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Daphnia behavioural responses to taste and odour compounds: ecological significance and application as an inline treatment plant monitoring tool

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Abstract Continuous monitoring of volatile organic compounds (VOC) in raw water is highly desirable for taste and odour management, but in most treatment plants this capacity is lacking. We used a bbe© *Daphnia* toximeter installed in the Zurich water treatment plant to determine if *Daphnia magna* could be used to monitor odour compounds in source-water. Trace levels of two widely distributed biogenic VOCs in freshwater: β-cyclocitral and 2(*E*),4(*E*),7(*Z*)-decatrienal were added to the raw water inflow to chambers containing test animals and their behaviour was recorded using a high resolution camera. We observed that each compound elicited a marked short-term increase in *Daphnia* swimming velocity, but the effect was brief and an acclimation to the compounds was observed after a time period or with repeated additions. The results demonstrate that the toximeter has considerable potential as a tool to monitor certain VOCs in water, and that *Daphnia* perceive and react to 2(*E*),4(*E*),7(*Z*)-decatrienal and β-cyclocitral at concentrations between 2.5 and 25 μM
Keywords *Daphnia magna*; β-cyclocitral; decatrienal; repellent; toximeter; swimming velocity; taste and odour

Introduction

The majority of taste and odour (T&O) outbreaks in drinking water are neither anticipated nor traced to their origins (Bruchet, 1999). Few water treatment plants can afford continued analytical monitoring for T&O compounds, which are usually present in trace amounts and often episodic, showing rapid increases in raw water which allow little opportunity for proactive management. Many T&O outbreaks are caused by VOCs produced by cyanobacteria, algae or heterotrophic microbiota (Jüttner, 1995a; Watson, 2003; Zaitlin and Watson, 2006). These outbreaks often appear to bear little relationship to traditional models of plankton dynamics, or respond to efforts to control them using nutrient-based management approaches.

Traditionally, the water industry has tended to rely on treatment as a means of controlling T&O outbreaks. Forward-thinking managers now recognise that while effective removal remains a primary goal, early warning and prevention is essential for the long-term delivery of high quality water. This requires a far better understanding of T&O "from source to tap" (CCME, 2002). In fact, these events can provide vital diagnostic clues to immediate and long-term issues in drinking water sources and supplies. T&O can signal treatment or distribution system malfunction, changes in the distribution or activity of certain biota, or chemical/biological hazards in the source-water. T&O can also provide an early warning of deep-seated changes in surface waters resulting from human activity (Watson, 2003).

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Recent evidence, mostly from marine research, suggests that some VOCs act as chemical signals which modify cell and food web processes at sub-molar concentrations (e.g. Watson and Cruz-Rivera, 2003). In particular, trace levels of some cyanobacterial and algal VOCs may act as repellents towards their planktonic and benthic grazers (Jüttner, 2005). To date, however, we have little information about the extent or relative importance of T&O compounds in such chemical interactions because it has been extremely difficult to find an effective protocol to demonstrate this phenomenon, which involves short-lived behavioural changes (e.g. in activity or swimming velocity/patterns) in response to non-lethal VOC levels.

Static bioassays with *Daphnia* and fish have long been used by the water industry to test for toxicity, but these assays cannot detect subtle responses to sub-lethal or dynamic contaminant levels. More recently, inline biomonitoring systems (or "toximeters") have been employed in water treatment plants across Europe, using continual behavioural measures of organisms such as *Daphnia* and small fish to monitor changes in water quality. These systems also offer a potential new method of evaluating the response of such organisms to specific contaminants against the natural background variation in water quality encountered in natural systems. In this paper, we describe the results of bioassays with the model odour compounds 2(*E*),4(*E*),7(*Z*)-decatrienal and ß-cyclocitral, carried out using an inline *Daphnia* toximeter that is in operation at the groundwater plant Hardhof, Zürich Water Supply.

Materials and methods

Organisms

We tested the response of the freshwater crustacean *Daphnia magna* to 2(*E*),4(*E*),7(*Z*)-decatrienal, an important VOC produced by some diatoms and chrysophytes (Watson *et al.*, 2001; Jüttner, 2005) and β-cyclocitral, which is produced in large amounts by members of the cyanobacterium genus *Microcystis* (Jüttner, 1984, Jüttner and Höflacher, 1985). The animals were raised in batch cultures with the green alga *Scenedesmus acuminatus* as a food source. The *Scenedesmus* strain was obtained from the Max-Planck-Institute of Limnology at Plön, Germany, and cultured in a defined inorganic growth medium which supports the growth of planktonic green algae and is well tolerated by crustaceans. This algal strain supports excellent growth of *Daphnia* and does not stick to glass walls (Hawkins and Lampert, 1989). It was also used as a food source in the toximeter.

Daphnia toximeter

The experiments were performed with *D. magna* in two replicate glass toximeter cells (36 mL net volume; 21 × 45 × 68 mm) of a *bbe*© *Daphnia* Toximeter (bbe Moldaenke, Kiel-Kronshagen, Germany), diagrammed in Figure 1 (Lechelt *et al.*, 2000 and bbe operation manual[1]). An inflow of oxygen-saturated river bank filtered water from the Limmat River (non-chlorinated) was split between the two parallel flow-through cells at a constant supply rate to each of 9 mL min^{-1} and held at a temperature of 20 °C ± 0.2 °C by inline heating / cooling elements.

Each cell was supplied with eight individuals of *D. magna*, raised from a single strain. The animals were replaced each week and allowed a 2–3 day period of acclimation, during which they were maintained on a controlled supply of the algal food source added continually to the cell inflow line from a fermentation unit. Experiments were carried out over a period of 1 day, after this period. We used 3–4 day-old individuals rather than

[1] htpp://www.bbe-moldaenke.de.

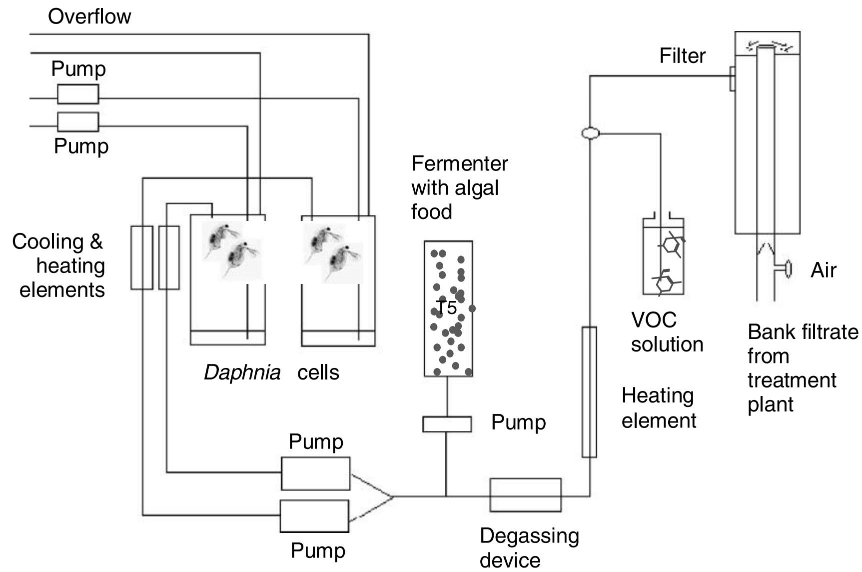

Figure 1 Schematic diagram of bbe© toximeter operating inline at the groundwater plant Hardhof, Zürich Water Supply

younger animals; preliminary experiments had shown a stronger response in older *Daphnia*. The initial number of animals in each of the cells was eight; however, this number could transiently be reduced in the course of the observation period as the animals moulted several times per week. During this process, *Daphnia* became inactive and could remain at the bottom of the chambers, showing no detectable movement.

The behaviour of the animals was generally very sensitive and responded to very small changes in the composition and temperature of the inflow. This produced a noisy baseline activity which tended to mask responses to test compounds, particularly in smaller or recently molted individuals. Both 2(*E*),4(*E*),7(*Z*)-decatrienal and β-cyclocitral are lipophilic VOCs, and a suitable solvent was required to mediate their solution in water. Initially the choice of solvent was a problem; trials indicated that ethanol and particularly methanol had significant effects on swimming velocity above 5–10 $\mu L.L^{-1}$ ($p < 0.008$, $p < 0.042$; Figure 2).

We selected ethanol, because it showed no effects on *Daphnia* below 5 $\mu L.L^{-1}$ (86 μM), and because the VOCs dissolved easily in this solvent. Initial trials showed that the response was affected by the variations in the ethanol between injections and intervening periods of bank-filtrate only. In later tests we controlled for this source of variability by exposing the *Daphnia* to riverbank filtrate spiked with 5 $\mu L.L^{-1}$ ethanol in the periods between each VOC application, thereby sustaining the same solvent level throughout the entire observation period. Washing water applied between the experiments to rinse any residual compound from the system also contained this ethanol concentration.

Each *Daphnia* was continuously tracked by a camera which recorded 25 images per second. These periodic values were then averaged for each chamber for all animals to provide an observation per minute, and recorded to an electronic database. Images were analysed by software which calculated a series of response parameters averaged each minute for all animals in each cell (providing one mean value per cell per minute for each measure). These parameters included the maximum linear dimension of the *Daphnia* presented to the camera, mean swimming velocity and trajectory (fractal dimension), location (height) in the cell, dispersion and velocity class index (Table 1). These values

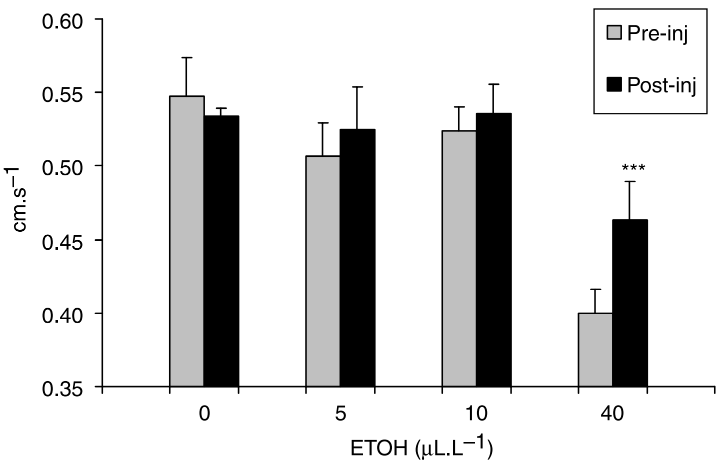

Figure 2 Mean swimming velocity of *Daphnia magna* exposed to different levels of ethanol (ETOH) injected directly inline to the toximeter; pre- and post-injection swimming velocities and standard deviation shown. Significant differences between pre- and post-responses indicaed by asterisks above ($p > 0.001$)

were also used to calculate the maximum % change in velocity following the injection, relative to the preceding baseline. Data were segmented into three periods for each trial: (1) prior to the injection (baseline), (2) injection and lag period for the compound to reach the chambers and (3) period during which the animals were exposed to the treatment (Figure 3). Grouped data from each of the two experimental series were analysed using least squares means comparisons (SAS© 1969) between period 1 (baseline) and period 3 from repeat trials, for each of the behavioural parameters. This was also repeated with the entire dataset.

Response to 2(E),4(E),7(Z)-decatrienal and β-cyclocitral

We tested the response of *Daphnia* to injections of the two test compounds, (2(*E*),4(*E*),7(*Z*)- decatrienal obtained from material synthesised by P. Rüedi, Institute of Organic Chemistry, University of Zürich; see Jüttner, 2005) and β-cyclocitral (BASF, Ludwigshafen, Germany). For each treatment, primary solutions of the compounds were prepared in ethanol (≥99.8% v/v, No 02860, Fluka, Buchs, Switzerland) and then added to 1 L of gently stirred bank filtrate water in glass bottles. The primary solutions were injected just below the surface of the water with a glass syringe, giving a small cloud that dissolved rapidly. For each trial, the spiked water was added from the bottle at a constant rate for 30 min; in the second experimental series, this was followed by a period of 180 min of river bank filtrate spiked with 5 $\mu L.L^{-1}$ ethanol. The water flow (72 $mL.L^{-1}$) in the inlet tube (Teflon) was not interrupted during the transfer of the inlet tube from

Table 1 Summary of *Daphnia* behavioural parameters monitored or calculated by the bbe® inline toximeter system, Hardhof, Zürich Water Supply

Parameter*	Unit	Description
Velocity	$cm\,s^{-1}$	
Height	cm	Vertical displacement
Distance	cm	Dispersion measure between *Daphnia*
Velocity class index	%	Index of % animals swimming above average velocity
Fractal dimension*	unit-less	Curvilinearity of swimming trajectory (turning, circling)

*See Lechelt *et al.* (2000) for calculation methods and detailed description of parameters

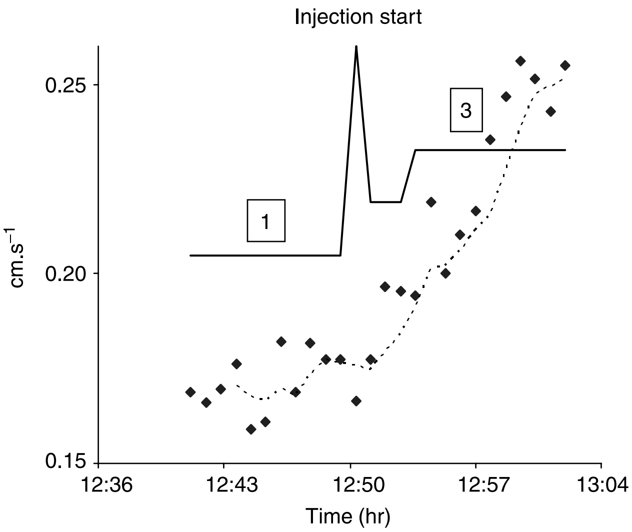

Figure 3 Change in *D. magna* swimming velocity (symbols; dashed line shows 3-point running average following injection of 2.5 μM 2,4,7-decaterienal into inline taximeter. Solid black line shows periods 1 and 3 used for comparative statistical analysis

one bottle to the next. Any air bubbles introduced by this procedure were removed completely by the degassing unit (Figure 1).

During the first series of experiments, a range of VOC levels between 250 nM and 250 μM were used, but in many trials the primary solutions were not adjusted to maintain the same final level of ethanol and these have not been included in the final statistical analysis. For 2(*E*),4(*E*),7(*Z*)-decatrienal, two levels (2.5 and 25 μM) had associated final ethanol concentrations of 5 $\mu L.L^{-1}$ and are included in this analysis. For β-cyclocitral, only one level (25 μM) used in the early trials had this same solvent concentration and is included here.

Results

Initial experiments showed that if a response occurred, it happened during the 10 min period after the compound reached the chamber, and then returned to a baseline level. With repeated closely-timed experiments, the *Daphnia* showed a decrease in the magnitude of their response (Figure 3), and it was determined that to minimise this acclimating effect, a maximum of three injections per day should be applied with an interval of at least 1.5 h between applications. Changes of linear velocity was the most sensitive response measure to changes in water quality and clearly evident in the graphical traces (Figure 4; Table 2); changes in other behavioural parameters were often less apparent but also statistically significant.

β-Cyclocitral

Daphnia magna showed a significant behavioural change to 25 μM β-cyclocitral in both experimental series. This response was reproduced in all trials with sufficient (1.5 h) intervals between injections where the final ethanol levels did not exceed 5 $\mu L.L^{-1}$, even though these were carried out on different days (up to a year apart) with different *Daphnia* clutches (Table 2). Combined data showed that there was a highly significant increase in the mean swimming velocity ($p < 0.001$; Table 2). When β-cyclocitral reached the toximeter cell, the animals' linear swimming velocity peaked rapidly and

Table 2 Summary statistics for LSMEANS comparisons between behaviour responses of *Daphnia magna* before (period 1) and after (period 3) exposure to 25 μm β cyclocitral; combined and individual datasets 1 (no ethanol during intervals) and 2 (sustained ethanol levels)

Variable	Period	N	All data		N	Data set 1		N	Data set 2	
			Mean	SD	N	Mean	SD		Mean	SD
Velocity	1	54	0.54	*0.04*	31	0.47	*0.02*	23	0.5	*0.02*
	3	54	0.66***	*0.01*	31	0.54***	*0.04*	23	0.6***	*0.11*
Box count	1	54	1.34	*0.06*	31	1.35	0.05	23	1.33	*0.06*
	3	54	1.31**	*0.06*	31	1.31	*0.06*	23	1.29*	*0.05*
Fractal	1	54	1.41	*0.05*	31	1.43	*0.05*	23	1.41	*0.05*
dimension	3	54	1.39*	*0.06*	31	1.39	*0.06*	23	1.37*	*0.06*
Distance	1	54	2.5	*0.3*	31	2.5	*0.3*	23	2.5	*0.3*
	3	54	2.5	*0.3*	31	2.4	*0.3*	23	2.7**	*0.2*
Height	1	54	2.4	*0.6*	31	2.0	*0.5*	23	2.8	*0.3*
	3	54	2.0***	*0.5*	31	1.6***	*0.4*	23	2.14***	*0.3*
Velocity	1	54	32.7	*6.7*	31	26.0	*7.7*	23	35.1	*6.3*
class	3	54	44.3***	*13.3*	31	44.8***	*10.4*	23	43.6***	*16.7*

SD: standard deviation. Significant difference between periods 1 and 3 indicated by: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

then declined to the baseline while the spiked water was still being supplied, indicating an acclimatisation to the VOC levels (Figure 4). Similarly, the velocity-class also showed a highly significant increase ($p < 0.001$), demonstrating that the increase in mean velocity was due to a higher proportion of individuals swimming at rates above the cell average velocity (Table 2). Other parameters indicated shifts in swimming pattern and trajectory. The mean distance between animals showed a small (non-significant) increase, while their vertical displacement in the cell (height) declined as they moved towards the bottom ($p < 0.0001$). Significant decreases in both fractal dimension and box count indices following exposure to the compound reflected a change to more linear trajectories, suggestive of more directional movement, which under natural conditions would result in a more rapid movement away from the source of the compound. The combined 'TOX' index was found to be insensitive to the behaviour changes induced by the VOC spikes.

2(*E*),4(*E*),7(*Z*)-Decatrienal

Daphnia magna showed a highly significant response to applications of 2.5 and 25 μM of 2(*E*),4(*E*),7(*Z*)-decatrienal (Figure 3 and Table 3). As seen with β-cyclocitral, there was a highly significant increase in mean swimming velocity and decrease in the curvilinearity of the swimming trajectory, as indicated by the lower mean box count and fractal dimension (Table 3). With exposure, there was a decrease in height as the animals moved downward in the cell.

Discussion

Our results have important ecological and applied significance. They not only demonstrate that some biologically derived T&O compounds have significant effects on other aquatic organisms, and may play a key role as repellents towards predators such as *Daphnia*, but our experiments also provide a careful evaluation of the application of inline toximeter units in T&O (and source water) research and monitoring.

The VOCs studied are not liberated continually by intact cells, but formed by catalytic enzymes activated upon cell damage. These compounds would, therefore, only be encountered at sufficient levels to elicit behavioural changes among grazers following the disruption of the algal or cyanobacterial cells, such as via grazing, parasitism or

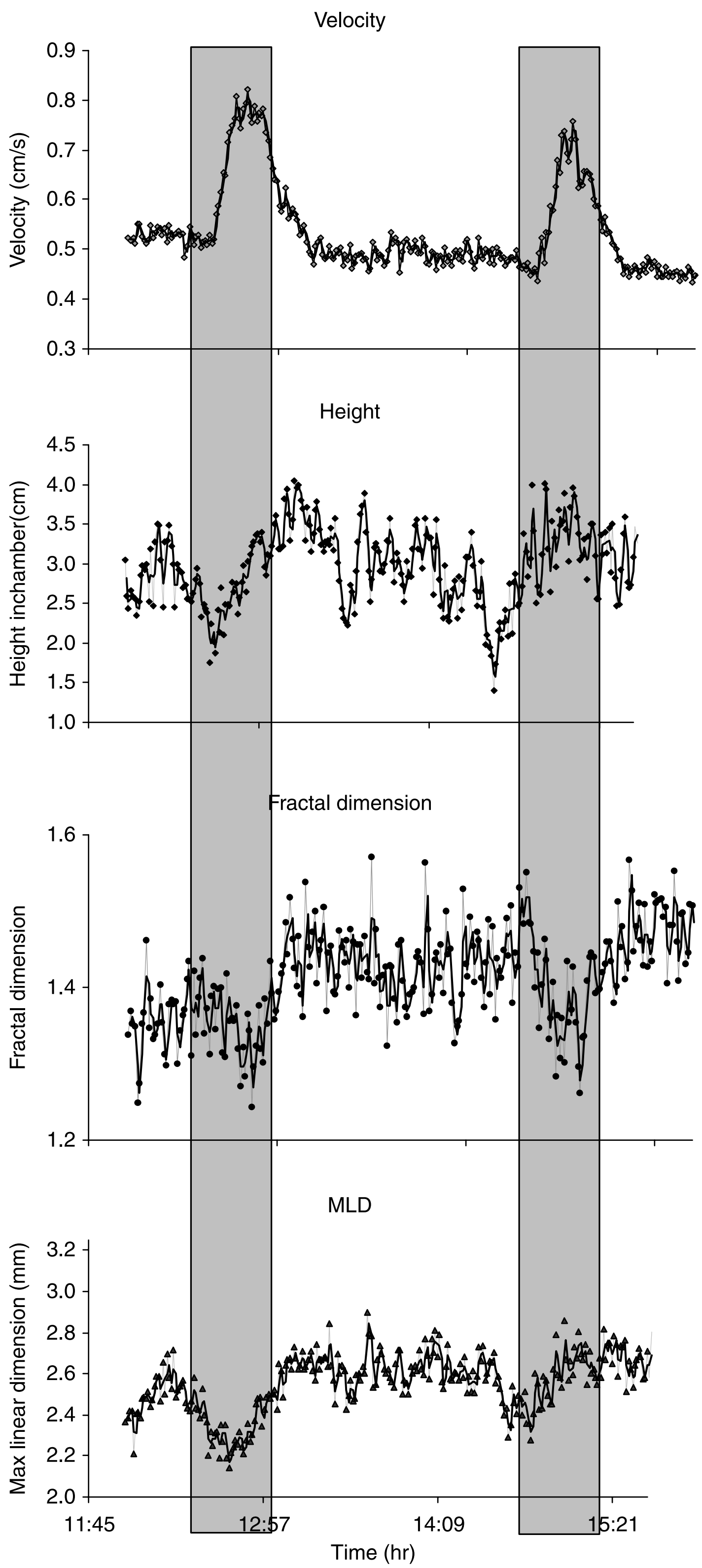

Figure 4 Example of changes in behavioural responses of *Daphnia magna* to two consecutive applications of 25 μM β-cyclocitral. Periods of exposure indicated by shaded areas

Table 3 LSMEANS comparison of *Daphnia magna* behavioural measures, pre- (period 1) and post- (period 3) application of 2.5 and 25 μM 2(*E*),4(*E*),7(*Z*)-decatrienal

Decatrienal (μM)			2.5		25	
Parameter	Period	N	Mean	SD	Mean	SD
Velocity	1	5	0.41	0.02	0.49	0.03
	3	6	0.51****	0.02	0.523**	0.03
Velocity class	1	5	10.72	4.19	21.21	6.94
	3	6	33.22****	4.50	24.83	6.04
Fractal dimension	1	5	1.41	0.05	1.35	0.03
	3	6	1.34**	0.04	1.38*	0.03
Box count	1	5	1.38	0.13	1.30	0.04
	3	6	1.28****	0.04	1.31	0.04
Height	1	5	1.92	0.37	1.94	0.24
	3	6	1.72	0.31	1.5****	0.19

Levels of significance as in Table 2; with ****$p < 0.0001$

photo-oxidative damage in *Microcystis* surface scums. The production of both VOCs represents an efficient use of cell resources to produce grazer deterrents (and in the case of the aldehyde, a potential toxin at high levels; cf. Jüttner, 2005). Beta-cyclocitral is derived from β-carotene and 2,4,7-decatrienal from polyunsaturated fatty acids. These compounds therefore act as both VOC precursors and constitutive elements of the cell photosystems and membrane structures, and very little additional cell resources are required for the VOC production.

Extensive research continues to improve our ability to characterise and treat these VOCs and identify some of the major producers (see e.g. Jüttner, 1995a,b; Hargesheimer and Watson, 1996; Bruchet and Laine, 2005; Cooke and Newcombe, 2004; Jung *et al.*, 2004; Izaguirre and Taylor, 2004). Yet why these compounds are produced (i.e. their biological significance) and how their production and release are modified by large- and small scale abiotic and biotic processes has not been clearly determined, and this understanding is essential for successful management.

A number of toxicological studies have shown that some VOCs, including 2(*E*),4(*E*),7(*Z*)-decatrienal, are lethal, carcinogenic or teratogenic at high levels, such as would be encountered in biofilms and grazer guts (Gagne *et al.*, 1999; Miralto *et al.*, 1999; Jüttner, 2005). However, as noted above, there has been very little work on non-lethal interactions, particularly in freshwater planktonic environments, even though this could play a key role in the plankton interactions that modify species dominance and succession patterns (Watson, 2003). This mechanism could explain why biologically derived source-water T&O outbreaks can bear little apparent relationship to traditional nutrient-based models of plankton dynamics, and VOC increases can be well underway before they are detected.

Some authors have used a Y tube set up to investigate grazer responses to algal metabolites. With this type of experiment it is difficult to control and manipulate VOC levels, since the levels encountered by the organisms can change over time as the compounds diffuses and become diluted. Nevertheless, van Gool and Ringelberg (1996) reported differences in the migratory responses by *Daphnia galeata* to intact "edible" and "inedible"[2] algal and cyanobacterial species, and interpreted this behaviour as an ability to discriminate between the two prey types based on unidentified sensory cues. Using a more elaborate tube set-up that allowed replicate observations, Jüttner (2005) observed more direct evidence of an avoidance response by crustaceans following exposure to 2(*E*),4(*E*),7(*Z*)-decatrienal.

[2] Defined by these authors as microcystin-producing species.

We conclude that the inline toximeter system offers a potentially effective tool to study these subtle interactions and behavioural changes under a natural variability in source water. We note, however, that reproducibility was only obtained following a series of initial trials and adjustments in the setup and experimental design, since these planktonic crustaceans are highly sensitive to such factors as changes in temperature, flow patterns, air bubbles and other contaminants. Replicable results were also dependent on using animals of the same age, which had not recently undergone moulting. Using the toximeter, we demonstrated that both β-cyclocitral and 2(*E*),4(*E*),7(*Z*)-decatrienal elicit measurable behavioural changes in *Daphnia,* such as increased swimming velocity, activity level and linearity of movement, all indicative of an avoidance response.

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