SD$	$\overline{S}_i\%$	$\sum\overline{S}_i\%$
Diplolaimelloides meyli (average similarity: 47.56)					
Clostridium sp.2	0.23	6.78	1.67	14.25	14.25
Arcobacter sp.	0.19	4.87	1.27	10.24	24.49
Unknown 3	0.11	3.53	2.45	7.42	31.92
Unidentified 1	0.14	3.44	1.28	7.23	39.15
Clostridium sp.1	0.1	2.8	1.22	5.88	45.02
Diplolaimelloides oschei (average similarity: 47.54)					
Clostridium sp.2	0.25	8.62	2.3	18.13	18.13
Unknown 8	0.19	6.41	2.18	13.49	31.61
Arcobacter sp.	0.19	5.44	1.32	11.45	43.06
Unidentified 1	0.11	3.3	2.1	6.95	50.01
Clostridium sp.1	0.12	3.06	1.23	6.43	56.44
Panagrolaimus paetzoldi (average similarity: 42.48)					
Clostridium sp.2	0.28	10.57	3.66	24.88	24.88
Unidentified 5	0.12	3.66	1.24	8.62	33.49
Clostridium sp.1	0.12	3.02	1.04	7.12	40.61
Anaerofilum sp.	0.1	2.41	0.92	5.67	46.28
Arcobacter sp.	0.14	2.29	0.51	5.38	51.66
Diplolaimella dievengatensis (average similarity: 52.74)					
Clostridium sp.2	0.28	10.52	3.29	19.95	19.95
Arcobacter sp.	0.27	9.23	2.45	17.5	37.45
Unidentified 7	0.13	4.35	1.92	8.05	45.7
Oceanospirillum sp.	0.13	3.54	1.2	6.71	52.41
Clostridium sp.1	0.12	3.27	1.44	6.2	58.6
control (average similarity: 48.83)					
Clostridium sp.2	0.28	9.53	2.57	19.52	19.52
Arcobacter sp.	0.21	6.72	2.36	13.77	33.29
Unknown 5	0.15	4.06	1.23	8.32	41.61
Oceanospirillum sp.	0.14	3.96	1.64	8.11	49.72
Clostridium sp.1	0.11	3.27	1.34	6.7	56.42

Table 4. Results of the Simper analysis giving the dissimilarities between treatments. Five species which contribute most to the dissimilarity are listed. $\overline{\delta}_i$: contribution of the i th species to the total dissimilartity; $\overline{\delta}_i/SD$: the value of the i the species as a discriminating species; $\delta\%$: percentage contribution of the i th species to the total dissimilarity; $\sum\overline{\delta}_i\%$: cumulative contribution to the total dissimilarity.

	$\overline{A}$	$\overline{\delta}_i/SD$	$\overline{\delta}_i\%$	$\sum\overline{\delta}_i\%$
D. meyli versus *D. oschei* (average dissimilarity 68.27)				
Unknown 8	3.93	2.72	5.75	5.75
Arcobacter sp.	2.74	1.38	4.02	9.77
Unknown 18	2.45	1.12	3.58	13.35
Unknown 1	2.37	1.1	3.47	16.82
Clostridium sp.1	2.29	1.28	3.35	20.17
D. meyli versus *P. paetzoldi* (average dissimilarity: 75.46)				
Arcobacter sp.	3.54	1.44	4.69	4.69
Unidentified 1	2.82	1.55	3.74	8.43
Unidentified 5	2.59	1.75	3.44	11.87
Unidentified 2	2.51	1	3.32	15.19
Unknown 1	2.38	1.15	3.15	18.34
D. meyli versus *D. dievengatensis* (average dissimilarity: 68.91)				
Arcobacter sp.	2.96	1.41	4.29	4.29
Unknown 1	2.37	1.11	3.43	7.72
Unknown 14	2.34	1.64	3.4	11.12
Clostridium sp.1	2.28	1.18	3.31	14.43
Unknown 3	2.25	2.84	3.27	17.69
D. meyli versus control (average dissimilarity: 68.96)				
Anaerofilum sp.2	2.95	1.46	4.27	4.27
Unidentified 7	2.75	1.55	3.99	8.27
Arcobacter sp.	2.56	1.43	3.71	11.97
Clostridium sp.1	2.51	1.27	3.63	15.61
Unknown 1	2.37	1.1	3.44	19.04
D. oschei versus *P. paetzoldi* (average dissimilarity: 70.59)				
Unknown 8	3.88	2.41	5.5	5.5
Arcobacter sp.	3.53	1.46	5	10.51
Unidentified 5	2.59	1.72	3.67	14.18
Unidentified 2	2.55	1	3.62	17.8
Unknown 18	2.43	1.08	3.45	21.25
D. oschei versus *D. dievengatensis* (average dissimilarity: 61.80)				
Unknown 8	3.64	2.27	5.89	5.89
Arcobacter sp.	2.78	1.31	4.5	10.39
Unknown 18	2.4	1.07	3.88	14.28
Unknown 14	2.38	1.64	3.86	18.13
Unidentified 5	2.09	1.11	3.38	21.51
D. oschei versus control (average dissimilarity; 67.39)				
Unknown 8	3.91	2.6	5.8	5.8
Anaerofilum sp.2	2.9	1.43	4.3	10.1
Arcobacter sp.	2.39	1.34	3.55	13.65
Unknown 18	2.37	1.09	3.52	17.17
Unidentified 7	2.31	1.14	3.43	20.61
P. paetzoldi versus *D. dievengatensis* (average dissimilarity: 61.61)				
Arcobacter sp.	4.11	1.49	6.67	6.67
Unidentified 7	2.78	2.12	4.51	11.18
Unidentified 2	2.57	1	4.18	15.36
Unknown 14	2.46	1.54	4	19.35
Oceanospirillum sp.	2.43	1.51	3.94	23.3
P. paetzoldi versus control (average dissimilarity: 67.37)				
Arcobacter sp.	3.56	1.67	5.28	5.28
Anaerofilum sp.2	2.67	1.33	3.96	9.24
Unidentified 5	2.64	1.74	3.92	13.17
Unidentified 2	2.56	1	3.79	16.96
Oceanospirillum sp.	2.54	1.6	3.77	20.73
D. dievengatensis versus control (average dissimilarity: 61.20)				
Anaerofilum sp.2	2.71	1.36	4.43	4.43
Unidentified 7	2.71	2.12	4.43	8.86
Arcobacter sp.	2.43	1.31	3.97	12.83
Unidentified 5	2.11	1.1	3.45	16.28
Unknown 14	2.1	1.44	3.43	19.71

not be represented on a DGGE-gel (Muyzer *et al*., 1993). The presence of multiple heterogeneous rRNA operons might result in multiple bands from one bacterial species (Nübel *et al*., 1996) and co-migration of sequences might also bias the banding profile (Vallaeys *et al*., 1997). Therefore, the diversity index calculated from the DGGE banding patterns should not be interpreted as an absolute measure, but rather as an indication of bacterial diversity (Eichner *et al*., 1999). Nübel *et al*. (1999) showed that the number of bands and the Shannon–Wiener diversity index can be used as an estimate of the bacterial diversity. Because all the samples in our experiment were treated in the same way, it is expected that bias operated uniformly on all samples allowing proper comparison of the results (Schauer *et al*., 2000; Fromin *et al*., 2002).

Bacterial diversity is represented by the Shannon–Wiener Index (Fig. 3), which integrates information on the number of bands and on the band-intensities. The number of bands (data not shown) showed a similar pattern through time as the Shannon–Wiener Index.



Diplolaimelloides meyli

Diplolaimelloides oschei

Panagrolaimus paetzoldi

Diplolaimella dievengatensis

Control

Fig. 2. DGGE fingerprints with indications of the band classes. Numbers refer to Table 2.

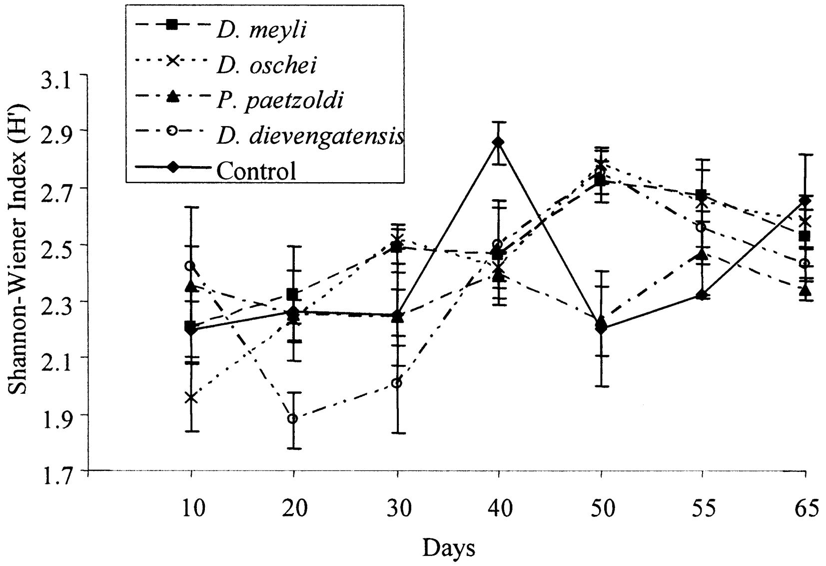


Fig. 3. Shannon–Wiener Index (H′) during the course of the experiment (average of three values ± one standard error).

Figure 3 shows that diversity reached a peak after 40 days in the controls and after 50 days in the presence of the monhysterids. Diversity was rather constant in the *P. paetzoldi* treatment. A Tukey HSD pairwise posthoc test only showed significant differences within treatments in the presence of *D. oschei*, in which the index on day 10 was significantly lower than on day 50 ($P < 0.01$) and day 55 ($P < 0.05$), and in the *D. dievengatensis* treatment where the index on day 50 was significantly higher than on day 20 ($P < 0.01$) and day 30 ($P < 0.05$). No significant differences between treatments could be found at any sampling occasion.

The generally lower bacterial diversity from 30 days onwards in the presence of *P. paetzoldi* compared with the treatments with Monhysteridae could be ascribed to the significantly higher densities of the former species. From that moment onwards, *P. paetzoldi* reached densities up to 10^5 individuals per microcosm, whereas the monhysterid nematode species (*D. meyli, D. oschei, D. dievengatensis*) attained densities of a few thousand individuals (De Mesel *et al.*, 2003). The high densities of *P. paetzoldi* could have led to overgrazing of the bacterial community. This effect will have been intensified by the unselective feeding habit of this species, enabling it to eliminate a large proportion of the bacterial community. A high grazing pressure in the presence of *P. paetzoldi* was confirmed by measurements of respiration and proteolytic activity of the microbial community (Fig. 4A) (De Mesel *et al.*, 2003). A reduction of diversity at the bacterial level in the presence of high protist densities has been observed before (van Hannen *et al.*, 1999). Most information on the relationship between grazer densities and prey diversity, however, originates from studies using herbivores and plants as a model (Valiela, 1995 and references therein; Hillebrand, 2003). These studies confirm low diversity under high grazing pressure, and suggest that highest prey diversity develops under intermediate grazing pressure. Applying this to our experiment, the highest bacterial diversity would be expected in the presence of the monhysterid species. This however, was not unambiguously confirmed by our findings. Only on day 50 and day 55 was bacterial diversity higher in all monhysterid treatments versus the control; with none of these differences being statistically significant. We assume that the monhysterid densities were not high enough to reach an ‘intermediate disturbance’ level as the numbers of monhysterid nematodes obtained in our experiments were situated at the lower end of the range found in field conditions. Furthermore Herman and Vranken (1988) showed that monhysterids only affected a small subset of the standing stock of bacteria, even at higher densities than those reached in this experiment. Additionally we found that in these treatments diversity was linked to nutritional value (N-content) of the leaves (Fig. 4B), rather than to nematode densities. The Shannon–Wiener Index was lowest at the beginning and at the end of the experiment, and reached a peak around 50 days. Initially, few opportunistic bacterial species will have benefited from the availability of high concentrations of labile compounds. As these easily degradable compounds became depleted, opportunists became replaced by a more diverse community of decomposers. Towards the end of the experiment, the detritus mainly consisted of refractory substances, which can only be broken down by few specialized species. Similar dynamics in bacterial diversity were seen in the control, however, at the end of the experiment an unexpected increase of bacterial diversity occurred. This coincides with a peak of nitrogen content in the organic matter (De Mesel *et al.*, 2003), which may

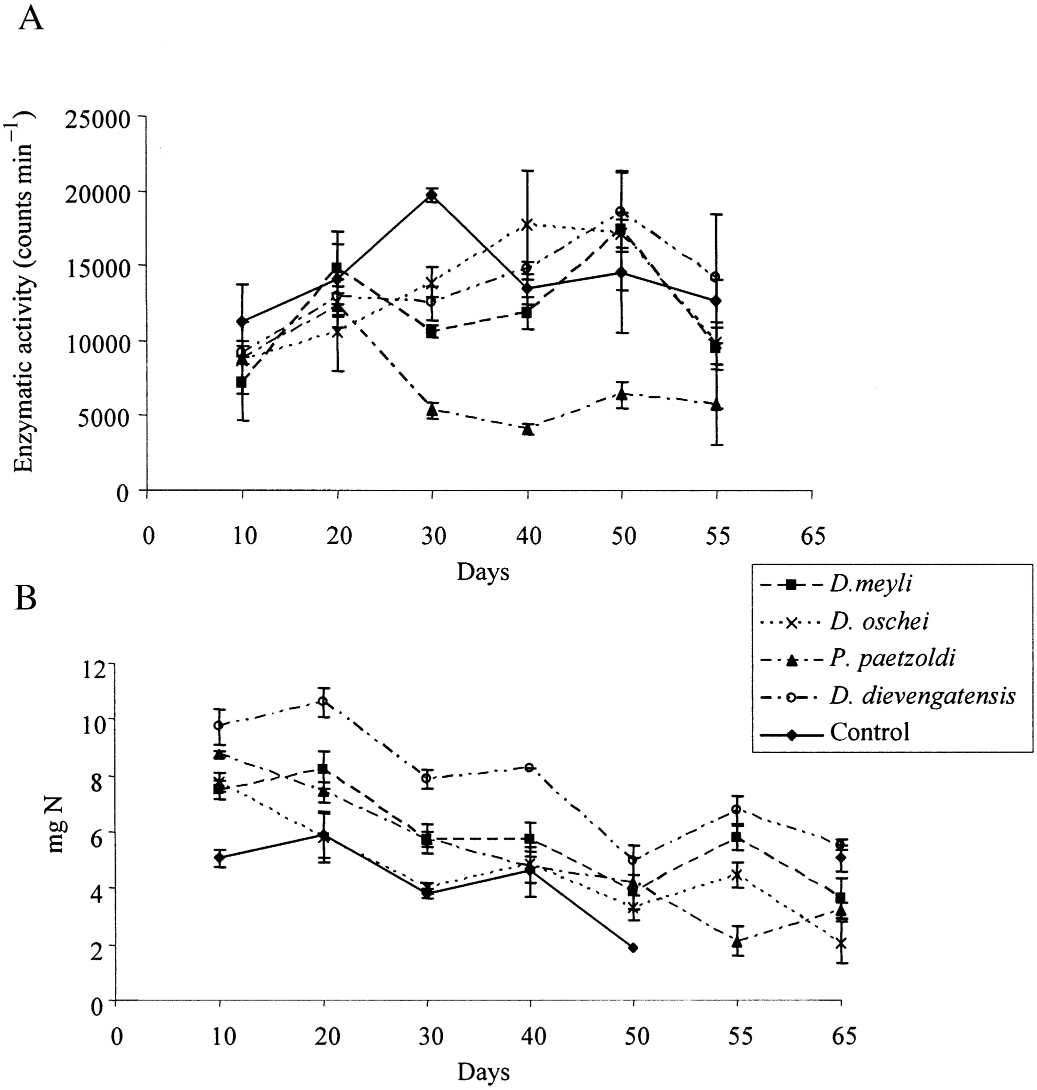


Fig. 4. A. Bacterial activity expressed as proteolytic activity (average of three samples ± one standard error).
B. N-content of the cordgrass leaves, expressed as mg per sample (average of three replicates ± one standard error) (De Mesel *et al.*, 2003).

be a result of the binding of nitrogen-rich compounds to the cordgrass detritus (White and Howes, 1994).

Conclusion

Our results suggest that the bacterial community composition in the microcosms was shaped by a combination of bottom-up and top-down effects: the same bacterial species appeared in different treatments, indicating that the types of bacteria occurring were primarily determined by the substrate. However the composition and the relative importance of different members of this 'pool' of bacteria was severely modified by the grazing activities of the bacterivorous nematodes, even at relatively low densities. A nematode effect on bacterial diversity was only evident under high grazing pressure, as in the presence of *P. paetzoldi*.

Experimental procedure

Experimental setup and sampling

The experimental set up has been described in detail in De Mesel *et al.* (2003). Briefly, green cordgrass leaves (*Spartina anglica*) collected in the Paulina Salt Marsh (Westerscheldt, the Netherlands) were cleaned with ethanol (70%) and artificial sea water (ASW) (Dietrich and Kalle, 1957), put in Petri dishes (Ø 90 mm) on cleaned sediment saturated with ASW with a salinity of 20, and inoculated with a microbial inoculum. This inoculum consisted of bacteria from fresh and decomposing cordgrass leaves, sediment and habitat water and from the nematode cultures. This mixture was filtered over Whatman GF/C filters to remove flagellates and other small eukaryotes. All Petri dishes were inoculated with 2 ml of this suspension. After an incubation of 24 h nematodes were added to the microcosms.

Four nematode species, commonly found on decaying cordgrass in the Paulina Salt Marsh (T. Moens, unpublished) were selected: *Diplolaimelloides meyli*, *Diplolaimelloides oschei*, *Diplolaimella dievengatensis* and *Panagrolaimus paetzoldi*. These species are available in monospecific cultures in the Marine Biology Section. Each microcosm, except for the controls, received 36 individuals of a single nematode species. Microcosms were incubated in the dark at 20°C. Three replicate microcosms per treatment were destructively sampled after each of the following incubation times: 10, 20, 30, 40, 50, 55 and 65 days. However, for the following days and treatments, only two replicates were successfully analysed: *D. meyli*: day 55; *D. oschei*: day 30, 50 and 65; *P. paetzoldi*: day 40; control: day 40 and 55. Detritus fragments were homogenized in sterile ASW with a salinity of 20 to collect the bacterial community. Four millilitres of this suspension was centrifuged at 13000 r.p.m. After removing the supernatant the pellet was preserved at −80°C.

DNA-extraction and -purification

DNA was extracted as described by Zwart *et al.* (1998) using the bead-beating method concomitant with phenol extraction and ethanol precipitation. The DNA was purified on a Wizard column (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

PCR and DGGE

The amplification of DNA fragments and the DGGE analysis were performed as described by Van der Gucht *et al.* (2001). DNA fragments of about 250 bp were amplified using primers for the V3-region: F357-GC primer (5′-CGCCCGCCGCGC CCCG CGCCCGGCCCGCCG CCCCCGCCCCCCTACGGG AGGCAGCAG-3′) and the R518 primer (5′-ATTACCGCGG CTGCTGG-3′) which are specific for most bacteria (Muyzer *et al.*, 1993). The temperature cycling started with a preincubation of 5 min at 94°C. Twenty cycles were performed consisting of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, with the temperature decreasing 0.5°C every cycle until a temperature of 56°C was reached, and primer extension at 72°C for 1 min. During the next five cycles the annealing temperature stayed at 55°C for 1 min. Finally temperature was held at 72°C for 10 min

DNA concentrations were checked visually by analysing 5 µl of the product on 1% agarose gel, staining with ethidium bromide, and comparison with a molecular weight marker (Smart ladder, Eurogenetic). Thirty-five µl of the samples were loaded on the DGGE gels. The linear gradient on the DGGE-gels increased from 40% at the top to 75% at the bottom. To compare the banding patterns between gels a marker was loaded and some experimental samples were run on different gels. Additionally the information on the DNA sequence of a number of bands was used to make a reliable alignment of the gels.

The software package Bionumerics 5.1 (Applied Maths BVBA, Kortrijk, Belgium) was used to analyse the banding patterns. The Bionumerics software measures an optical density profile through each lane (corresponding to a single sample), identifies band positions, and calculates the percent contribution of the intensity of each band to the total intensity of the lane. This procedure yielded a matrix with the relative intensity of each band in all samples.

Sequencing of excised DGGE bands

The procedure used was based on Van der Gucht *et al.* (2001), but with some small modifications. Individual bands were excised from the DGGE-gel and put in a sterile recipient to which 50 µl 1X TE was added. DNA could diffuse form the acrylamide gel to the TE-buffer during incubation overnight at 4°C. Five µl of the buffer was used for amplification (PCR mix and temperature cycle as described above) and the position of the band was checked on a DGGE gel. DNA from the bands was then amplified with the F357GC primer (5′-CG CCCGCCGC GCCCCGCGCCCGGCCCGCCGCCCCCG CCCCCCTACGGGAGGCAGCAG-3′) and the R518 + primer (5′-GCGTTCTTCATCGTTGCGAGATTACCGCGGCTGCTGG-3′) using the temperature cycling as described by Van der Gucht *et al.* (2001), followed by a sequencing reaction with a Big Dye^{TM} terminator ready reaction kit (Applied Biosystems, Foster City, CA, USA) and the Stef1Tex primer (5′-GCGTT CTTCATCGTTGCGAG-3′). The temperature cycling was as described by Zwart *et al.* (1998). Sequencing reactions were analysed on an Applied Biosystem 3100 genetic analyser.

Bands were identified by comparing the partial 16S rRNA gene sequences with GenBank and EMBL sequences with BLAST (see Table 1) (Altschul *et al.*, 1990).

Bacterial diversity

The Shannon Wiener Index (H′) was used to express bacterial diversity and was calculated as explained by Magurran (1988), based on the relative intensities of the bands:

$$H' = -\sum_{i=1}^{n} p_i \ln p_i$$

n represents the number of bands in the sample and p_i the relative intensity of the ith band. The index indicates how the intensity of the bands is distributed within a sample.

Statistical analysis of the DGGE fingerprints

The banding profiles of the DGGE gels were analysed by means of Multi Dimensional Scaling (MDS) using the PRIMER software package and based on the arcsin squareroot transformations of the relative band intensities. The Bray-Curtis index was chosen as similarity coefficient. Multi dimensional scaling analysis places samples in a multi dimensional space, based on the similarities between them. This multidimensional space can be represented by a 2D ordination plot, with the stress value indicating how faithfull the high-dimensional relationships between the samples are represented in the 2D plot. This plot is very easy to interpret as similar samples are placed close together, and distance between samples increases as similarity decreases (Clarke, 1993; Clarke and Gorley, 2001; Clarke and Warwick, 2001).

Analysis of similarities (ANOSIM) is a non-parametric permutation procedure, based on the similarity matrix underlying the ordination of the samples. ANOSIM was used to test for significant differences between treatments (Clarke, 1993; Clarke and Gorley, 2001; Clarke and Warwick, 2001).

Similarity percentage analysis (SIMPER) was used to weight the contribution of each band to the similarity/dissimilarity within/between treatments and to seek for those bands which were important during the succession in each treatment (Clarke and Gorley, 2001; Clarke and Warwick, 2001).

Univariate 2-way analysis of variance (ANOVA) and the Tukey HSD post hoc test were used to test for differences in the Shannon-Wiener Index (H′) between treatments, times and treatment × time (Statistica software).

Nucleotide sequence accession numbers

All partial sequences have been deposited in the EMBL database under accession nos AY510428-AY510447.

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