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Odor compounds from cyanobacterial biofilms acting as attractants and repellents for free-living nematodes

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Abstract

Nematodes can both taste and smell an array of compounds, but whether and how these senses effect their capacity to locate microhabitats in aquatic environments is not known. Cyanobacterial biofilms may offer structure, shelter, and food for nematodes and are known to produce a variety of odor compounds. We studied the chemotaxis response of the freshwater nematode *Bursilla monhystera* (Rhabditidae) and the terrestrial model organism *Caenorhabditis elegans* (Rhabditidae) to odors of cyanobacterial biofilms. We used gas chromatography–mass spectometry ultra-trace analysis to identify odor compounds produced by two epilithic cyanobacterial biofilms of Lake Zurich. We also studied artificial, axenic biofilms of *Plectonema* sp., *Calothrix* sp., and *Calothrix parietina*, to assign these compounds to the metabolism of the cyanobacteria. The axenic cyanobacteria and epilithic biofilms had many odor compounds in common. *B. monhystera* was significantly attracted to *Plectonema* sp. and *C. parietina* but not to *Calothrix* sp. *C. elegans*, in contrast, was not attracted to any of these cyanobacterial biofilms. Furthermore, we applied a multicomponent mixture of odor compounds and found significant attraction for both nematodes. Although *C. elegans* was also attracted by a variety of single odor compounds, *B. monhystera* was not attracted to any of the volatiles tested. ß-ionone even repelled this species. Our experiments demonstrate that aquatic nematodes are attracted to cyanobacterial biofilms using odor compounds as chemical cues. In contrast to the model organism, *C. elegans*, the chemotaxis of the aquatic nematode is elicited by a multicomponent odor rather than by single compounds.

Many interactions among aquatic organisms are governed by infochemicals. Food webs are effectively regulated by this kind of interaction, when chemical signals of food and prey organisms trigger attraction or repulsion of grazers and predators (Wolfe 2000; Zimmer and Butman 2000). As in mammals, odor and taste receptors are well developed in invertebrates, and both senses affect animal behavior (Bargmann and Mori 1997).

Cyanobacteria produce and release a number of volatile organic compounds (VOCs), which may be infochemicals (Jüttner 1987; Höckelmann and Jüttner 2004). They are well known for their off flavors, geosmin and 2-methylisoborneol, earthy smelling compounds with high economic relevance in the fish industry and drinking water production (Wnorowski 1992), but the ecological roles of cyanobacterial VOCs remains to be studied (Watson and Cruz-Rivera 2003).

On hard substrates in the littoral zone of lakes and running waters, filamentous cyanobacteria often form a dense network of entangled trichomes (Stal and Caumette 1994). Cyanobacteria secrete extracellular polymeric compounds that facilitate attachment to surfaces and protection against environmental fluctuations and desiccation (Decho 1994; Potts 1999). Cyanobacterial biofilms may also play a role as a safe habitat in which other organisms are protected against drifting or desiccation. Some aquatic insects locate biofilms, in which they deposit their eggs, through cyanobacterial odor compounds (Evans 1982; Rejmankova et al. 2000).

Nematode communities inhabiting cyanobacterial biofilms have been poorly documented, except for some microbial mat communities from extreme environments (Gerdes et al. 1985; Gerdes and Krummbein 1987; Farmer 1992). However, it would appear, from the limited information available, that nematode densities in cyanobacterial biofilms are in the

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same order as in sediments, peaking at values up to 1.6×10^6 individuals m^{-2} (Farmer 1992). Whether and how nematodes may influence microbial biofilms remains a matter for debate. Several authors have suggested that meiofauna in general and nematodes in particular affect mat communities through bioturbation and grazing (Bauld 1984; Walter and Heys 1985; Farmer 1992), but poor knowledge of the feeding habits of most aquatic biofilm–inhabiting nematodes hampers any proper assessment of their trophic roles (Moens and Vincx 1997).

The patchiness of aquatic nematodes in relation to microbiota is specific and may involve chemotaxis (Moens et al. 1999*b*). However, the chemical sense of orientation in aquatic nematodes has not been well investigated. To our knowledge, only one study has addressed the chemical background for the observed taxis of an aquatic nematode (Riemann and Schrage 1988). Most information on chemical orientation in nematodes stems from work on terrestrial species. *Caenorhabditis elegans* can distinguish between olfactory and taste sense (Bargmann and Mori 1997). In this organism, odor compounds are detected by the AWA, AWB, and AWC receptors in picomolar and water-soluble compounds, in micromolar concentrations, by eight taste receptors (Bargmann and Mori 1997).

Studies on the chemotaxis of terrestrial nematodes have shown that attraction or repellence is elicited by single odor compounds (Bargmann et al. 1993; Troemel et al. 1997). However, most organisms such as the marine sponge *Ircinia muscarum* secrete multicomponent VOCs (De Rosa et al. 2002), which raises the question of whether chemotaxis responses are triggered by single compounds or by multicomponent mixtures. To our knowledge, this question has been addressed for the first time in insect ecology (Silverstein and Young 1976). These authors initiated the discussion that a pheromone can be a multicomponent odor. In aquatic systems, the relevance of multicomponent mixtures has been reported by Zimmer-Faust (1989).

In the present study, we focused on the chemical orientation of free-living nematodes to cyanobacterial biofilms. We identified VOCs from natural epilithic biofilms, and used artifical biofilms of monoxenic and axenic cyanobacterial cultures to verify the cyanobacterial origin of these odor compounds. Chemotaxis assays were performed to investigate the short-distance movement (5.5 cm) of two nematode species, the freshwater nematode *Bursilla monhystera* and the terrestrial model organism *C. elegans*, towards or away from these odor compounds.

Materials and methods

Cultivation of microorganisms and collection of biofilms—Axenic strains of *Plectonema* sp. (PCC 7410), *Plectonema notatum* (PCC 6306), *Calothrix* sp. (PCC 7507), and *Calothrix parietina* (PCC 6303) were cultivated as described elsewhere (Höckelmann and Jüttner 2004). Monoxenic cultures of *Phormidium* sp., *Tolypothrix distorta*, and *Rivularia* sp. were isolated from epilithic biofilms in Lake Zurich (Switzerland) and cultivated according to the same reference. Two epilithic cyanobacterial biofilms—a *Rivularia* sp./*C. parietina* community (biofilm A) and an encrusted layer (biofilm B)—were obtained from the shore of Lake Zurich in October 2002. Organisms of both biofilms were identified by light microscopy. The *Escherichia coli* strain OP50, which was used as food for *C. elegans* (see below), was cultivated for 2 d at 37°C, in the dark, on a rotary shaker (120 rpm).

Cultivation of nematodes—A wild-type strain (N2 variety, Bristol) *C. elegans* (Rhabditidae) culture was grown at 20°C in the dark on Nematode Growth Medium (NGM) agar with excess *E. coli* (strain OP50) as food (Sulston and Hodgkin 1988). Crowding was avoided by regular subculturing. The freshwater nematode *Bursilla monhystera* (Rhabditidae) was isolated from epilithic biofilms in a small pond in the botanical garden at Ghent University, Belgium. *B. monhystera* was cultivated at 20°C in the dark on a half-concentration cyanobacterial medium with 0.7% agar (pH 7.0; Jüttner et al. 1983).

VOC analysis—Closed-loop stripping, thermodesorption, and gas chromatography–mass spectrometry (GC/MS; GC 8000 Top, MD 800; Fison Instruments) were applied to analyze VOCs produced by cyanobacteria (Jüttner 1988; Höckelmann and Jüttner 2004). Biofilms were immediately analyzed in the laboratory for VOCs. For VOC analysis of biofilm B (but not A), cells were minced with a knife. The resulting cell lysis activates specific enzymes such as the lipoxygenase. The VOCs that are produced by these enzymes cannot be found without prior activation.

Tested cyanobacterial strains and concentration of odor compounds—The culture of *Plectonema* sp. used in bioassays with *C. elegans* was 2 months old and, in assays with *B. monhystera*, 9 months old. We used 30 and 10 μl of the cyanobacterial suspension in assays with *C. elegans* and *B. monhystera*, respectively. The culture of *C. parietina* was 3 months old, and two bioassays were performed: one with 10 μl of suspension (live biomass with growth medium) and one with activated biomass. For activation, the biomass was treated with liquid nitrogen to lyse cells and activate enzymes. A similar amount of activated biomass and of live biomass was used in the bioassays. The culture of *Calothrix* sp. was 9 months old and was tested only as activated biomass. Furthermore, VOCs found in the epilithic biofilms of Lake Zurich and in the different axenic and monoxenic cyanobacterial cultures were tested as potential odor compounds (Table 1). Additionally, a ''multicomponent odor,'' a mixture of cyanobacterial VOCs primarily of *Plectonema* sp. (Table 2), was tested (Höckelmann and Jüttner 2004; Höckelmann and Jüttner in press).

Chemotaxis assays: C. elegans—Chemotaxis assays were performed on square plates (12×12 cm) that contained 20 ml of the corresponding nematode cultivation agar. Bioassays with *C. elegans* were performed as population assays with at least 10 individuals. These assays were similar to those described by Bargmann et al. (1993) and Troemel et al. (1997), with the exception that plates were divided into four equal sectors (labeled A, B, C, and D) instead of six,

Table 1. Concentrations of the single odor compounds used in the bioassays with *C. elegans* and *B. monhystera*. — not tested.

Odor compound	*C. elegans* (μmol)	*B. monhystera* (μmol)
2-butanone	11.0	0.1
2-pentanone	9.1	—
2-pentanol	9.2	0.09
3-pentanone	9.7	—
3-pentanol	9.3	—
2,3-pentandione	—	0.09
2,3-hexandione	7.4	0.07
2-hydroxy-3-hexanone & 3-hydroxy-2-hexanone	2 μl	0.03 μl
3-pentanol	9.3	—
3-methyl-3-buten-1-ol	9.6	0.1
3-methyl-2-buten-1-ol	9.7	—
6-methyl-5-hepten-2-one	6.7	0.07
	0.07	—
6-methyl-5-hepten-2-ol	6.6	—
	0.07	—
β-ionone	4.7	0.5
dihydro-β-ionone	4.8	0.5
1,2,3-trimethyl-2,7-dioxabicyclo (2,2,1) heptane (TDH)	6.3	0.6
(−)-geosmin	0.0001	0.0001
skatol	0.0001	0.0001
indol	0.001	0.001
heptadecane	3.2	—
7-methylheptadecane	3.2	—
8-methylheptadecane	3.2	—

and the odor was spotted in a line (2 cm long) on the lid (and not on the agar surface). A potential disadvantage of this experimental design is that concentration gradients of VOCs may be affected by advection and turbulence, transporting odors over the Petri dish surface in basically unpredictable patterns. To determine the distribution of VOCs over time, we performed an iodine-starch test. An iodine crystal was fixed with grease on the lid of the Petri dish at the same position where VOCs were spotted in the bioassay. The iodine vapor was traced at 5-min intervals by the formation of the blue iodine clathrate in the underlying agar, which had been supplemented with a 1% soluble starch. We always observed a radial distribution that, at room temperature, typically had reached a diameter half that of the square plate after ~1 h. Summarizing these results, the highest concentration of the odor compound was always found where it was spotted. The taxis results reported here can therefore be conservatively interpreted as referring to the organisms' capacity to locate (or move away from) the highest concentration of a chemical over a basically radial gradient.

Table 2. Concentration of VOCs in 1 μl of the multicomponent odor.

VOCs of the multicomponent odor	Amount (μmol)
2-butanone	0.002
3-methyl-3-buten-1-ol	0.001
2-pentanol	0.001
2,3-pentandione	0.001
2,3-hexandione	0.001
2-hydroxy-3-hexanone/3-hydroxy-2-hexanone*	0.001 μl
6-methyl-5-hepten-2-one	0.001

* Concentration was not determined on the purified mixture, and 1 μl of a 1 : 100 dilution was taken.

Sodium azide (1 μl, 1 mol L^{-1}) was spotted on the agar surface at the attractant (sector A) and the counter attractant (sector D) side to anesthetize and kill *C. elegans,* which were attracted to or repelled by the odor. Because we did not continuously record the nematodes' positions and nematodes do not remain in ''attractive'' spots unless food is available, the poison was needed as a trap to allow an exact quantification of the total number of nematodes reaching a (counter) attractant spot.

The chemotaxis index (I_C) was calculated according to the method of Bargmann et al. (1993), using the difference in numbers of worms in sectors A and D (after 1 and 24 h) and dividing this by the total number of worms found in all sectors. Each odor compound was tested in at least five independent assays with 10–30 nematodes each. For odor compounds that had not been tested before, 12 independent assays were performed on at least 2 different d. Additionally, a series of 12 control assays (117 worms in total) were performed without any odor compound on the lid.

Chemotaxis assays: B. monhystera—A suitable assay to test for chemotaxis of aquatic nematodes to odor compounds was not available, so we started from a slightly modified version of the standard population assay for *C. elegans,* which belongs to the same nematode family (Rhabditidae) as *B. monhystera.* Preliminary experiments with population assays yielded highly variable results, partly because intraspecific attraction based any chemotactic response to the odor compounds. We therefore established a single-worm assay to study the chemotaxis behavior of *B. monhystera.*

Plates were divided into six equal sectors according to the

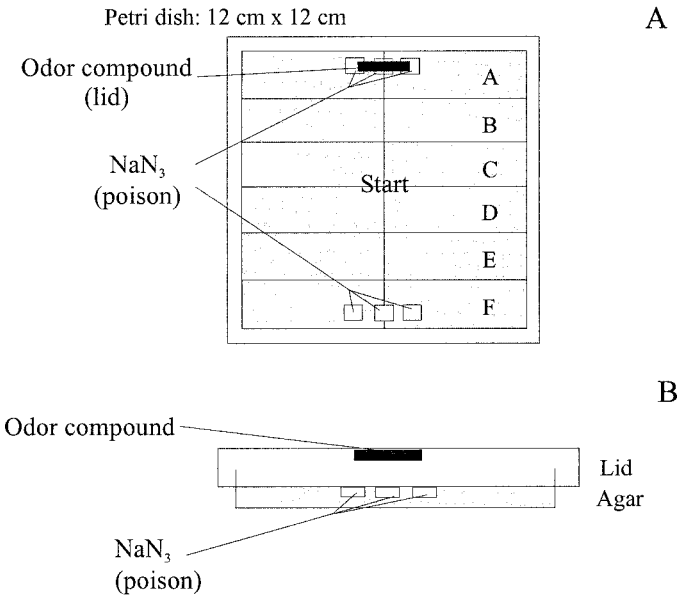

Fig. 1. (A) View of the square petri dish from above. In the chemotaxis assay of *B. monhystera*, sectors are labeled A–F. The starting point was in the center of the plate. Sector A is the attractant, and sector F the counterattractant side. In both sectors sodium azide was spotted on the agar. (B) Side view. Square petri dish at the attractant side (sector A) with the odor compound as a line on the lid and the poison (sodium azide) on the agar surface.

method of Troemel et al. (1997), with the odor compound in sector A as a line on the lid (Fig. 1). Animals from stock cultures were transferred in an agar drop to sterile agar plates. Mobile worms moved out of the drop within 1 h and were transferred with a needle to the center of the assay plate (the borderline between sectors C and D). Because *B. monhystera* proved very resistant to sodium azide, more of the anesthetic was used (three 2-μl drops of a 1 mol L^{-1} NaN_3 at distances of 1 cm; Fig. 1).

The chemotaxis behavior was scored as for the avoidance assays of *C. elegans* (Troemel et al. 1997). In brief, sectors received the following scores: A (+3), B (+2), C (+1), D (−1), E (−2), and F (−3). Worms that migrated directly from inoculum to sector A were scored with a value of +6 (and vice versa for sector F, −6). Worms that first moved away from the attractant but then migrated into sector A were scored with a value of +5 (if they initially reached sector D), +4 (E), or +3 (F). Worms that moved back toward or into the center after having reached a further sector retained the score associated with their maximal migration. Scores of +6 to +3 were considered to be a positive response, +2 to −2 no response, and −3 to −6 a negative response. Observations were made every 5–10 min during the first 4 h and again after 24 h. At least 18 independent assays were performed on 2 different d. Furthermore, we performed a series of 29 single-worm control assays without any odor compound. We used the following formula to calculate a specific chemotaxis index (I_B):

$$I_B = (\text{number of worms with positive chemotaxis} - \text{number of worms with negative chemotaxis}) \div \text{total number of worms in assay}$$

Statistical analysis—Mean values and SDs of the population assays of *C. elegans* were calculated using standard methods of analyzing normal distribution. The Kolmogorov-Smirnov test was used to test for normality, and the means were compared with a control data set (without any odor) using a two-tailed *t*-test ($p < 0.05$). In the single-worm assay of *B. monhystera*, the chemotaxis responses were tested with a two-tailed Mann-Whitney *U*-test ($p < 0.05$). The statistical program GraphPad-InStat (version 3; GraphPad) was used to compare the chemotaxis data.

Results

VOCs of natural biofilms and isolated cyanobacteria—Epilithic biofilms obtained from Lake Zurich near the Limnological Station at Kilchberg consisted of the benthic cyanobacteria species *C. parietina, Phormidium* sp., *Rivularia*, sp. and *T. distorta.* Diatoms (*Diatoma* sp.) and green algae (*Ulothrix zonata* and *Cladophora* sp.) were also present. Table 3 shows the VOCs from two biofilm samples, the ''*Rivularia* sp./*C. parietina* community'' (biofilm A) and the ''encrusted layer'' (biofilm B), composed of *T. distorta*, *Phormidium* sp., *Rivularia* sp., and *C. parietina.* Characteristic VOCs of biofilm A were geosmin, 2-methylisoborneol, ß-cyclocitral, and dihydro-ß-ionone. In biofilm B, the aliphatic hydrocarbons heptadecene, heptadecane, 7-methylheptadecane, and 8-methylheptadecane were the main compounds. To assign the origin of the observed VOCs to particular cyanobacteria, monoxenic cultures of *Phormidium* sp., *T. distorta*, and *Rivularia* sp. were isolated from these epilithic biofilms. The aliphatic hydrocarbons 7-methylheptadecane and 8-methylheptadecane were found in the culture of *T. distorta* and heptadecene in *Rivularia* sp. and in the axenic strain of *C. parietina* (Table 3). The nor-carotenoid ß-ionone was detected in all monoxenic cultures, whereas dihydro-ß-ionone was missing in *T. distorta* (Tables 3, 4). Geosmin was not found in any of the applied monoxenic cultures but was present in the axenic strain of *Calothrix* sp. (Tables 3, 4). Because of bacterial contamination of monoxenic cultures, we analyzed and used artificial, axenic cyanobacterial strains of *Plectonema* sp., *P. notatum, C. parietina*, and *Calothrix* sp. to assign the odor compounds to the metabolism of the cyanobacteria (Table 3).

A further problem is that some products detected in natural cyanobacterial biofilms can be artifacts of the analytical procedure. Recently, it was shown that the aliphatic aldehydes (octanal, nonanal, and decanal), limonene, and 1,2,3-trimethyl-2,7-dioxabicyclo(2,2,1) heptane (TDH) are products of the analytical procedure, rather than accruing from the metabolism of cyanobacteria (Höckelmann and Jüttner 2004; Höckelmann and Jüttner in press).

Concentration of VOC in cyanobacterial cultures—In the cultures of *Phormidium* sp., high concentrations of dihydro-ß-ionone (0.03 μmol L^{-1}) and lower concentrations of ß-

Table 3. VOCs of the two epilithic biofilms *A* (*Rivularia* sp./*C. parietina* community) and *B* (incrusted layer). The compounds are given with their molecular mass (M) and retention time (Rt) on a capillary column (DB-1301). +, present; 0, absent. VOCs we found in our cultures are designated as *C. parietina* (1), *Calothrix* sp. (2), *P. notatum* (3), *Plectonema* sp. (4), *Phormidium* sp. (5), *T. distorta* (6), and *Rivularia* sp. (7). Compounds, which we classify to be metabolites of cyanobacteria, are indicated with x (Jüttner 1987, Jüttner 1995; Höckelmann and Jüttner 2004; Höckelmann and Jüttner in press).

VOC	M	Rt (min)	Biofilm *A*	Biofilm *B*	VOC of cultures	VOC of cyanobacteria
Fatty acid pathway						
Octatriene	108	16.38	+	0		x
2,4-heptadienal	110	19.97	+	0		
2-heptanone	114	15.77	0	+		x
2,4-octadienal	124	23.17	+	0		x
Octadien-1-ol	126	14.40	+	0		
1-octen-3-one	126	18.74	+	+		x
Octa-1,5-dien-3-ol	126	19.08	+	0		x
Octanal	128	19.45	+	+	1–7	
2-octanone	128	19.24	0	+		
1-octen-3-ol	128	19.25	+	0		x
2,4-nonadienal	138	24.80	+	0		x
Nonanal	142	19.92	+	+	1–7	
Ectocarpene	148	23.27	+	0		
2,4,7-decatrienal	150	29.04	+	0		
2-decenal	154	27.87	0	+		
3-decanone	156	25.39	+	0		
2-decanone	156	25.56	0	+		
Decanal	156	25.91	+	+	1–7	
2-tridecanone	212	36.32	0	+	3	x
Heptadecene	238	37.36	0	+	1, 7	x
Heptadecane	240	37.60	0	+	1–7	x
8-methylheptadecane	254	38.59	0	+	3, 4, 6	x
7-methylheptadecane	254	38.63	0	+	3, 4, 6	x
Isoprenoid pathway						
6-methyl-5-hepten-2-one	126	19.15	0	+	1, 3, 4, 5, 6	x
6-methylheptan-2-one	128	18.01	0	+		x
Limonene	136	19.10	−	+	1–7	
α-pinene	136	15.60	0	+		
2,6,6-trimethylcyclo-hex-2-en-1-one	138	21.21	+	0	1, 3, 4, 5, 7	x
TDH	142	15.41	+	+	1–7	
β-cyclocitral	152	26.42	+	+	1, 3, 4, 7	x
2-methylisoborneol	168	25.09	+	0		x
Geosmin	182	31.20	+	+	2, 7	x
β-ionone	192	33.90	+	+	1, 3, 4, 5, 6, 7	x
Dihydro-β-ionone	194	32.51	+	0	1, 3, 4, 5, 7	x
β-ionone-5,6-epoxide	208	34.28	+	+	1–7	x

ionone (0.004 μmol L^{-1}) were found (Table 4). In the other cultures, the ß-ionone concentrations were equivalent to or higher than those of dihydro-ß-ionone (e.g., in *P. notatum* the concentrations were 0.01 and 0.001 μmol L^{-1}, respectively) (Table 4). Skatol was only present in *C. parietina*, with a concentration of ~0.38 μmol L^{-1} and geosmin only in *Calothrix* sp. at ~1.81 μmol L^{-1} (Table 4). The 2-month-old *Plectonema* sp. culture had odor compounds in the nanomolar concentration range, whereas, in the 9-month-old culture, the odor compounds had accumulated, and micromolar concentrations of 6-methyl-5-hepten-2-ol (4 μmol L^{-1}), 6-methyl-5-hepten-2-one (3 μmol L^{-1}), and 2-butanone (3 μmol L^{-1}) were found (Table 5).

Chemotaxis assays: C. elegans—We first tested the response of *C. elegans* to single odor compounds found in cyanobacterial biofilms. Compounds of the isoprenoid pathway elicited differences in nematode chemotaxis with exposure time (1 or 24 h) (Fig. 2). Irrespective of exposure time, worms were attracted to the hemiterpene 3-methyl-3-buten-1-ol, TDH, and the nor-carotenoid dihydro-ß-ionone. In contrast to 3-methyl-3-buten-1-ol, another hemiterpene, 3-methyl-2-buten-1-ol, did not prove to be attractive after 24 h of exposure. In the presence of TDH, *C. elegans* almost immediately moved to the odor, yielding an I_C of 0.76 ± 0.16 ($p < 0.0001$) after 1 h. The attraction to dihydro-ß-ionone was weaker than that to TDH (I_C 0.22 ± 0.16 after 1 h ($p < 0.05$). *C. elegans* showed no attraction to ß-ionone. The repellence by geosmin was found after 24 h (I_C −0.22 ± 0.34; $p < 0.05$). No response to geosmin was found during the first hour of exposure (Fig. 2) in contrast to the nor-carotenoids, which caused attraction after 20 min, with a I_C

Table 4. Concentrations of β-ionone, dihydro-β-ionone, skatol and geosmin in the cultures of *Calothrix* (cultures used in the bioassays), *Phormidium* sp., *T. distorta*, *Rivularia* sp., and *P. notatum*. The fragment ion (*m/z*) is given. M, molecular mass; <, below the detection limit.

Cyanobacteria, VOC	M	Ion *m/z*	Concentration (μmol L^{-1})
C.parietina (PCC 6303)			
β-ionone	192	177	0.001
Dihydro-β-ionone	194	43	<
Skatol	131	130	0.38
Calothrix sp. (PCC 7507)			
β-ionone	192	177	<
Geosmin	182	112	1.81
Phormidium sp.			
β-ionone	192	177	0.004
Dihydro-β-ionone	194	43	0.03
T. distorta			
β-ionone	192	177	0.0002
Rivularia sp.			
β-ionone	192	177	0.0004
Dihydro-β-ionone	194	43	0.0005
P. notatum (PCC 6306)			
β-ionone	192	43	0.01
Dihydro-β-ionone	194	43	0.001

of 0.35 ± 0.34 ($p < 0.005$) for 6-methyl-5-hepten-2-one and 0.4 ± 0.3 ($p < 0.0005$) for 6-methyl-5-hepten-2-ol (data not shown). The I_C for both nor-carotenoids decreased after 1 h exposure; after 24 h, the worms were repelled. Testing the response to both odors with a dilution of 10^{-2} (data not shown), *C. elegans* showed no chemotaxis over the entire exposure time.

In general, compounds of the acetate pathway such as 2-butanone, 2-pentanone, and 2-pentanol were strong attractants for *C. elegans* after 1 h of exposure (Fig. 3). The fermentation products 2,3-hexandione and hydroxyhexanones exhibited an attractive odor for *C. elegans* (Fig. 3) ($p < 0.05$). The natural hydroxyhexanones, which were enriched, extracted, and purified from a culture of *Plectonema* sp. (Höckelmann and Jüttner in press), were stronger attractants than their precursor compound 2,3-hexandione. Lipoxygenase products (e.g., 3-pentanone and 3-pentanol) were also attractive to *C. elegans* (Fig. 3). Furthermore, we found no response to the aliphatic hydrocarbons heptadecane, 7-methylheptadecane, and 8-methylheptadecane and to products of the shikimat pathway, such as skatol and indol (Fig. 3). The

Table 5. Concentrations of VOC (μmol L^{-1}) in two different cultures of *Plectonema* sp. (PCC 7410) that were tested in bioassays with nematodes. For *C. elegans*, the tested culture was 2 months, and that for *B. monhystera* was 9 months. The fragment ion (*m/z*) is given. M, molecular mass; <, below detection limit.

VOC	M	Ion *m/z*	Concentration *C. elegans* (μmol L^{-1})	Concentration *B. monhystera* (μmol L^{-1})
2-butanone	72	43	<	3.0
2-pentanone	86	43	0.2	1.5
2-pentanol	88	45	0.1	0.9
3-pentanone	86	57	0.06	0.3
3-pentanol	88	59	0.04	0.4
2,3-hexandione	114	71	<	0.3
1-hexanol	102	56	<	0.1
6-methyl-5-hepten-2-one	126	43	0.1	3.0
6-methyl-5-hepten-2-ol	128	67	0.1	4.0
Heptadecane	240	57	0.003	0.06
7-methylheptadecane	254	112	0.003	0.03
8-methylheptadecane	254	126	0.004	0.03

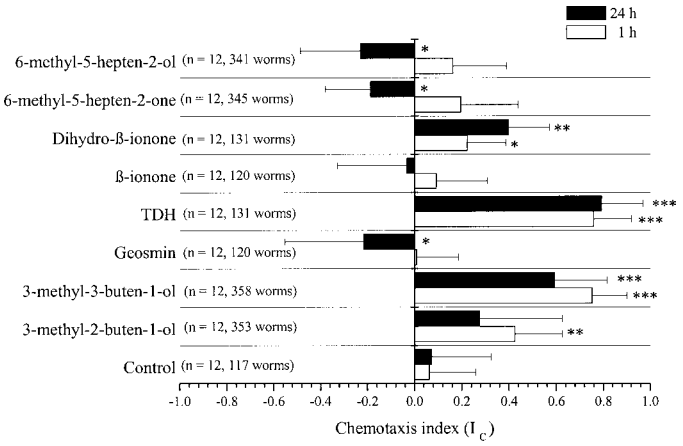

Fig. 2. Population assays of *C. elegans*. Chemotaxis responses after 1 and 24 h with compounds of the isoprenoid pathway (n = number of assays, total number of worms). Values significantly different from control experiments: ***$p < 0.0005$, **$p < 0.005$, and *$p < 0.05$. Error bars show the SD.

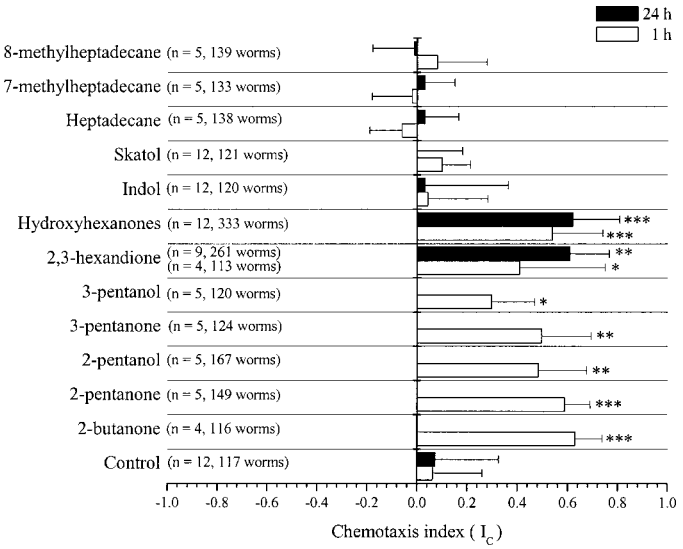

Fig. 3. Population assays of *C. elegans*. Chemotaxis responses after 1 and 24 h to compounds of the acetate and shikimat pathway (n = number of assays, total number of worms). Values significantly different from control experiments: ***$p < 0.0005$, **$p < 0.005$, and *$p < 0.05$. Error bars show the SD.

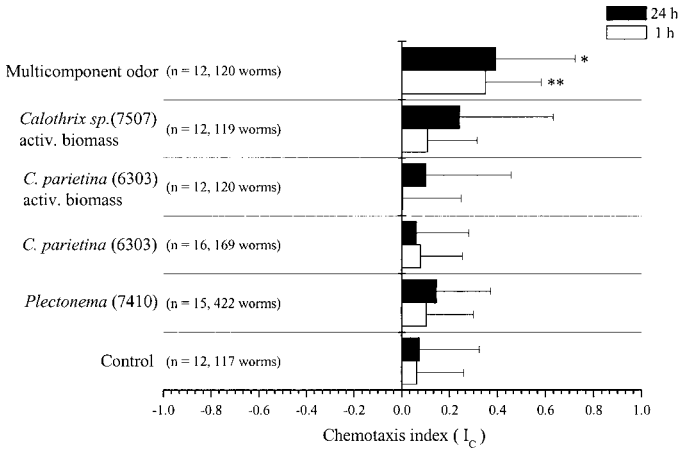

Fig. 4. Population assays of *C. elegans.* Chemotaxis responses after 1 and 24 h to the cyanobacteria and to the multicomponent odor (n = number of assays, total number of worms). Values significantly different from control experiments: ***$p < 0.0005$, **$p < 0.005$, and *$p < 0.05$. Error bars show the SD.

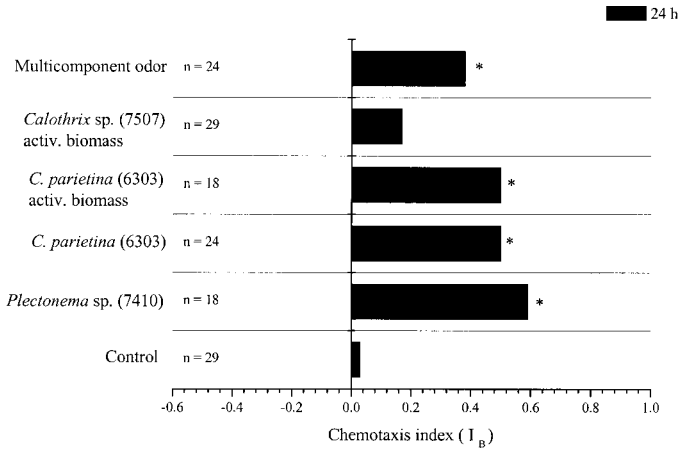

Fig. 5. Single-worm assays of *B. monhystera.* Chemotaxis responses after 24 h to axenic cultures of cyanobacteria and to the multicomponent odor (n = number of assays). Values significantly different from control experiments: *$p < 0.05$.

latter compound was found to be the main odor compound in the *E. coli* culture that served as food for *C. elegans* (data not shown).

In a further experiment we tested the response to a ''multicomponent odor,'' a mixture of fermentation products (2-butanone, 2-pentanol, 2,3-pentandione, 2,3-hexandione, and hydroxyhexanones) and compounds of the isoprenoid pathway (3-methyl-3-buten-1-ol, and 6-methyl-5-hepten-2-one) (Table 2). *C. elegans* was attracted to this multicomponent odor with an I_C of 0.35 ± 0.23 ($p < 0.005$) and 0.39 ± 0.33 ($p < 0.05$) after 1 and 24 h, respectively (Fig. 4). However, it showed no response to the axenic biofilms of *Plectonema sp.* or to the biofilms of *C. parietina* and *Calothrix* sp. (Fig. 4). These biofilms essentially differ in their composition and concentrations of VOCs (Tables 3, 4). *Plectonema* sp. and *C. parietina* (PCC 6303) were a rich source of fermentation products, nor-carotenoids, and aliphatic hydrocarbons, whereas high concentrations of geosmin (1.8 μmol L^{-1}) were characteristic of *Calothrix* sp. (Tables 4, 5) (Höckelmann and Jüttner 2004). Observations with epifluorescence microscopy showed that *C. elegans* indeed ingested filaments of *Plectonema* sp.

Chemotaxis assays: B. monhystera—After applying the odor compound, we typically observed a searching behavior. Worms tended to move in circles, with a velocity of ~1 cm min^{-1}. After this initial ''orientation'' (which typically lasted ~10 min), worms started moving toward the odor spot.

In contrast to *C. elegans, B. monhystera* showed a positive response to the axenic biofilm of *Plectonema* sp., with an I_B of 0.59 ($p < 0.05$). The worm moved at a rate of ~1.5 cm min^{-1} to *Plectonema* sp. Although we did not observe the ingestion of *Plectonema* sp. by *B. monhystera,* this nematode browsed the cyanobacterial filaments, probably in search of bacteria or exopolymeric substances. The chemotaxis of this nematode to *C. parietina* was also positive (I_B 0.50, $p < 0.05$) (Fig. 5). There was no difference between live and activated biomass. In contrast, *B. monhystera* was not attracted to the activated biomass of *Calothrix* sp. (I_B 0.17) (Fig. 5). Furthermore, *B. monhystera* was attracted to the *Plectonema* sp. multicomponent odor, with an I_B of 0.38 ($p < 0.05$) (Fig. 5), but it showed no positive response to any single chemical compound. VOCs belonging to the isoprenoid pathway, such as 3-methyl-3-buten-1-ol, TDH, geosmin, 6-methyl-5-hepten-2-one, and dihydro-ß-ionone, were not attractive, and ß-ionone was a repellent, with an I_B of −0.35 ($p < 0.05$; Fig. 6). *B. monhystera* exhibited no significant response to 2-butanone, 2-pentanol, the hydroxyhexanones, 2,3-pentandione, 2,3-hexandione, skatol, and indol (Fig. 7).

Discussion

Microbial biofilms in aquatic habitats are typically populated with high nematode densities. In intertidal soft sediments, a close spatial coupling between microphytobenthos patches and nematodes has been observed repeatedly (Blanchard 1990; Pinckney and Sandulli 1990; Moens et al. 1999*a*). Bacterial biofilms on macroalgae and other sub-

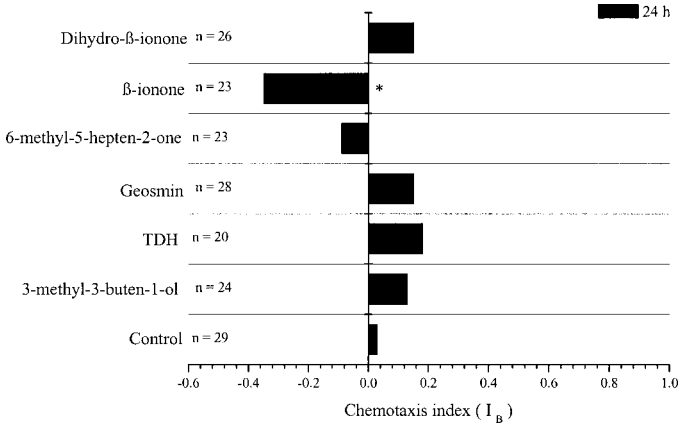

Fig. 6. Single-worm assays of *B. monhystera.* Response to odors of the isoprenoid pathway after 24 h (n = number of assays). Values significantly different from control experiments: *$p < 0.05$.

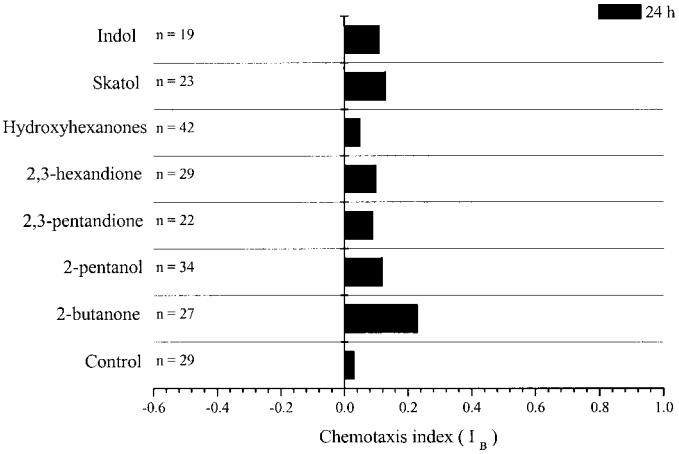

Fig. 7. Single-worm assays of *B. monhystera.* Response to odors of the acetate and shikimat pathway after 24 h (n = number of assays). There were no values significantly different from control experiments.

strates also attract high densities of nematodes (Trotter and Webster 1983; Bouwman et al. 1984). However, there have been no studies examining the chemoecological basis of patch-finding in aquatic nematodes.

Moreover, identifying the exact origin of compounds in complex natural biofilms is not easy. In the present study, we performed VOC analyses of natural biofilms, as well as of monoxenic and axenic cultures of the individual cyanobacterial strains constituting the biofilms. This allowed us to correlate odor compounds to the cyanobacterial metabolism (Jüttner 1987; Höckelmann and Jüttner 2004). Many of the compounds detected by GC/MS are well known as bacterial catabolites that result from different fermentation reactions. The VOC analysis of axenic cyanobacteria clearly showed that this group also releases VOCs that are generally attributed to heterotrophic bacteria (e.g., 2-butanone and 2,3-pentandione; Höckelmann and Jüttner 2004; Höckelmann and Jüttner in press).

Designing a proper bioassay for the study of chemotaxis to VOCs in a (semi)aquatic environment is not straightforward. On the one hand, the distribution of VOCs in an experimental microcosm may be affected by advection and turbulence, transporting odors in basically unpredictable patterns. The results of our iodine starch test, however, provide evidence that our experimental design does allow for the formation of radial gradients emanating from a point source. On the other hand, we specifically aimed to study the nematodes' response to volatiles. Anatomical and neurophysiologic studies have shown that the chemosensory neurons of smell and taste are separated in *C. elegans* (Bargmann and Mori 1997) and most likely in other nematodes. Thus, the advantage of applying candidate attractants on the lid of the dishes rather than on the agar surface is in the clear separation of the air phase from water phase, which allows our results to be unambiguously explained in terms of olfactory (and not taste) sense. Indeed, our assay is based on a design that has been successfully used to select a large number of odor receptor mutants of *C. elegans* (Bargmann et al. 1993). Additional advantages of the square-plate design over alternative designs, such as Y-tubes (e.g., Baumgärtner et al. 2002) or T-mazes (VanHouten et al. 1982) are (1) the ease with which behavioral observations of small organisms can be made during the course of the experiment and (2) the extensive mixing zone of candidate attractants, in which test organisms can reorient continuously as they move along gradients (Ingvarsdóttir et al. 2002). However, further work is needed to investigate the exact influence of flow, distance (see below), and odor concentrations on the behavior of nematodes in aquatic environments, and this may require yet different assay methods.

The present article is first to demonstrate chemotaxis of a meiofaunal-sized invertebrate to VOCs in an aquatic environment. Previously, studies on invertebrate chemotaxis have not focused on the differentiation between odor and taste compounds. Much more information exists on the behavior of microfauna- and, to a lesser extent, meiofauna-sized aquatic organisms in cues and gradients of dissolved substances. Moens et al. (1999*b*) showed highly species-specific response patterns of estuarine monhysterid nematodes to the presence of different bacterial strains. However, they did not provide unequivocal proof that these patterns resulted from chemotaxis; it is possible that nematodes accumulated inside patches with suitable bacterial food after a ''random walk.'' Applying the candidate attractant on the microcosm lid, as in the present study, instead of on the agar surface avoids this ambiguity. The marine nematode *Chromadorita tenuis* emerges from sediment and swims to the surface of submerged macrophytes, probably in response to chemical compounds released by the macrophytes or their epiphytes (Jensen 1981). However, no characterization of the infochemicals involved has been done. Riemann and Schrage (1988) demonstrated chemotaxis of the brackish-water species *Adoncholaimus thalassophygas* along gradients of CO_2. These gradients may guide nematodes to sites of intense decomposition that may provide copious food. However, photoautotrophic cyanobacterial biofilms exposed to light are carbon sinks rather than sources. Hence, different odor mechanisms are likely to mediate the response to microbial mats, and we propose that strong allelochemical interactions may be involved.

Moens et al. (1999*a*) suggested that nematodes actively search for optimal food patches but feed rather nonselectively once they are inside such a suitable patch. Studies on ciliates (belonging to different feeding types), however, have shown that noncontact chemical recognition is not only involved in finding prey aggregations (Ricci et al. 1996; Morelli et al. 1999) but also in selecting individual prey within such aggregations (Hamels et al. 2004). The distances over which *C. elegans* exhibits strongest chemotaxis in response to chemicals are in the order of 5 cm (Bargmann et al. 1993), similar to the distances used in our study. This indicates that the observed chemotaxis of nematodes may affect both their capacity to locate nearby biofilms and to position themselves within a biofilm. However, more work is needed to assess hierarchies of different cues and distances at which they typically act. In particular, it remains to be studied how volatile and soluble cues ''interact'' to affect the behavior and microdistribution of animals in limnetic and intertidal habitats.

The capacity to locate cyanobacterial biofilms from a dis-

tance may provide aquatic nematodes with suitable food and shelter. Diatoms and bacteria are commonly considered the main microbial food sources of free-living aquatic nematodes (Moens and Vincx 1997; Montagna 1995). Much less information exists on the possible contribution of cyanobacteria to the nutrition of nematodes. Jensen (1987) presented a picture of an estuarine *Monhystera* sp. ingesting filaments of *Oscillatoria,* and Hellwig-Armonies et al. (1991) found cyanobacteria (*Microcoleus/Oscillatoria*) to be a major component of the gut contents of the large, omnivorous *Enoplus brevis* inhabiting a Wadden Sea salt marsh. Moens and Vincx (1997) described how several estuarine intertidal nematode species browse filamentous microalgae and cyanobacteria in benthic biofilms, probably feeding on associated bacteria and mucus. Some epistrate feeders (equipped with one or more teeth) are able to puncture and feed on the filaments. The morphometry of the stoma of the nematodes used in the present study indicates that small filaments of *Plectonema* sp. are ingestible. In the present study, epifluorescence microscopy demonstrated that *C. elegans* ingests small filaments of *Plectonema* sp. We also observed several nematode species such as the predacious/omnivorous *Mononchus truncatus* and *Dorylaimida* sp. from epilithic biofilms of Lake Zurich feeding on cyanobacterial filaments, which suggests that a capacity to graze on cyanobacteria may be more widespread among biofilm-inhabiting nematodes.

In addition to food, biofilms composed of filamentous microorganisms may offer structure and shelter for a variety of nematode species (Moens and Vincx 1998), analogous to fungal mats which provided a suitable substrate for the growth and reproduction of several marine and brackish-water nematodes (Meyers and Hopper 1967). Finally, cyanobacterial biofilms produce copious amounts of extracellular polymeric substances which may facilitate attachment of organisms and provide protection against, for example, desiccation and pH fluctuations (Decho and Lopez 1993; Decho 1994).

The model organism *C. elegans* showed a positive chemotaxis to a variety of single odor compounds. Our results add a number of cyanobacterial VOCs to the list of known attractants for *C. elegans* (Bargmann et al. 1993; Troemel et al. 1997). *C. elegans* exhibited no uniform chemotaxis response to nor-carotenoids. Although ß-ionone did not elicit any response, *C. elegans* was attracted to dihydro-ß-ionone, which illustrates that minor differences in the molecular structure of a compound have informational relevance for nematodes (Troemel et al. 1997). Surprisingly, *C. elegans* was not attracted by indol, the main VOC produced by its food source, *E. coli.* Indol is a typical odor for *E. coli* strains (Yu et al. 2000) and has also been described as cyanobacterial VOC (Tsuchiya et al. 1979). The absence of attraction to indol in the present experiments does not necessarily imply that *C. elegans* is not capable of sensing this compound. *C. elegans* has been shown to display both adaptation and habituation, two elementary types of olfactory learning (Bernhard and van der Kooy 2000). Hence, although it can associate a single odor compound with food, cultivation on *E. coli* lawns may lead to adaptation and habituation to indol.

C. elegans was attracted to the multicomponent odor, but this chemotaxis was weak compared with those to the single odor compounds. Hence, the different attractants did not have an additive effect. It is unlikely that the chemotaxis to the multicomponent odor was a consequence of the presence of 6-methyl-5-hepten-2-one, because this compound only became repellent after prolonged incubation and at high concentrations. Its effect, if any, in the multicomponent odor was probably masked or counteracted by the presence of attractive odor compounds. Similarly, although we expected repellence to the biofilm of *Calothrix* sp. because of its geosmin production, such a negative response was not found. The presence of both attractants and repellents in a multicomponent of volatiles can help microorganisms to maintain a delicate balance between dependence on nematodes and other potential grazers for mineralization and dispersal and a threat of overgrazing (Farmer 1992; Polz et al. 2000; Traunspurger 2000).

The observed chemotaxis response of the aquatic nematode *Bursilla monhystera* to cyanobacteria and their VOCs was substantially different from that of *C. elegans.* Although *C. elegans* did not show a statistically significant attraction to any of the cyanobacterial strains, *B. monhystera* was attracted to *Plectonema* sp. and *C. parietina* but not to *Calothrix* sp. *Z*5-heptadecene was an important compound that was primarily detected in activated biomass of *C. parietina* (Höckelmann and Jüttner 2004). *Z*5-Heptadecene is a decarboxylation product of petroselinic acid (Jüttner 1991), and its role as an odor compound has not been described before. In spite of its positive chemotaxis to cyanobacteria and to a multicomponent odor, none of the single VOCs tested proved to be attractive to *B. monhystera,* and ß-ionone was even repulsive. The negative response to ß-ionone may have been concentration-dependent (Bargmann et al. 1993). The lack of positive response to any individual VOC clearly indicates that the chemotaxis response of *B. monhystera* to *Plectonema* biofilms was caused by a multicomponent odor rather than by any single compound. Preliminary studies with another aquatic nematode, *Plectus cirratus,* isolated from epilithic biofilms of Lake Zurich, yielded results very similar to those for *B. monhystera*: *P. cirratus* was attracted to the axenic biofilm of *C. parietina* but not to single compounds such as dihydro-ß-ionone (Höckelmann and Jüttner in press).

The ecological relevance of the difference in chemotaxis response between *B. monhystera* and *C. elegans* is not well understood. The response of the freshwater nematodes *B. monhystera* and *P. cirratus* is consistent with insect studies, which emphasizes that, in many cases, chemotaxis is elicited by multicomponent odors (Silverstein and Young 1976; Vinson 1984; El-Sayed et al. 1999). Chemotaxis responses to single compounds, shown for *C. elegans,* can be interpreted as a more specialized strategy of food-finding. However, the microhabitat preferences and feeding ecology of the nematodes used in our study are not well known. *C. elegans* feeds on a variety of microbial foods but shows clear preferences (Grewal and Wright 1992). However, the responses observed here to cyanobacterial VOCs may be linked to factors other than food and may further have been affected by culture conditions. More work is needed to fully establish the functional implications of the nematode's chemotaxis. However,

it is obvious from our results that data on the model organism *C. elegans* can not readily be generalized for aquatic nematodes.

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