

***Isolation and identification of kairomone(s) in
the Daphnia-Scenedesmus system***

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**Isolation and identification of kairomone(s) in
the *Daphnia-Scenedesmus* system**

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Ga nu maar liggen liefste in de tuin,
de lege plekken in het hoge gras, ik heb
altijd gewild dat ik dat was, een lege
plek voor iemand, om te blijven

Een lege plek om te blijven. Rutger Kopland

Voor mama
Zonder jou was ik nooit in Wageningen terecht gekomen

Chapter 1. Chemical communication in aquatic systems

Parts of this chapter are based on:

van Holthoon, F.L., T.A. van Beek, M. Lüring, E. Van Donk, and Ae. de Groot, 2003. *Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone*. *Hydrobiologia*. **491**: p. 241-254.



Introduction

Simplified, predation is the consumption of one organism by another. Fish eat *Daphnia* (waterfleas) and *Daphnia* graze on *Scenedesmus* (green algae). It has been found that changes in one trophic level affect other trophic levels, surprisingly though the effect was not always the expected one. In this simplified foodchain of fish, *Daphnia* and *Scenedesmus*, it would be expected that increased predation on *Daphnia* by fish would also increase *Scenedesmus* biomass, because of reduced grazing, or that lowered predation on *Daphnia* results in lower algal biomass. The magnitude of change in biomass is, however, not always in agreement with general food-chain theoretical predictions^{1,2}. Apparently some other mechanisms are at work.

Recently this mechanism has been identified over a wide variety of trophic levels as a defence mechanism of the prey. These defence mechanisms include changes in life history (e.g. age at maturity), behaviour (e.g. migration), morphology (e.g. formation of spines and colonies) and biochemistry (e.g. toxins and repellents). Generally these changes are very small, because larger changes involve higher costs. Investing in defence only when a predator is near can further reduce these costs and prevents the development of resistance to toxins³.

The type of cue used to detect the presence of a predator depends on the environment. Copepods (in clear water) react to visual, acoustical or mechanical cues from their predator, while other copepods (in turbid water) have developed a reaction to a chemical cue. Chemical cues have an advantage over mechanical cues because they can accumulate and can prevent errors in activation by requiring a threshold to be exceeded before defence is initiated. Additionally, the chemical cue remains longer in the environment, can be more specific and can provide reliable information about the predator even in darkness⁵.

Models are often used to generate hypotheses, which are subsequently tested with controlled experiments to gain further insight in the interaction of foodchains and their trophic interactions. One of the biggest simplifications in current models is the assumption that all prey items can be caught and eaten. This does not hold true if defence mechanisms exist, which seems to be the rule rather than the exception². It is important to identify these chemical cues to create more accurate models concerning food webs and prey-predator interactions leading to a significant contribution of the scientific theory^{2,4,5}.

Predator-prey interactions are very important processes in the food web as they represent the move of energy through the trophic levels and have strong consequences for individuals, populations and communities. Therefore, the mechanisms regulating important ecological interactions, such as the predator-prey interaction, need to be

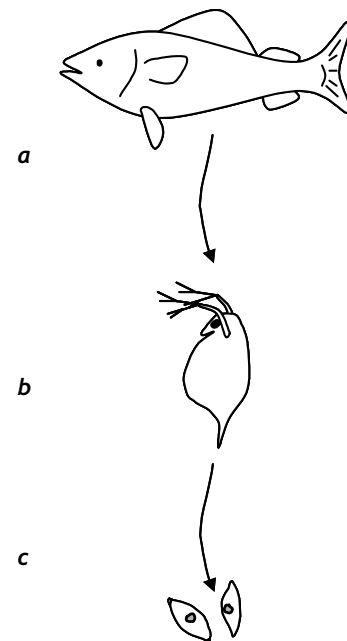


Figure 1. Food chain

a. Fish; b. *Daphnia*; c. *Scenedesmus*



elucidated. Chemical characterisation of the kairomones will allow controlled experimentation demonstrating their regulating role as well as the investigation of production and dispersal and the underlying sensory, physiological and genetic capabilities in the receiving algae.

Although the presence of chemical cues has been confirmed in many systems, the chemical structures remain predominantly elusive. Especially in fresh water environments very few structures have been elucidated. Nonetheless three kairomones, produced by *Lembadion* (predatory ciliate), *Amoeba* (microorganism), and *Stenostomum* (flatworm) affecting a freshwater ciliate, *Euplotes*, have been characterised⁶⁻⁸. These compounds are complex proteins varying in molecular weight from 4.5 kDa to 31.5 kDa⁶. In marine environments more progress has been made. C₁₁ hydrocarbons (feeding deterrents and gamete attracting compounds, 1-6, Fig. 2a)⁹ and phlorotannins (feeding deterrents, 7, Fig. 2b)¹⁰⁻¹² have been isolated from brown algae. Inosine, glutamic acid, uric acid, 5-methyl-3-heptanone, 3,5-octadiene-2-one and nereithione (sex pheromones, 8-16, Fig. 3) have been found in *Nereis* and *Platynereis*¹³⁻¹⁶.

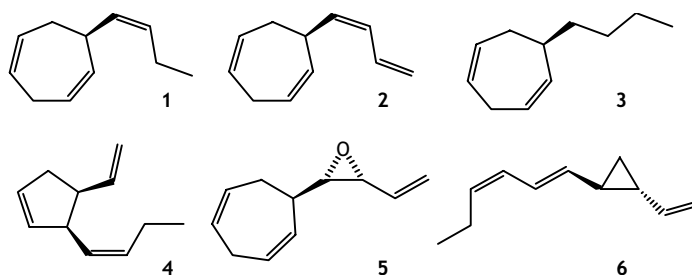


Figure 2a. Pheromones from marine brown algae⁹.

1. ectocarpene; 2. desmarestene; 3. dictyotene; 4. multifidene; 5. lamoxirene; 6. hormosirene

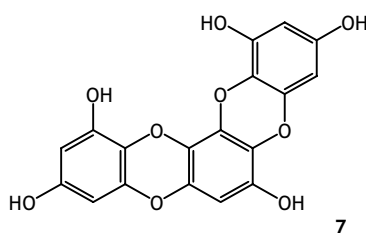


Figure 2b. Induced defence compounds from marine brown algae¹⁰⁻¹².

7. phlorotannin A

Cladocerans like *Daphnia* have proved to be excellent model organisms in many fields of biology. There are few other groups of organisms where the relationship between genotype and the effect of the environment can be studied so easily. Their reproductive system makes them well suited for studies of environmentally induced variability (e.g. cyclic parthenogenesis)¹⁷. This is one of the reasons why much research has been devoted to *Daphnia* (Table 1, a through g). So far research has shown that *Daphnia* reacts to at least 10 different cues⁴.

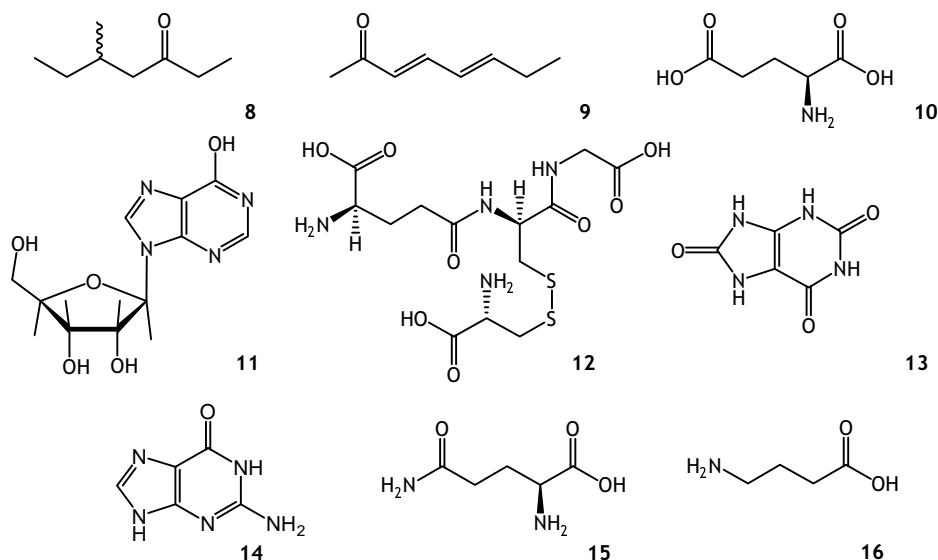


Figure 3. Pheromones from marine polychaetes, *Nereis* and *Platynereis*¹³⁻¹⁶.

8. 5-methyl-3-heptanone; 9. 3,5-octadiene-2-one; 10. glutamic acid; 11. inosine; 12. nereithione; 13. uric acid; 14. guanosine; 15. L-glutamine; 16. γ -amino butyric acid

***Daphnia* and the predator**

Daphnia (waterflea) is not only a good food source for many species of planktivorous fish but also for invertebrate predators, such as *Chaoborus* larvae (midge) and *Notonecta* (backswimmer). *Daphnia* has developed several defence mechanisms to avoid predation by these predators.

One of the most studied mechanisms is diel vertical migration (DVM). During the day *Daphnia* migrates down to darker depths to avoid fish. During the night they will resurface to the warmer top layers of the lake. Although light plays an important role, DVM seems to be triggered by a chemical signal¹⁸. Ringelberg and van Gool found evidence that it is not the fish themselves but bacteria associated with them that produce the cue. When treated with antibiotics, the fish water showed significantly less DVM than a positive control. Nonetheless some significant biological activity still remained. Although bacteria do seem to play a part, the effect cannot be explained completely by them⁶⁶. The observed DVM when *Daphnia* is in the presence of *Chaoborus* is completely opposite to DVM when *Daphnia* was exposed to fish^{19,67,68}.

Additionally, it has also been shown that *Daphnia* can change morphologically after being exposed to chemical cues from predators. These changes occur to a degree naturally when the season progresses and temperature changes, but some were shown to be a direct response to chemical cues (cyclomorphosis). Neckteeth, helmets and crests were formed when *Daphnia* is exposed respectively to *Chaoborus*⁶⁹, planktivorous fish⁶⁸, and a notonectid predator³⁴. Furthermore, Slusarczyk reported that diapause is also regulated by chemical cues. He reported evidence for two chemical cues that regulate synchronisation of sexual reproduction (i.e. formation of males and production of ephippial eggs or resting eggs) to protect the genome during high predation risk⁷⁰. So far no evidence was found for the presence of sex pheromones⁷¹.



Table 1a. Studies on the isolation and identification of infochemicals involved in the Fish-Daphnia system.

Fish: Lep = *Lepomis*, Gas = *Gasterosteus*, Esx = *Esox*, Car = *Carassius*, Rhd = *Rhodeus*, Lup = *Leucaspis*, Leu = *Leuciscus*, Rut = *Rutilus*, Per = *Perca*, **Daphnia:** Dob = *D. obtusa*, Dpa = *D. parvula*, Dre = *D. retrocurva*, Dpc = *D. pulicaria*, Dpx = *D. pulex*, Dga = *D. galeata*, Dma = *D. magna*, Dhy = *D. hyalina*, Dgh = *D. galeata x hyalina*.

Producer Fish	Receiver Daphnia	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Lep	Dam, Dga, Dob, Dpa, Dpx, Dpc, Dre			¹⁸
Gas, Esx, Car, Rhd, Lup	Dma			pH stable; insensitive to pronase E and proteinase; < 0.5 kDa; heat stable; non-volatile; degraded by microbes ¹⁹
Leu, Rut, Car	Dma			enrichment on lipophilic sorbent (C ₁₈ , C ₈ , phenyl); insensitive to hydrogenation; anionic; not cationic; insensitive to derivatisation (OPA, fluorescamine, FMOC); sensitive to reversible acetylation; HPLC (eluting between 68-84% methanol) ²⁰
Leu	Dhy	trimethylamine 24		²¹
Gas, Esx, Car	Dma		cellulose 29, laminarin 30, xylan 31	enrichment on lipophilic sorbent (C ₁₈); anionic; not cationic; pH stable; insensitive to phosphatase, sulphatase, glucuronidase, proteases, driselase (contains cellulase, pectinase, laminarinase, xylanase, and amylase activities), hyaluronidase (depolymerase of mucopolysaccharides, hyaluronic acid, chondroitin sulfates A and C), diazomethane; sensitive to reversible acetylation; HPLC (eluting between 68-84% methanol) ²²
Leu, Lup, Car	Dma		trimethylamine 24	lipophilic ²³
Leu	Dga, Dgh		trimethylamine 24	²⁴
Leu, Rhd	Dma, Dga, Dhy		trimethylamine 24	²⁵
Leu, Per	D.	6-hydroxy-5-tetradec-2-enolide 25, erythro-6-acetoxy-5-hexadecanolide 26, 5-hydroxy-4-decanolide 27, phenylalanine-proline diketopiperazine 28		²⁶

Table 1b. Studies on the isolation and identification of infochemicals involved in the Fish-Insect system.

Fish: Lep = *Lepomis*, Gas = *Gasterosteus*, Pim = *Pimephales*, Sca = *Scardinius*, Pun = *Pungitius*, Pom = *Pomoxis*, **Insects:** Cha = *Chaoborus*

Producer Fish	Receiver Insects	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Lep, Pim	Cha			²⁷
Gas	Cha			²⁸
Sca, Pun	Cha			non-volatile ²⁹
Pom	Cha			³⁰



Table 1c. Studies on the isolation and identification of infochemicals involved in the Insect-Daphnia system.

Insects: Cha = Chaoborus, Ani = Anisops, Not = Notonecta. **Daphnia:** Dca = D. carinata, Dce = D. cephalata, Dlo = D. longicephala, Dan = D. angulata, Dmg = D. magniceps, Dwa = D. wankelata, Dob = D. obtusa, Dpa = D. parvula, Dpx = D. pulex, Dga = D. galeata, Dpc = D. pulicaria, Dpx = D. pulex, Dga = D. galeata, Dam = D. ambigua.

Producer Insects	Receiver Daphnia	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Cha	Dam			easily extracted, heat stable, low molecular weight, single compound ³¹
Cha	Dpx			heat and pH stable; <0.5 kDa; sensitive to acetylation and incineration; insensitive to derivatisation (fluorescamine), sodium borohydride and pronase (cleaves peptide bonds); LPLC (eluting between 25-50% methanol) ³²
Cha	Dpx		glucose 43	enrichment on lipophilic sorbent (C ₁₈ , C ₈ , not phenyl); heat and pH stable; <0.5 kDa; anionic; acidification increases positive charge; sensitive to reversible acetylation; insensitive to derivatisation (OPA, fluorescamine, FMOC); insensitive to glucosidases; HPLC (eluting between 65-80% methanol) ³³
Ani	Dca, Dce, Dlo, Dan, Dmg, Dwa			³⁴
Not	Dam, Dga, Dob, Dpa, Dpx, Dpc, Dre			¹⁸
Daphnia	Insects			
Dpa	Cha			³⁰

Table 1d. Studies on the isolation and identification of infochemicals involved in the Algae-Daphnia system.

Daphnia: Dma = D. magna, Dpx = D. pulex, Dghy = D. galeata x hyalina. **Green algae:** Act = Actinastrum, Sce = Scenedesmus, Chl = Chlamydomonas. **Cyanobacteria:** Osc = Oscillatoria, Mic = Microcystis.

Producer Algae	Receiver Daphnia	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Sce, Osc	Dgh			³⁵
Sce, Osc, Mic	Dpx			³⁶
Sce	Dgh, Dpx, Dma			³⁷
Daphnia	Algae			
none	Sce	glucose 43		³⁸
other: Moina	Sce, Chl			³⁹
none	Sce	indole-3-acetic acid 44, gibberellic acid 51	indole-2-propionic acid 49, indole-3-butyric acid 52, naphthalene acetic acid 50	⁴⁰
none	Sce		glucose 43	⁴¹



Producer	Receiver	Proposed	Rejected	Chemical constituents/ chemical and physical properties
<i>Dma</i>	<i>Sce, Mic</i>		urea 32 , ammonia 33	⁴² <0.5 kDa; extracted on lipophilic sorbent (C ₁₈) but not desorbed with methanol; insensitive to pronase E (cleaves peptide bonds), proteases; heat and pH stable; non-volatile; sensitive to incineration ⁴³
<i>Dma</i>	<i>Sce</i>		indole-3-acetic acid 44 , phenylacetic acid 45 , p- chlorophenoxacetic acid 46	⁴⁴
<i>Dma</i>	<i>Sce</i>	SDS 87 , FFD-6 85	urea 32 , ammonia 33 , ascorbic acid 34 , citric acid 39 , fructose 40 , cellulose 29 , pectin 42 , glucose 43 , methyl dodecylsulfonate 75 , 2- butanone 76 , 2-hexanone 77 , Brij 35 ⁴⁵ , ethyl acetate 79 , 4- heptanone 80 , Triton® X-100 82 , 1- hexa-decanol 84 , Extran MA02 (containing kathon 81 , marlopon 86 , marlipal 83 , dodecylbenzenesulfonic acid 74)	^{45,46}
<i>Dma</i>	<i>Sce</i>		fatty acids	enrichment on lipophilic sorbent (C ₁₈), lipophilicity increased at low pH; ionic moiety; insensitive to acetylation; olefinic double bonds; insensitive to sulphatase, phosphatase, pronase E, proteases; HPLC (eluting between 77-100% methanol) ⁴⁷
<i>Dma</i>	<i>Sce</i>	urea 32	cyclic AMP 47	⁴⁸
<i>Dma</i>	<i>Sce</i>	nucleic acids, oligonucleotides, deoxyribonucleotides, peptides		⁴⁹
<i>Dma</i>	<i>Act</i>		acetic acid 34 , butanoic acid 35 , lactic acid 36 , ascorbic acid 37 , citric acid 38 , amino acids 10 , 53 - 67	extractable by ion exchange, HPLC (C ₁₈ , 80 % MeOH fraction), carboxylic acid ⁵¹
<i>Dma</i>	<i>Sce</i>		farnesic acid (four stereoisomers) 41 , fatty acids 68-73	⁵²
<i>Dma</i>	<i>Sce</i>		20-hydroxyecdysone 48	⁵³
<i>Dma</i>	<i>Sce</i>		jasmonic acid 39	HPLC: eluting between 42-52% methanol (Kromasil C ₁₈ , end-capped); eluting between 60-68% methanol (Lichrospher C ₁₈ , non-endcapped); eluting between 61-67% methanol (Spherisorb ODS, end-capped) ⁵⁴
<i>Dma</i>	<i>Sce</i>		urea 32	⁵⁵
<i>Dma</i>	<i>Sce</i>			non-volatile, enrichment on SPE sorbent (C ₁₈ , CN, ENV ⁺ , Oasis TM), extractable by anion exchange ² , HPLC (eluting between 42-69% acetonitrile) ⁵⁶



Table 1e. Studies on the isolation and identification of infochemicals involved in Daphnia-Daphnia interactions.

Daphnia: Dma = *D. magna*, Dpc = *D. pulicaria*, Dpx = *D. pulex*, Dhy = *D. hyalina*, Dga = *D. galeata*, Dcu = *D. cucullata*, Dam = *D. ambigua*, Dla = *D. laevis*, Dlu = *D. lumholztzi*

Producer Daphnia	Receiver Daphnia	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Dma, Dpx	Dma, Dpx			unstable (<i>Dma</i> compound), stable (<i>Dpx</i> compound) ^{57,58}
Dma,Dpc, Dpx,Dhy, Dga	Dma,Dpc, Dpx, Dcu, Dhy,Dga, Dam,Dla, Dlu		ammonia 33	⁵⁹
Dpx, Dcu	Dpx, Dcu		ammonia 33, urea 32 (not solely responsible, possibly synergistic with unknown compound)	⁶⁰

Table 1f. Studies on the isolation and identification of infochemicals involved in Macrophyte interactions.

Daphnia: Dma = *D. magna*, Dro = *D. rosea*. **Cryptophyceae:** Cry=Cryptomonas; Rhd=Rhodomonas. **Green algae:** Chl = *Chlamydomonas*, Sce = *Scenedesmus*, Nan = *Nannochloris*, Sti = *Stigeoclonium*. **Cyanobacteria:** Ana = *Anabaena*, Scy = *Synechocystis*, Sco = *Synechococcus*, Tri = *Trichormus*. **Macrophyte:** Myr = *Myriophyllum*, Nul=Nuphar, Nit = *Nitella*, Cer = *Ceratophyllum*, Pot = *Potamogeton*, Elo = *Elodea*.

Producer Macrophytes	Receiver Daphnia	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Elo, Pot, Myr, Nit, Cer	Dma, Dro			^{61,62}
Nul	Dma	resorcinol 23		⁶³

Macrophytes Algae

Myr	Sce, Ana, Scy, Sco, Sti, Tri, Nan	tellimagrandin II 22, gallic acid 20, ellagic acid 21, other polyphenols		⁶⁴
Nul	Sce, Ana, Chl, Cry, Rhd	resorcinol 23		⁶³

Table 1g. Studies on the isolation and identification of infochemicals involved in microorganism interactions.

Green algae: Chl = *Chlamydomonas*. **Cyanobacteria:** Ana = *Anabaena*. **Dinoflagellate:** Oxy = *Oxyrrhis*, **Haptophyte:** Emi = *Emiliana*

Producer Microorganism	Receiver Microorganism	Proposed	Rejected	Chemical constituents/ chemical and physical properties
Chl	Ana			>5 kDa, <15 kDa; hydrophilic; heat sensitive; proteinase sensitive ³
Oxy	Emi	dimethylsulphonio- proponate 17, dimethylsulphide 18, acrylic acid 19		⁶⁵



Not only does *Daphnia* react to chemical cues released from the predator, but also to a chemical signal released by injured conspecifics. Filtered water that had contained crushed *Daphnia* induced individuals to remain deeper in the water column. They also aggregated more frequently in the presence of the cue^{72,73}. Closely related species reacted differently to the presence of predators, some species needed the added presence of the conspecific cue to react adequately to predation^{72,73}. Infochemicals released from competitors such as conspecifics⁷⁴ and congeners⁷⁵ may affect feeding, growth, reproduction and morphology in *Daphnia*. Conspecific-associated infochemicals improved the reproduction in *D. cucullata*⁷⁶ and in *D. magna*^{77,78}. This contrasts with intraspecific substances reported for *D. magna*^{57,79,80}, *D. carinata*⁷⁵ and *D. hyalina*⁷⁷ that give a reduction in reproduction. Crowded congeners also reduced the fecundity in *D. cucullata*, and *D. magna*^{76,78,81}. Recently, Burns demonstrated that water containing the moderate amount of 85 daphnids L⁻¹ depressed growth rate, lowered body size and clutch size in small-sized *Daphnia* species, but not in larger species⁵⁹.

When fish were fed with an alternative food source for 10 weeks prior to experiments with *Daphnia*, *Daphnia* did not show morphological changes. After these same fish were

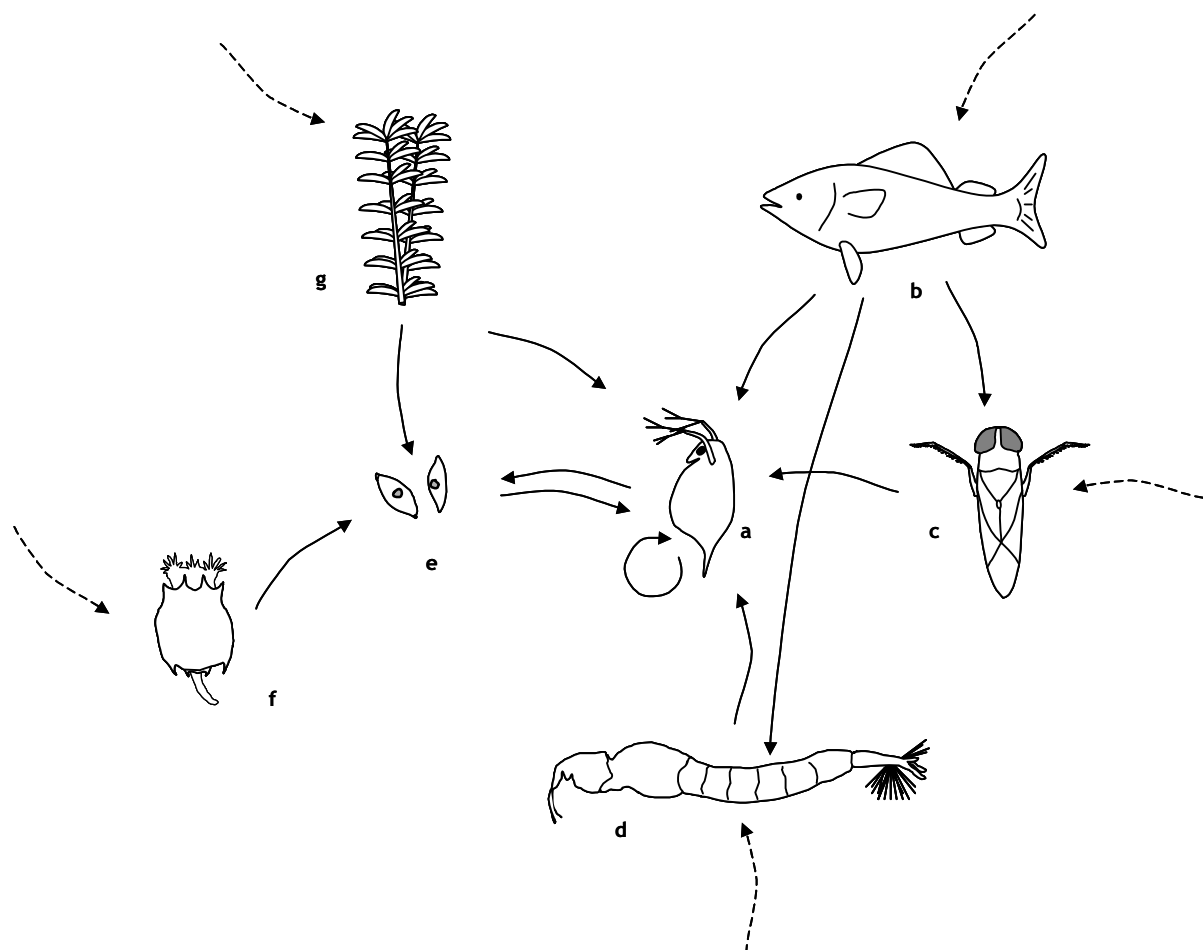


Figure 4. Predator-prey interactions involving *Daphnia*

a. *Daphnia*; b. Fish; c. Backswimmer (e.g. *Notonecta* or *Anisops*); d. Midget (e.g. *Chaoborus* larvae); e. Rotifer (e.g. *Brachionus*); f. Algae (e.g. *Scenedesmus*); g. Macrophyte (e.g. *Myriophyllum*)



fed their normal diet of *Daphnia*, the morphological changes reappeared⁸². Stabell et al. proposed therefore that passage of conspecifics through the intestine of the predator is necessary for the chemical signals to be activated. Technically these compounds would not be kairomones, but pheromones, according to the terminology described by Dicke and Sabelis⁸³ (Appendix I) since compounds of the ingested conspecifics are activated⁸². In all studies on crowding in *Daphnia* the animals reacted as if they were food limited⁸¹, because food uptake under crowded conditions may be lower than when not crowded^{81,84}. This may be the result of mechanical interference and allelochemical interactions⁷⁵. A similar mechanism to DVM is diel horizontal migration (DHM), where *Daphnia* migrate horizontally into the vicinity of water plants. The macrophytes create shelter for them from fish during the day^{61,62,85}. Paradoxically these macrophytes have been shown to produce chemicals that repel *Daphnia*. Life history changes were observed when *Daphnia* was exposed to these chemicals from macrophytes. Only the presence of chemical cues exuded by fish overrides the repellent effect of the macrophyte compounds⁶². The presence of macrophytes does not only affect *Daphnia*. It also has an impact on phytoplankton, because macrophytes directly influence predation on *Daphnia* and indirectly on phytoplankton^{86,87}. Macrophytes also produce chemical cues that deter phytoplankton, probably because both compete for the same sources of light and nutrients⁸⁷. Leu et al. suggested that even a moderate reduction of photosynthetic activity may impair growth of co-occurring epiphytes and phytoplankton⁸⁸. These compounds seem to affect cyanobacteria more than green algae^{64,86,87}.

Daphnia and the prey

Many prey of *Daphnia* have developed mechanisms to avoid being ingested (toxins, spines, colony formation) and digested (thick cell walls, mucous). Most of these have a morphological nature, however Hansson et al. have reported evidence for another type of defence mechanism for phytoplankton. They found that recruitment of algae from the sediment into the water phase was correlated with predator abundance (recruitment increased at low predation abundance and decreased at high predation abundance). Hence they could not reject the hypothesis that presence of predators induced shifts in behaviour of some algal groups⁸⁹⁻⁹².

Although Mikheeva and Kruchkova reported about morphological changes in green algae in the presence of a cladoceran predator (*Moina*) in an inaccessible Russian journal thirteen years earlier³⁹, Hessen and van Donk were the first to show that filtered *Daphnia* water induced colony formation in the green alga, *Scenedesmus subspicatus*, but not in the cyanobacterium, *Microcystis aeruginosa*⁴². However this latter organism may be toxic and possibly does not need additional defence^{93,94}. Nonetheless, the toxin production did increase when the cyanobacteria came in contact with a predator or chemicals released by the predator and may still have an inducible component⁹⁵. The formation of colonies in response to grazing-associated cues seems not to be unique to the *Chlorophyceae* (green algae), as it has been reported that phytoplankton (such as *Phaeocystis*, a haptophyte) from other taxonomic groups also exhibit colony formation in the presence of a predator or predator-associated cue^{96,97}.



Extracts of crushed *Daphnia* and *Scenedesmus* did not show colonising activity^{43,47}. Because mechanically crushing *Daphnia* and *Scenedesmus* did not free the active compound, it may be that an interaction between *Scenedesmus* and *Daphnia* is necessary to initiate production of the active compound⁹⁸. Additionally Fink found that a methanolic extract of the digestive tract (corrected for dry weight) showed four times more biological activity than extracts of other parts of the body of *Daphnia*⁵⁴.

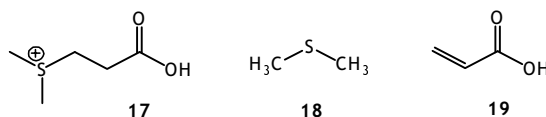


Figure 5. Proposed structures for compounds that induce the defence mechanism of *Emiliana huxleyi*⁹⁹.

17. dimethylsulfoniopropionate; 18. dimethylsulphide; 19. acrylic acid

The fact that *Daphnia* has to be fed in order to produce the chemical cue⁹⁸ suggests a mechanism as with *Emiliana-Oxyrrhis* (microorganisms). In *Emiliana* dimethylsulfoniopropionate (DMSP, **17**, Fig. 5) is only converted to dimethylsulfide (DMS, **18**, Fig. 5) when it comes into contact with DMSP lyase, which is stored in other cells. Digestion by *Oxyrrhis* lyses *Emiliana* cells, mixes cell contents and thus produces DMS⁶⁵. As a second product acrylic acid **19** is formed, which deters other predators. The same strategy has been found in other defence mechanisms^{82,99}.

However observations by Von Elert and Franck do not support this for *Daphnia* and *Scenedesmus*. They found that colony formation activity was not linked to the ingestion rate, because colony formation did not increase with body mass of *Daphnia*, indicating that the cue originates from specific metabolic reactions that differ from the general metabolism⁴⁷. This was confirmed by Lüring who stated that colony formation did not seem to be related to biomass, but rather to the feeding activity of *Daphnia*, as starved animals did not induce colony formation⁴⁵. Comparable to the fish factor⁶⁶, it might be bacteria, living in symbiosis with *Daphnia*, which are responsible for the formation of the kairomone.

Chemical cues from potential food algae could be important because they provide information on the food quality of a particular organism⁴. So, according to van Gool and Ringelberg, algae-associated odours could be detected by *Daphnia*³⁵. *Daphnia* moved in the direction of edible green algae (*Scenedesmus*), but did not move when the offered alternative (cyanobacteria, *Oscillatoria*) was toxic. Studies by Haney et al. revealed that *Daphnia* did evade toxic cyanobacteria or reduce food intake when exposed to chemicals released by cyanobacteria^{100,101}. In fact, one would expect *Daphnia* to avoid areas with dangerous and potentially lethal cyanobacteria. In contrast, in food gradient experiments *Daphnia* showed the strongest aggregative response at intermediate food levels, and avoided high food levels¹⁰². The mechanism used by *Daphnia* to locate these regions is probably related to the concentration of algal cells rather than the presence of algal odours^{102,103}. Furthermore, in studies done by the groups of Porter and Roozen, *Daphnia* appeared unable to locate and detect algal patches^{37,104}. Additionally diaptomid copepods



did not respond to algal odours¹⁰⁵. On the other hand, *Daphnia* may perceive signals from toxic cyanobacteria as information associated with danger that may result in immediately reduced food intake¹⁰¹ and altered swimming behaviour¹⁰⁶.

It is well known that some cyanobacteria produce toxins that affect several organisms (e.g. *Daphnia* and green algae). However what is little known is that *Chlamydomonas* (green algae) appears to produce a compound that actually increases anatoxin but decreases microcystin production in *Anabaena* (Cyanobacteria). The compound was hydrophilic, proteinase and heat sensitive, and between 5 and 15 kDa in size (possibly a peptide)³. Possibly the elicitation of microcystin toxins plays a role in the reduction of losses associated with herbivory⁹³. Mutual interactions were also described for *Peridinium* (dinoflagellate) and *Microcystis* (Cyanobacteria). *Peridinium* related compounds induced sedimentation and subsequent cell lysis in *Microcystis*. Simultaneously biosynthesis of microcystin toxins was increased. These toxins then influenced cell death and division in *Peridinium*¹⁰⁷. The contrasting results (decreased³ and increased¹⁰⁷ microcystin production) underline the complexity of the biotic interactions in water bodies.

Daphnia and the signal

Macrophyte- Daphnia / Algae

Although the compound produced by *Myriophyllum* (macrophyte) that acts as a repellent on *Daphnia* has not yet been elucidated, several compounds were identified that acted on

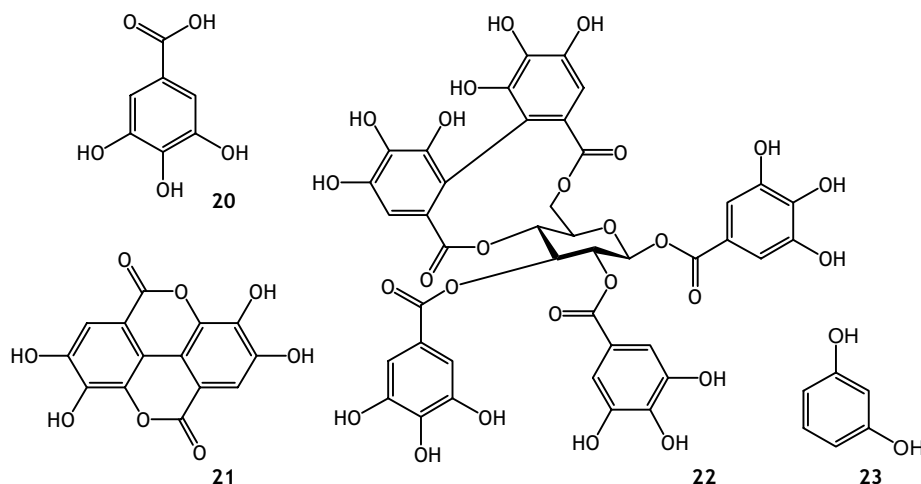


Figure 6. Proposed structures from macrophytes that affect phytoplankton and zooplankton.

20. gallic acid⁶⁴; 21. ellagic acid⁶⁴; 22. tellimagrandin II⁶⁴; 23. resorcinol⁶³

phytoplankton (algicides). Especially tellimagrandin II (**22**, Fig. 6) was very effective in decreasing the exoenzyme activity of the algae. Other polyphenols (e.g. gallic acid **20** and ellagic acid **21**, Fig. 6) were also investigated and they showed some activity, but not as strong as tellimagrandin II⁶⁴. Furthermore another macrophyte (*Nuphar*) has been found to exude resorcinol **23** that does have an effect on *Daphnia*⁶³.



Fish-Daphnia

Most research at present is aimed at the characterisation of the kairomones excreted by predators of *Daphnia*. Boriss et al. suggested that trimethylamine (TMA, **24**, Fig. 7) produced by fish was the compound responsible for the defensive behaviour of *Daphnia*²¹. However, when Pohnert and Von Elert (2000) repeated the experiments of Boriss et al. they found that *Daphnia* reacted to TMA only at unrealistically high concentrations. Furthermore, diel vertical migration (DVM) activity remained even when TMA was removed from the fish water²³. Other research showed that the activity was lost when the cue was acetylated but this could be reversed by saponification. Because TMA cannot be acetylated, it should still show biological activity after acetylation. Clearly, this is in contradiction with the results found by Boriss et al. and gives further validation to the rejection of TMA as the kairomone. Other studies came to a comparable conclusion when they found that the response of *Daphnia* to fish kairomone and TMA is qualitatively similar

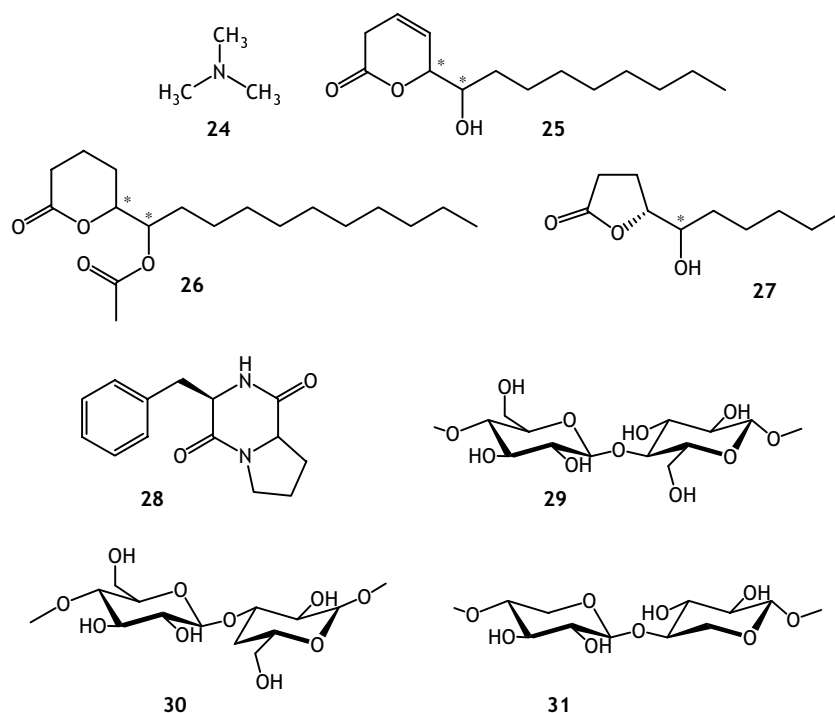


Figure 7. Proposed structures for compounds that induce the *Daphnia* defence mechanism

24. trimethylamine²¹; 25. 6-hydroxy-5-tetradec-2-enolide²⁶; 26. erythro-6-acetoxy-5-hexadecanolide²⁶; 27. 5-hydroxy-4-decanolide²⁶; 28. phenylalanine-proline diketopiperazine²⁶; 29. cellulose²²; 30. laminarin²²; 31. xylan²²

for some traits, but not for all^{24,25}. The loss of activity after acetylation indicates the presence of a nucleophilic group; esters could be excluded because activity was not lost by strong alkaline conditions.

Further, the kairomone showed no cationic characteristics (excludes TMA) but could interact with anion exchangers. The kairomone was incubated with several enzymes, but never lost its activity. Cellulose **29**, laminarin **30** and xylan **31** were rejected, based on



the inability of the driselase enzyme to destroy the biological activity²². Carboxylate groups could be excluded because the activity remained after methylation. Finally, the biological activity was restricted to one fraction when the sample was subjected to HPLC. Water from different species of fish yielded the same active fraction with an identical retention time^{19,20,22}. Thus far several compounds, 5-hydroxy-4-decanolide **27**, erythro-6-acetoxy-5-hexadecanolide **26**, 6-hydroxy-5-tetradec-2-enolide **25** and phenylalanine-proline diketopiperazine **28** (Fig. 7), have been identified from the active extract of fish water²⁶. The properties of 5-hydroxy-4-decanolide so far matched those of the compound described by Von Elert and Pohnert²². Although the synthetic compound showed some biological activity, the effect was less than unfractionated fish water²⁶. The hydroxylactones (**25-27**) possess two chiral centres and thus four stereoisomers exist of each compound. One of the stereoisomers from the mixture was more frequently found in nature. Since stereochemistry can influence bioactivity greatly, further investigations will be necessary to determine the exact stereochemistry of these hydroxylactones, only then can the right compounds be tested for biological activity. First results for 5-hydroxy-4-decanolide **27** indicate at an R-configuration at position 4²⁶.

Insects-Daphnia

The response of *Daphnia* to the *Notonecta* (backswimmer) cue resembled that of the fish factor^{67,68}. So far no work on the characterisation of the *Notonecta* cue has been done. The response of *Daphnia* to the chemical cue from *Chaoborus* was fundamentally different from the fish or *Notonecta* cue. The *Chaoborus* kairomone delays reproduction^{68,108}, reduces clutch size⁶⁸ (but increased clutch size has also been observed¹⁰⁸) and increases the age at maturity⁶⁸, whereas the fish and *Notonecta* kairomones induce opposite responses⁶⁸. Even the direction of DVM in the presence of *Chaoborus* is reversed to the migration pattern of *Daphnia* found in the presence of fish and *Notonecta*¹⁹. This indicates that at least two different chemical compounds are involved^{4,19,68,109}.

The *Chaoborus* cue has a molecular weight (MW) < 0.5 kDa. The activity of the chemical cue is not related to olefinic or aromatic double bonds, indicated by the inability to extract the active compound with a phenyl-SPE cartridge or to inactivate the cue by catalytic reduction. The compound was retained by strong cation and anion exchangers (although interactions with the anionic exchanger were stronger than with the cationic exchanger), indicating the presence of charged pH dependant moieties. A carboxylate group is essential for activity indicated by the results of several experiments³³. Acid and base treatment of the extract did not reduce activity, eliminating esters as the active moiety of the compound³². Tollrian and Von Elert found one active fraction after subjecting the sample to HPLC³³, whereas Parejko and Dodson found two active fractions after analysis of the extract on LPLC³². This is probably due to differences in their extraction methods. One³² extracted whole larvae while the other³³ extracted only the water that had contained larvae.



Daphnia -Algae

Less characterisation work has been done on the cue, released by *Daphnia*, which causes not only *Scenedesmus*⁴², but also *Actinastrum* (green algae)⁵¹ to colonise. Although

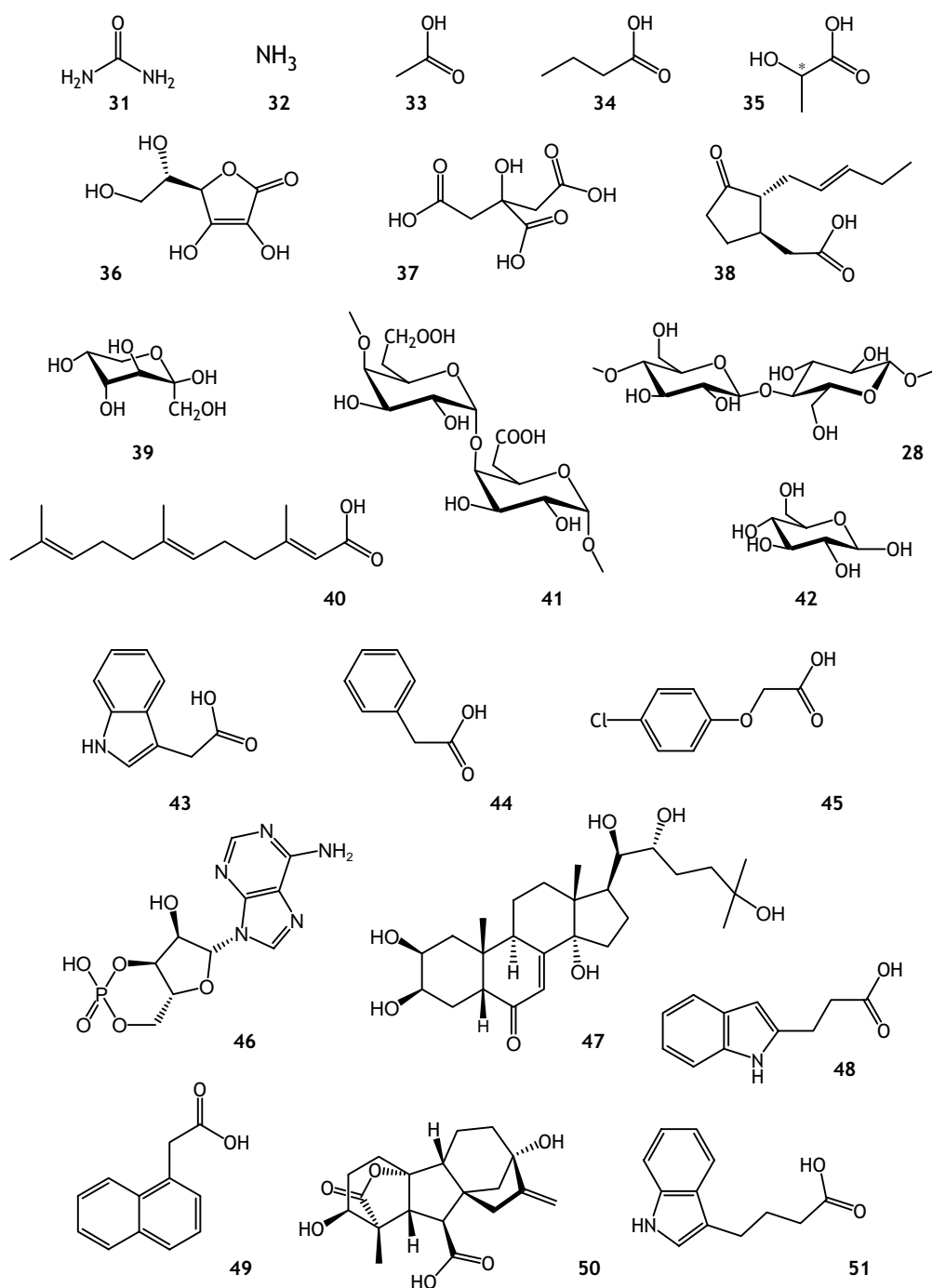


Figure 8a. Proposed structures for compounds that induce the colony formation in *Scenedesmus*

32. urea⁴⁸; 33. ammonia⁴⁵; 34. acetic acid⁵¹; 35. butanoic acid⁵¹; 36. lactic acid⁵¹; 37. ascorbic acid⁵¹; 38. citric acid⁵¹; 39. jasmonic acid⁵⁴; 40. fructose⁴⁵; 41. cellulose⁴⁵; 42. (E,E)-farnesoic acid⁵²; 43. glucose⁴⁵; 44. indole-3-acetic acid⁴⁴; 45. phenylacetic acid⁴⁴; 46. p-chlorophenoxyacetic acid⁴⁴; 47. cyclic AMP⁴⁹; 48. 20-hydroxyecdysone⁵³; 49. indole-2-propionic acid⁴⁰; 50. naphthalene acetic acid⁴⁰; 51. gibberellic acid⁴⁰; 52. indole-3-butyric acid⁴⁰



Lampert et al. had obtained negative results with several concentrations of urea⁴³, Wiltshire and Lampert reported that urea (**32**, Fig. 8a) could be the chemical cue that induced colony formation in *Scenedesmus*⁴⁸.

Lürling and Von Elert repeated these experiments and found that urea did not induce colony formation⁵⁵. Therefore the *Daphnia* factor is most likely not urea. Von Elert and Franck have collected most information on the nature of the abovementioned cue. In summary they found the cue to be a non-volatile organic substance with a low molecular weight (<0.5 kDa). It was heat and pH stable⁴⁷. This concurs exactly to what was reported by Lampert et al.⁴³ (but both studies were performed in the same institute).

The presence of a positively charged moiety could be excluded, but a pH-dependent negatively charged moiety was found, because the compound was retained by a strongly basic anion exchanger, which implies an anionic compound and excludes urea⁴⁷. The negative charge was lost at a low pH (pH=2). This points towards a carboxylic group. The presence of a carboxylate was also reported by Yasumoto et al.⁵¹.

The observed negative charge was not the result of phosphate or sulphate groups. Hydroxyl, carbonyl and amino groups could be excluded as functional groups essential for biological activity, but the presence of olefinic double bonds was necessary for colony formation. Although the possibility that the compound is a small peptide could not be excluded, it is less likely considering the presence of olefinic bonds. This is confirmed by the fact that peptide bonds in general and specifically Arg-Lys and Phe-Leu bonds were proven not to be essential for the biological activity of the cue⁴⁷. This is in conflict with

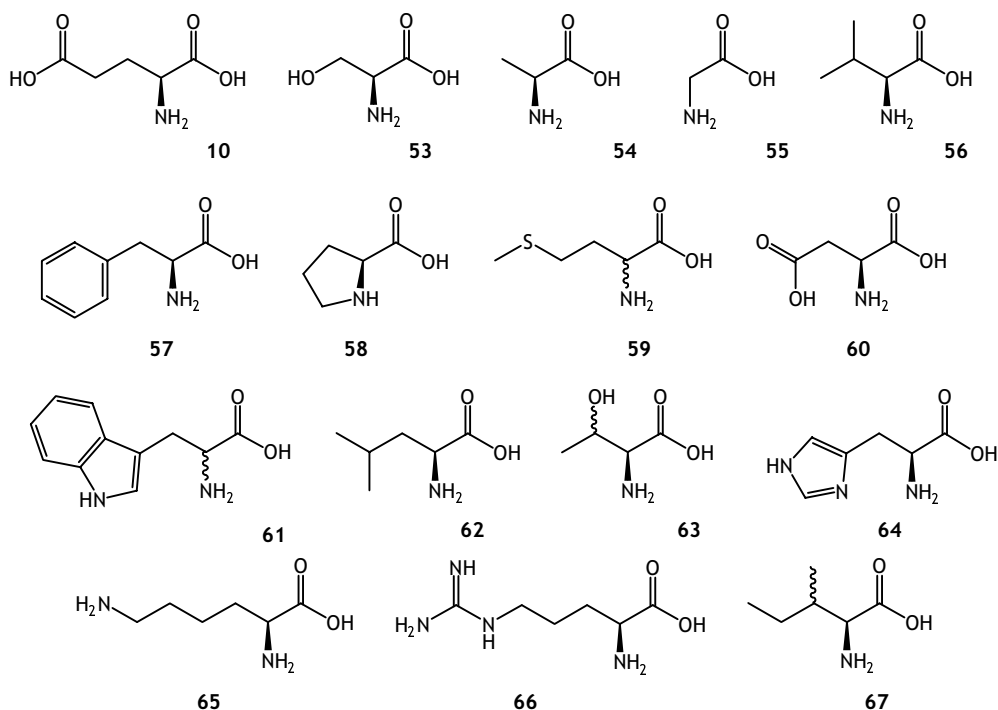


Figure 8b. Proposed amino acids that induce colony formation in *Scenedesmus*⁵¹

10. L-glutamic acid; 53. L-serine; 54. L-alanine; 55. glycine; 56. L-valine; 57. L-phenylalanine; 58. L-proline; 59. DL-methionine; 60. L-aspartic acid; 61. DL-tryptophan; 62. L-leucine; 63. L-threonine; 64. L-histidine; 65. L-lysine; 66. L-arginine; 67. L-isoleucine



the work by Kaler who proposed oligonucleotides or nucleic acids and possibly peptides as possible structures for the kairomone, although this is solely based on UV spectra⁵⁰. Moreover experiments carried out by the groups of Lampert and Von Elert do not support a peptide as a possible structure for the kairomone^{43,47}.

The cue could be enriched on C₁₈ solid-phase extraction material^{43,47,51,54}, interestingly the group of Lampert was unable to remove the cue from the C₁₈ material afterwards⁴³. Other studies have shown that the active compound could be eluted (either with methanol^{51,54}, ethanol⁴⁷, or acetonitrile^{51,54}) in a single active fraction from a C₁₈ HPLC column.

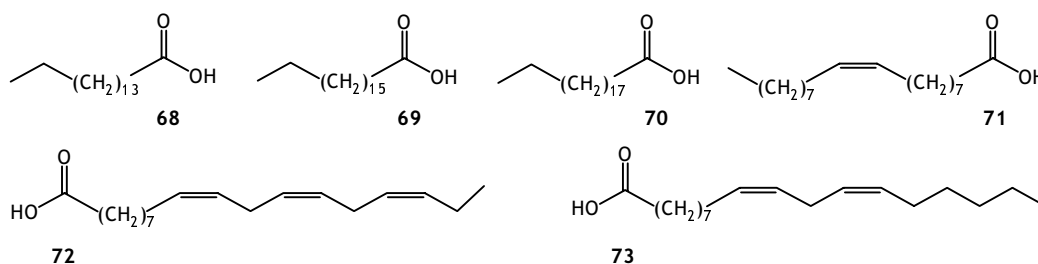


Figure 8c. Proposed fatty acids that induce colony formation in *Scenedesmus*⁵²
 68. palmitic acid; 69. stearic acid; 70. arachidic acid; 71. oleic acid; 72. α-linolenic acid;
 73. linoleic acid

Naturally occurring compounds such as free fatty acids (Fig 8c, 68-73⁴⁷), farnesoic acid 41 (four stereoisomers)⁴⁷, jasmonic acid 36⁵⁴, indole-3-acetic acid 44⁴⁴, phenylacetic acid 45⁴⁴, p-chlorophenoxyacetic acid 46⁴⁴, butanoic acid 35⁵¹, acetic acid 33⁵¹, amino acids (Fig 8b, 10, 53-67)⁵¹, urea 32^{43,48,55}, lactic acid 36⁵¹, citric acid 39^{45,51}, ascorbic acid 35^{45,51}, glucose 43^{38,41,45}, fructose 40⁴⁵, ammonia 30^{43,45}, cyclic AMP 47⁴⁹, 20-hydroxyecdysone 48⁵³, pectin 42⁴⁵ and cellulose 29⁴⁵ were assayed but showed no colony formation. Devi Prasad looked at some compounds known as growth hormones and found that indole-3-acetic acid 44 and gibberellic acid 51 promoted the formation of four-celled colonies, while naphthalene acetic acid 50, indole-2-propionic acid 49 and indole-3-butyric acid 52 did not have colony formation activity⁴⁰. Interestingly, 4-celled colonies in the presence of indole-3-acetic acid 44⁴⁰ were not found by Lürling⁴⁴, possibly due to different organisms, densities, concentrations and growth conditions.

Lürling and Beekman tested several extractable substances from membrane filters such as 1-hexadecanol 84⁴⁵, ethyl acetate 79, 2-butanone 76, 2-hexanone 77, 4-heptanone 80, Brij 35® 78, Triton® X-100 82, methyl dodecylbenzene sulfonate 76 (MDS) and Extran® MA02 (containing dodecylbenzenesulfonic acid 74, Kathon 81, Marlipal 83, Marlopon 85), but they showed no biological activity^{45,46}. However two detergents (FFD-6 containing mono- and didodecyl disulfonate diphenyloxide 86 and SDS = sodium dodecylsulphate 87) did induce colony formation (Fig. 8d)^{45,46}. These detergents are highly artificial and are not excreted by *Daphnia*, nevertheless they could give clues about the nature of the kairomone. Another hypothetical possibility is the presence of small impurities and contaminants in these two surfactants that might account for the colony formation activity.

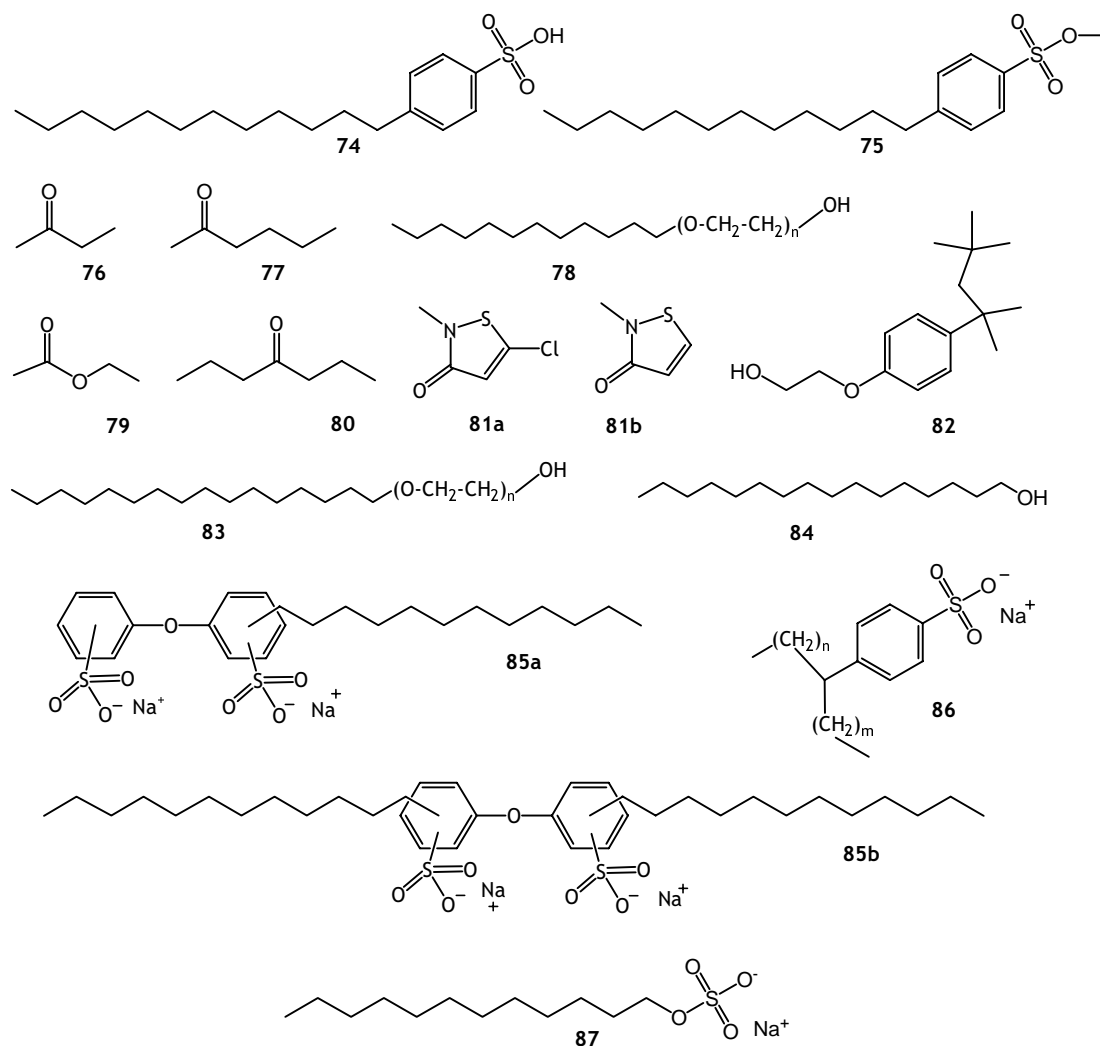


Figure 8d. Proposed surfactants that induce colony formation in *Scenedesmus*^{45,110}
74. dodecylbenzenesulfonic acid, 75. MDS (methyl dodecylbenzenesulfonate); 76. 2-butanone; 77. 2-hexanone; 78. Brij 35[®] (polyethyleneglycol lauryl ester); 79. ethyl acetate; 80. 4-heptanone; 81a. Kathon (5-chloro-2-methyl-4-isothiazolin-3-one); 81b. Kathon (2-methyl-4-isothiazolin-3-one); 82. Triton[®] X-100 (octylphenoxypolyethoxyethanol); 83. Marlipal; 84. 1-hexadecanol; 85a. FFD-6 (monododecyl disulfonate diphenyloxide); 85b. FFD-6 (didodecyl disulfonate diphenyloxide); 86. Marlopon; 87. SDS (sodium dodecylsulphate)

Scope of this Thesis

Ecological and biological research has proven the existence of chemical signals in many aquatic systems, through many innovative experiments. Once the structures are identified and isolated in larger quantities more detailed biological tests can be performed. Several biological questions can then be answered, such as the nature of the chemical cue (single compound or a blend), stability, origin (is it a kairomone, allomone or a synomone), specificity (activity in other algae species), activity (similar in laboratory and natural conditions) and occurrence. Often intricate computer models are made to facilitate the study of these food chain interactions. The fact that these cues exist, influences the way



we look at food chains. Some of the models will have to be adapted to incorporate the presence of chemical cues². A first step towards more reliable models is to identify the compounds responsible for the defence mechanisms. With these compounds ecologically relevant experiments can be performed to enhance the knowledge of specific food chain interactions, which can then be used to improve existing models.

Concluding, to improve our knowledge of aquatic ecology and chemical ecology it is essential to at least identify some of these chemical signals. Previous research has established that chemical cues excreted by *Daphnia* are responsible for the colony formation in *Scenedesmus*. This thesis focuses on the isolation and identification of this cue.

Bioassay-directed fractionation and analysis are the preferential techniques to determine the active compounds excreted by *Daphnia*. The bioassay is continuously used to give information on active and inactive fractions during the isolation process. This method depends highly on the performance of the bioassay, which is described in *Chapter 2*. Several experiments were performed to explore and identify problems with the bioassay. *Chapter 3* gives insight into uses of several popular and new extraction methods (such as liquid-liquid extraction, solid phase extraction, solid phase disk extraction and stir bar sorptive extraction). These methods were tested against each other, using a test mixture of 10 compounds. This way advantages and disadvantages of each method could be determined, leading to a prognosis which method would be most suitable for the extraction of *Daphnia* cues. Working with the best extraction method, *Chapter 4* describes the results obtained with biologically active *Daphnia* water extracts. Active fractions obtained from SPE and HPLC are described. Another method, namely differential diagnosis, was used to analyse the active water. This method involves identification of peaks unique to active extracts relative to inactive extracts and this will be described in *Chapter 5*. This chapter deals with the chromatographic aspects of this work. Results obtained on several different HPLC columns (Xterra C₈, C₈, C₁₈ and Phenyl), different elution protocols (methanol and acetonitrile) and different detectors (DAD, ELSD, MS and NMR) will be described. By using several different types of column more information about possible candidates can be obtained. Furthermore, attempts (NMR, HRMS and QTOF) to elucidate the structure of several peaks unique to *Daphnia* water will be reported. Finally, *Chapter 6* will conclude this thesis with a discussion of the major conclusions and will give suggestions for follow-up research.

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Chapter 2. Bioassay

Introduction

As described in Chapter 1, the alga *Scenedesmus* reacts to the presence of *Daphnia* by forming protective colonies (Fig. 1). Colony formation only occurs when unicellular test populations are exposed to either *Daphnia* or water that had contained *Daphnia*. Because these colonies suffer lower predation losses, this reaction is considered an induced defence mechanism¹. Lüring found that there was a trade-off between colony formation and sedimentation.

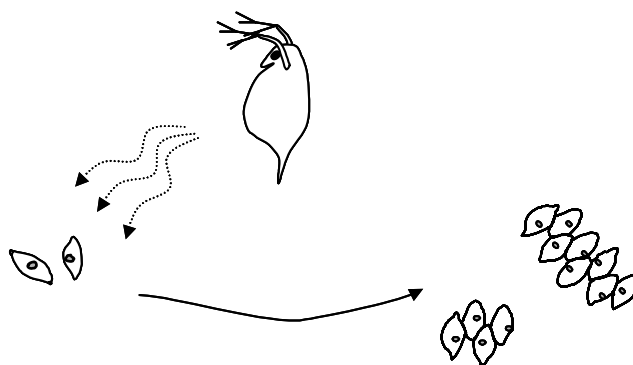


Figure 1. Inducible defence mechanism of unicellular *Scenedesmus* species by *Daphnia* species.

Colonies had higher settling properties than single cells, which will lead to enhanced sinking and consequently reduced growth due to lower light intensities and temperatures in deeper water layers^{2,3}. Since filtered *Daphnia* water also showed the colony formation activity, it was concluded that the responsible cue would have a chemical rather than a mechanical nature.

To enable the identification of these chemical cues, two methods are commonly used. The first method, differential diagnosis, involves identification of peaks unique to active extracts relative to inactive extracts, but this method lacks sensitivity in some cases. The other method, bioassay-directed fractionation and isolation, uses a bioassay to continuously identify active and inactive fractions during the isolation process. However, biologists and chemists often underestimate the effort necessary to develop a reliable bioassay⁴.

Although experiments investigating the *Daphnia* - *Scenedesmus* defence mechanism were performed as early^{5,6} as the 1980s, Lampert et al. were the first to develop a workable bioassay for this system based on the colony formation activity⁷. This assay has been further developed and improved by Lüring. The influence of biodegradation, incubation time, temperature, stoppers, carbon content, nutrients, medium, time, membrane filters, inoculum size, concentration of *Daphnia* water, *Scenedesmus* species, *Scenedesmus* strain and the physiological age of *Scenedesmus* cells have been investigated (see Table 1). Simultaneously the production of *Daphnia* test water was improved by exploring the influence of different predators, *Daphnia* species, incubation temperature, growth rate, biodegradation, density, body size, body mass, food concentration (*Scenedesmus*) and type of food (see Table 2). Generally the *Daphnia* test water is produced by incubating between 200 to 300 animals L⁻¹ in WC medium (modified



by Lürling and Beekman⁸). This medium is then placed in the dark for 24h with sufficient food; afterwards the water is filtered and used for experiments.

The basic protocol of the bioassay is shown in Figure 2. First *Daphnia* water (with kairomone) and WC-medium (without kairomone) are filtered to remove the animals from the water. An aliquot of this water is then diluted 10x with WC-medium and a certain amount of algae in uni-cellular state are added.

These flasks are put into an incubator at a specific rotation speed, light intensity and temperature. After 48 hours the flasks are removed from the incubator and a subsample

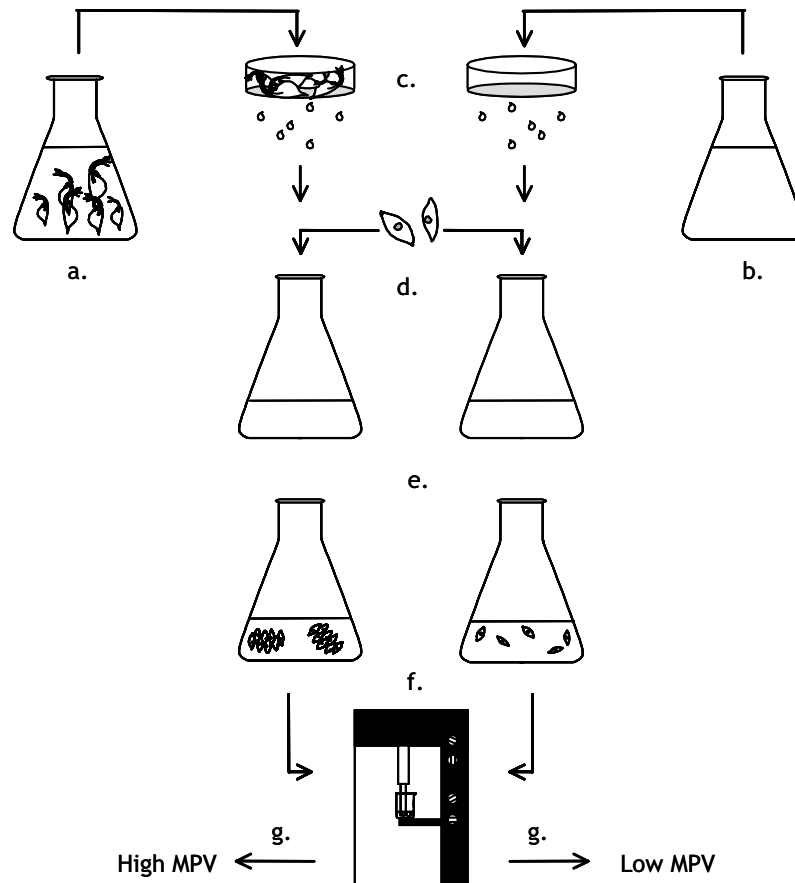


Figure 2. Principle of the *Scenedesmus* bioassay to test for presence of the *Daphnia* kairomone.

a. WC medium with *Daphnia*; b. WC medium without *Daphnia*; c. Filtrate medium; d. add unicellular *Scenedesmus*; e. Keep at constant temperature, rpm and light intensity; f. after 48 hours measure subsamples in a Coulter Counter; g. Mean Particle Volume correlates with colony formation.

is taken from each flask and diluted with electrolyte. These subsamples are then measured in a Coulter Counter. The subsample is drawn with a vacuum pump through an electrically charged tube with a tiny hole at one end. As it passes through the hole, each particle (e.g. algal cell) within the solution blocks the electric current for a moment.

The change in resistance as each particle passes through the opening generates a pulse proportional to the volume of the particle²³. After some calculations, a mean particle



Table 1. Optimisation of the bioassay.

Medium See Appendix II	Flask/Volume (ml)	Stopper	Algae	Temp (°C)	Light ($\mu\text{mol E m}^{-2} \text{s}^{-1}$)	Rotation (rpm)	Incub. Time/ Light regime (hrs.)	Other
20% Z8	100/		5.4×10^5 cells ml^{-1}	20		manually	0-168 /14:10	5
CHU 12	100/50	cellulose	1.25×10^5 cells ml^{-1}	22	250	80	48	7
20% Z8	100/50	parafilm		20	175		48 /16:8	1
COMBO	300/150	parafilm		20	125		69	9
20% Z8	100/50	parafilm		20	125		48	10
WC	300/150	cellulose	1.4×10^5 cells ml^{-1}	20	100	80	0-840	11
Modified WC [†]	100/50		1.3×10^4 cells ml^{-1}	22	115	90	48	12
Modified WC [†]	100/50	cellulose	$1.3-1.6 \times 10^4$ particles ml^{-1}	20	100	80	48	strains ¹³
WC	300/150	cellulose	1.4×10^4 cells ml^{-1}	9.5-29	125	80	0-28	14
Modified WC [†]	100/50	cellulose	$4.5 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$	20	100	80	48	strains ¹⁵
Modified WC [†]	100/50	none, cotton-wool, cellulose, silica- rubber, parafilm	$0.1-50 \times 10^4$ particles ml^{-1}	9.5-29	125	80	0-72	carbon, membrane filters, concentration <i>Daphnia</i> water, medium, nutrients, strains, species ¹⁶
Modified WC [†]	100/50	cellulose	$2.4-3.8 \times 10^4$ cells ml^{-1}	20	120	80	0-72	2
Modified WC [†]	100/50	cellulose	$0.1-50 \times 10^4$ particles ml^{-1}	20	125	80	48	biodegradation ¹⁷
Modified WC [†]	100/50		$1-2 \times 10^4$ cells ml^{-1}	20	105	100	48	detergents, ¹⁸
CHU 12	100/50	cellulose	1.6×10^4 particles ml^{-1}	20	175	80	0-120 /16:8	19
Modified WC [†] , Artificial Pond Water	100/50			20	120	100	48	19
Modified WC [†]	100/50		$1.6-4.5 \times 10^4$ particles ml^{-1}	20	175	80	48	membrane filters ⁸
Modified WC [†]	100/50	cellulose	$2.06 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$	20	146	80	0-528	physiological age ²⁰
Modified WC [†]	50/25	cellulose	2×10^5 particles ml^{-1}	18	175	80	48	several ²¹
Modified WC [†]	100/50	cellulose	$2 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$	20	125	80	48	22

[†] = modified by Lürting, ‡ = modified by Von Elert



Table 2. Optimisation of production of *Daphnia* water.
 Cho = *Chlorella*; Sce = *Scenedesmus*; Mic = *Microcystis*; Dcu = *D. cucullata*; Dma = *D. magna*; Dga = *D. galeata*; Dgh = *D. galeata* x *hyalina*; Bca = *Brachionus calyciflorus*;
 Cer = *Ceriodaphnia reticulata*; Egr = *Eudiaptomus gracilis*; Blo = *Bosmina longirostris*

Medium	Food	Species	Density Animals L ⁻¹	Food Dry Mass (mg C L ⁻¹)	Incub. Time (hrs)	Other
20% Z8	Sce, Mic	Dma	50			⁵
Filtered Schöhsee water	Sce	Dma	200	10	0-36	starvation ⁷
20% Z8	Sce	Dga, Dcu	0-200		24	<i>D. density, D. species</i> ¹
COMBO	Sce	Dma	200	2	24	⁹
20% Z8	Sce	Dga	200		24	<i>D. density, biodegradation, growth rate</i> ¹⁰
	Cho	Bca	1000		24	
	Sce	Egr	330		24	
	Sce	Blo	1000		24	
WC+RT	Sce	Dma	200	3.5	24	starvation, food type ¹¹
Modified WC [†]	Sce	Dma	300	7.5	24	body mass ¹²
Modified WC [†]	Sce	Dma	300		24	¹³
WC	Sce	Dma	200	3.5	24	¹⁴
Modified WC [†]	Sce	Dma	250		24	¹⁵
Modified WC [†]	Sce	Dma, Dpx, Dcu	0-600		21-24	starvation, body size, food concentration, food type, incubation temperature, other predators ¹⁶
Modified WC [†]	Sce	Dma	300		24	²
Modified WC [†]	Sce	Dma	300		24	¹⁷
Modified WC [†]	Sce	Dma	300	2 x 10	24	¹⁸
RT	Sce	Dma	250	10	24	location: NIOO-CL, the Netherlands ¹⁹
Filtered Lake Konstanz water and Modified WC [‡]	Sce	Dma	350		20	location: University of Konstanz, Switzerland ¹⁹
Modified WC [†]	Sce	Dma	250	9.4	24	²⁰
Modified WC [†]	Sce	Dma	200	10	24, 20 °C	²¹
RT	Sce Cer	Dma, Dga, Dgh, Cer, Byt, Lpt	200 0-500	10	24, 15 °C	other predators, fish ²⁰

[†] = modified by Lüring, [‡] = modified by Von Elert



volume (MPV) is obtained that indicates whether the sample is unicellular (low MPV) or multicellular (high MPV). Although the bioassay has been extensively described by Lürling and others some questions and problems still remain. The main problem described in this chapter is the gradual decline of the difference between positive and negative controls over time (see Figures 7 and 8), which raises the question as to why this gradual decrease occurs. Several conditions, such as time, temperature, algae strain, culture medium, location, incubator, Erlenmeyer size and bacterial growth were investigated. Additionally some general properties of the kairomone, such as thermal decomposition, biodegradation and dose response were tested for harmful effects on the ability to form colonies. The results of these experiments are described in this chapter.

Bioassay

Experiments to evaluate the bioassay were done using the general bioassay developed by Lürling¹⁶, unless otherwise stated.

Algae

The green alga used in this study, *Scenedesmus obliquus* (Turpin) Kützinger, was derived from the Max-Planck Institute (Plön, Germany). Harvested algae from the chemostat were transferred into a 50 ml stoppered Erlenmeyer flask containing 25 ml WC medium⁸ (Appendix II) and used as inoculum in the bioassay. This inoculum was kept in an illuminated ($175 \mu\text{mol quanta m}^{-2}\cdot\text{s}^{-1}$) and climate-controlled (20°C) orbital incubator (Sanyo, B203). The algae were diluted to 20000 particles ml^{-1} with fresh medium every Monday, Tuesday and Thursday.

Daphnia

Daphnia magna Straus was obtained from the Centre for Limnology (Nieuwersluis, The Netherlands) where it was cultured after isolation from Lake Zwemlust (the Netherlands). Animals were cultured at 20°C in 1 L jars. The test water with colony inducing chemicals was produced prior to the experiments by allowing 200 adult *D. magna* to feed for 24 h on a 1 L suspension of *S. obliquus* (i.e. 10 mg C L^{-1} , value obtained from algal suspension photometer measurements at 750 nm) in WC medium. After filtration (GF52, Schleicher & Schuell, Dassel, Germany) the test water was put directly into the freezer or immediately used for experiments. Before each experiment an aliquot of the incubation water was thawed by holding it under running hot water (57°C).

Bioassay

Tests were xenically run in batch cultures in 50 ml stoppered Erlenmeyer flasks. In a standard test, each flask contained 22.5 ml of fresh WC medium and 2.5 ml of medium (negative control) or test water (positive control or treatments). *S. obliquus* ($2.05 \times 10^6 \mu\text{m}^3 \text{ ml}^{-1}$) was added to these flasks. Flasks were incubated at 20°C in a climate-controlled orbital incubator (80 rpm) with continuous light from above ($175 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) for 48 h. All treatments and controls were run in triplicate.



Algal densities and particle size distributions were determined routinely in the size range 2.5 - 25 μm ESD (equivalent spherical diameter) using a Coulter Multisizer II Counter (100 μm capillary). The total algal volume ($\mu\text{m}^3 \text{ ml}^{-1}$) was then divided by the number of particles ml^{-1} to calculate the individual particle volume (μm^3). Mean particle volume (MPV) was highly correlated with the mean number of cells per colony (CPC)^{2,7,10,13,16}. Consequently, mean particle volumes were used for further experiments.

Statistical analysis

Statistical analyses were performed with SAS software (SAS system for Windows, version 8.0, SAS institute Inc.) using the PROC GLM procedure, followed by a Tukey *post hoc* comparison test to distinguish mean differences that are significantly different at $P = 0.05$.

Results

General Properties

In order to be sure that activity would not be lost during normal laboratory practice some simple experiments were performed to safeguard correct handling of the active *Daphnia* water.

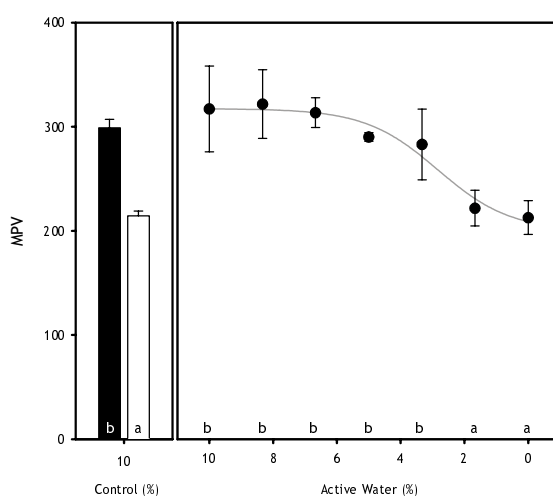


Figure 3. Dose response curve
Colony formation of several concentrations of active *Daphnia* water (●), expressed as mean particle volume (μm^3) with a positive control (■) and negative control (□). Error bars represent 1 SD ($n=3$). The solid (—) line represents a fitted curve ($r^2=0.966$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

First the final concentration of *Daphnia* test water in the actual bioassay was varied to obtain a dose response curve ($P<0.0001$). As can be seen in Figure 3 activity falls strongly between 1.67 and 3.33 % (v/v) added to medium in the bioassay. This is only slightly higher than what has been previously reported, a loss of activity between 0.2 and 2% (v/



v)¹⁶. To be on the safe side, 10% *Daphnia* water was added to the bioassay.

Furthermore it was important to know more about the shelf life of the *Daphnia* test water. Several studies have shown that chemical signals are subject to biodegradation by bacteria^{10,24-26}. Here colony formation activity of test water stored at 4 °C and 20 °C was compared. The potency of the *Daphnia* test water sharply decreases after storage at room temperature (20 °C) for one day, while the cooled (4 °C) test water retained its activity even after nine days ($P < 0.0001$, Fig 4). As a precaution all test waters were kept in the freezer and only thawed for use on the same day. After every experiment all fractions were immediately frozen and only thawed for processing in the bioassay.

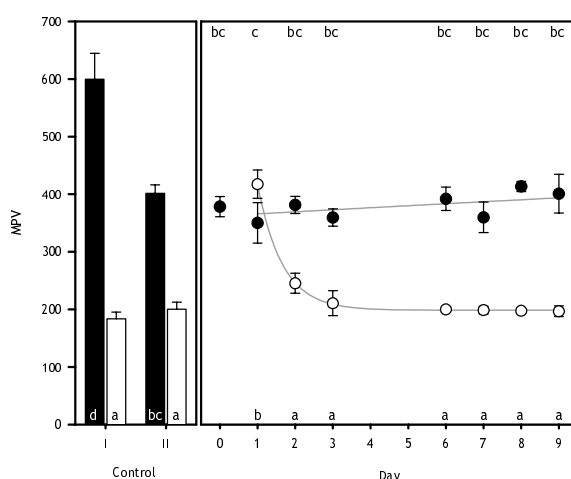


Figure 4. Degradation speed of the active compound

Colony formation of active *Daphnia* water kept refrigerated (4 °C, ●) or at room temperature (20 °C, ○), expressed as mean particle volume (μm^3), as a function of time. Positive control (■) and negative control (□) added to bioassay. Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey). Solid (—) lines represent fitted line at 4 °C ($r^2=0.348$) and fitted curve at 20 °C ($r^2=0.999$).

Daphnia water is not constant. When three different batches were produced within one week, one batch was significantly better than the other two ($P < 0.0001$). However this was reversed a month later (Fig. 5). Furthermore a month later all batches perform not as well as before ($P < 0.0001$), but still all three batches yielded a significantly higher MPV than the controls. Some natural variation is always observed when working with living organisms. However, it is best to minimise this variation by standardisation of the production of the *Daphnia* test water and all steps in the bioassay.

Finally, since most experiments will be of a chemical nature, most fractions will contain harmful organic solvents (i.e. methanol or acetonitrile). Algae are susceptible to these solvents, although they can handle small concentrations²⁷. The simplest way to avoid problems is to evaporate the solvents using a Speedvac[®] Plus evaporator (SC110A, Savant Instruments, Holbrook, NY operated with a PC 5 pump, Vacuubrand, Wertheim, Germany). After drying the samples the residues of the fractions were taken up in medium and subsequently they were tested in the bioassay. To ensure that no activity loss would occur during this drying step, *Daphnia* test water was dried using the



Speedvac® at three different settings (26, 40 and 60 °C). Statistically a significant temperature effect was observed ($P=0.043$, Fig. 6), albeit not very strong. Unfortunately, the small size of the experiment precluded the detection of significant differences between the three temperatures, although a slight trend could be observed that the

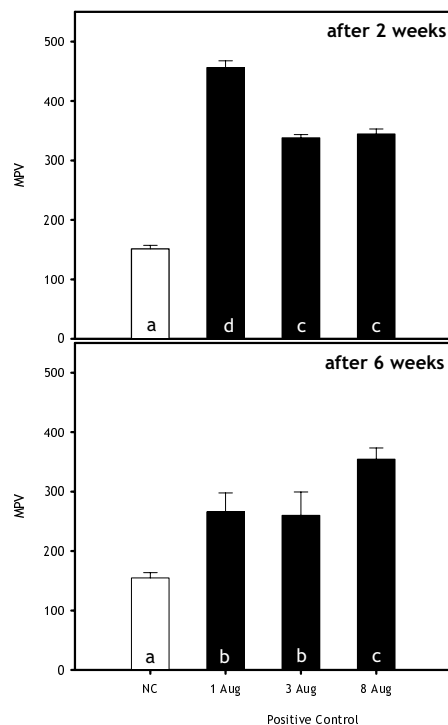


Figure 5. Quality of Daphnia water

Colony formation activity of different batches of Daphnia water, expressed as mean particle volume (μm^3) after 2 weeks ($n=3$) and after 6 weeks storage ($n=6$). Positive control (■) and negative control (□). Error bars represent 1 SD. Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

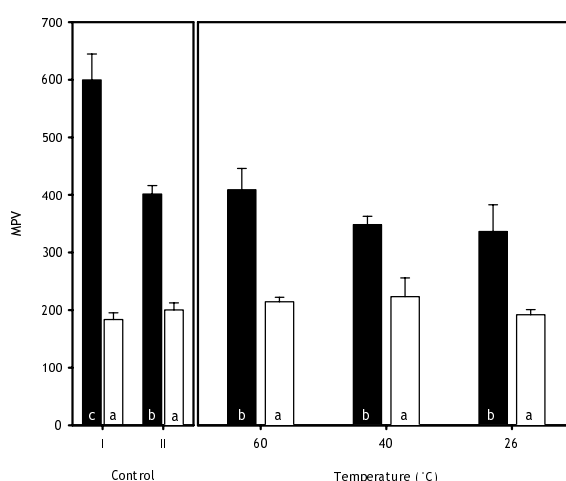


Figure 6. Thermal decomposition of the active compound during drying

Colony formation of active Daphnia water (■) and Control water (□) evaporated at different temperatures using a Speedvac®, expressed as mean particle volume (μm^3). Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



Daphnia test water seems to lose more activity at the lower evaporation temperatures possibly due to the longer evaporation times needed which increase biodegradation (see Fig. 4). Since evaporating at 60 °C was much faster than at the other temperatures this setting was chosen for all experiments. Based on this result it can be concluded that the kairomone does not have a volatile nature.

Troubleshooting Bioassay

Over time the performance of the bioassay appeared to deteriorate (Figures 7 and 8). As can be clearly seen the MPV of *Daphnia* test water has decreased, while the MPV of controls has remained the same or has marginally increased (Fig 7a). Unfortunately by the start of 2003, the confidence intervals of positive and negative controls almost overlapped. This resulted in difficulties in the positive identification of active and non-active fractions in the chemical experiments. When corresponding growth rates were determined (Fig 7b), it was found that these also decreased for the positive and negative

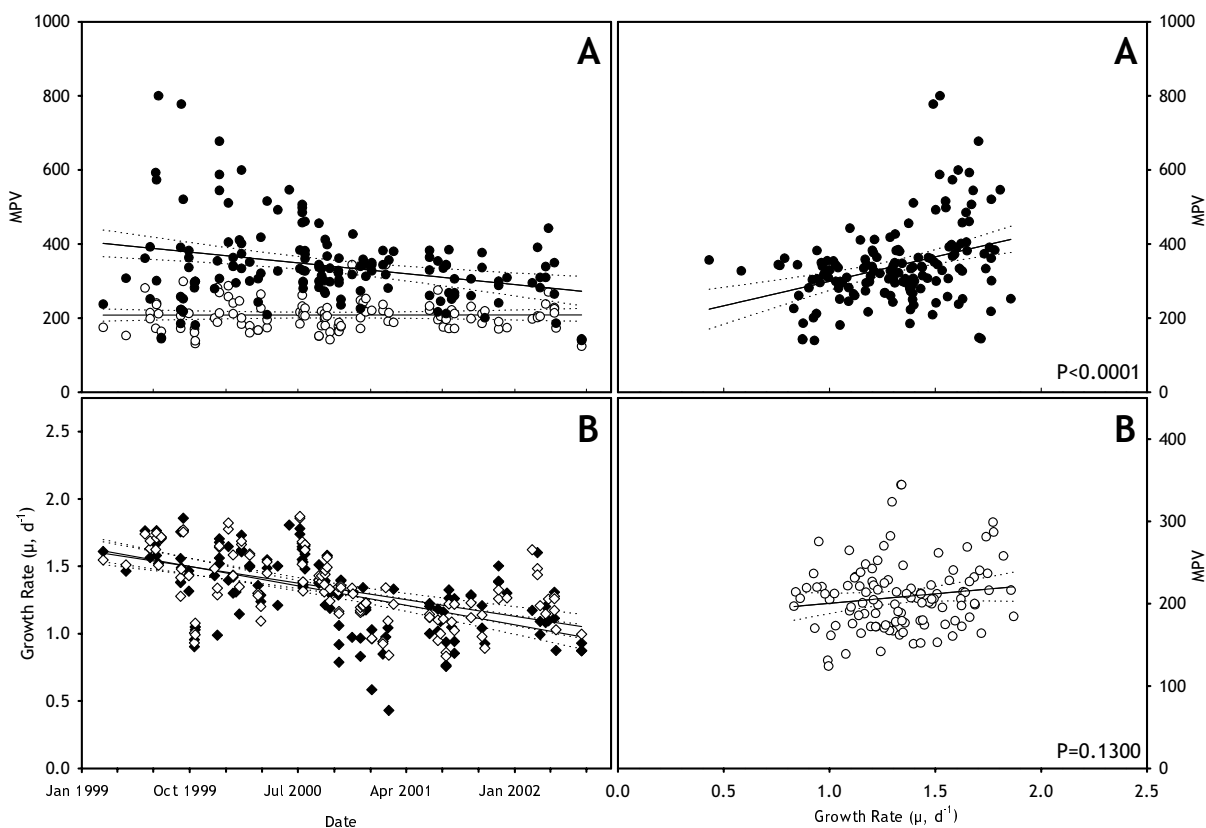


Figure 7. Bioassay performance over 3 years.

A. Colony formation of positive control (●, $R=-0.288$, $P=0.0003$) and negative control (○, $R=0.004$, $P=0.963$), expressed as mean particle volume (μm^3), as function of time. B. Growth Rate (μ, d^{-1}) of positive control (◆, $R=-0.552$, $P<0.0001$) and negative control (◇, $R=-0.543$, $P<0.0001$) as function of time. The solid (—) lines represent linear regression. The dotted lines (...) represent the 95% confidence intervals.

Figure 8. Correlation of MPV (μm^3) with Growth rate (μ, d^{-1}).

A. Positive control (●, $R=0.348$, $P<0.0001$) and B. Negative control (○, $R=0.143$, $P=0.1300$). The solid (—) lines represent linear regression. The dotted lines (...) represent the 95% confidence intervals.



controls over time, but there was no significant difference between positive and negative controls ($P=0.1530$). When looking closer at the relationship between MPV and growth rate a significant correlation was found only for positive controls ($P<0.0001$) but not for the negative controls ($P=0.1300$, Fig 8). Something appeared to affect the growth rate of the algae and thus the ability to form colonies.

When growth rate was manipulated in the presence of *Daphnia* water, for example by changing the P-content, reduced resource acquisition and biomass build-up led to less colonies. As colony formation is the result of a reproductive process, the organisms have to grow to reproduce and to form colonies. When they exhibit high growth, sufficient biomass can be gathered for the formation of either unicells or colonies depending on the environment²⁸. However when observations were made without *Daphnia* test water, but with varied levels of carbon content, a positive correlation between cell volume (μm^3) and growth rate and a negative correlation between MPV and CPC (cells per colony) with growth rate was found. Although no actual 8-celled colonies were observed. Here the carbon content was probably a contributing factor to the observed correlations, because in subsequent experiments no such correlations were observed¹⁶ and on the whole the general tendency is to discount a relationship between growth rate and colony formation²⁹⁻³². Still a positive correlation between growth rate and MPV is observed. A gradual decline in growth rate may explain why less difference was observed between controls and treatments after some time.

Potential factors, such as temperature, algae, and incubator that may have changed over time, could be responsible. However it should not be forgotten that it could also be the sum of more than one factor that determines the phenotype being produced (interaction of several factors). *Scenedesmus* cells have to grow and build up a certain amount of protoplasm before they can divide, a lower growth rate could imply that the experiment was too short for the colony formation to be expressed completely. Alternatively the availability of the kairomone to the dividing cells can become reduced when dividing cells cause less contact between the kairomone and algal cells with every division cycle. Furthermore the kairomone can become absorbed on the cell surface thus reducing its availability to the dividing cells¹⁷. Several aspects of the bioassay were reinvestigated to determine the source of this deterioration, such as incubation time, temperature, algae, bacteria, microevolution, medium, flasks, location and position inside the incubator.

Temperature

Over the past three years one of the parameters that has been changed several times was temperature. The results are given in Figure 9. Since the interest in temperature was *post hoc*, the replicates are not very evenly distributed and other factors such as medium, inoculum density and light intensity may also have varied. The 20 °C class is over represented ($n=206$) and the 21 °C class under represented ($n=45$). The original temperature (20 °C) seemed to perform better than the other two (18 and 21 °C). Moreover growth rate was significantly higher at this temperature than the other two temperatures ($P<0.0001$), however this could be due to the imbalance of the replicates in this experiment, because Lüring and van Donk found an optimal growth rate around 24 °

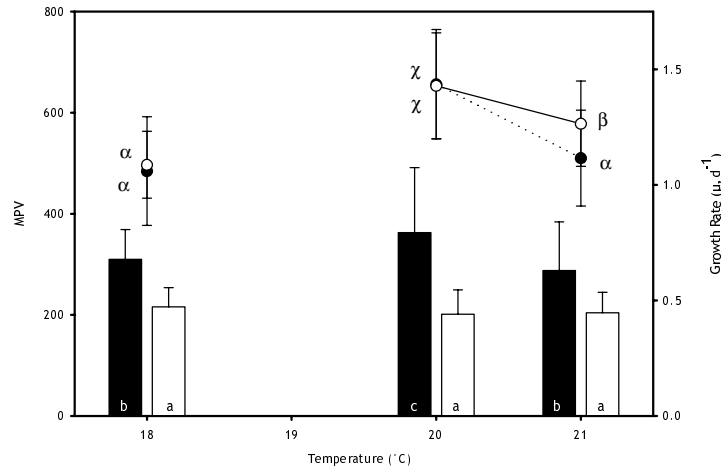


Figure 9. Influence of temperature on bioassay performance.

Colony formation (■, □) and Growth Rate (●, ○) of positive control (closed symbol) and negative control (open symbol), at different temperatures (°C). Error bars represent 1 SD. Similar symbols (a-c, α-χ) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

C¹⁴. Furthermore they found that CPC and MPV decreased at higher temperatures and reached a minimum plateau around 18 °C and cell volume was negatively correlated with temperature¹⁶, then again these observations were made at the end of the exponential growth phase and not after 48 hours. No advantage could be gained by changing incubation temperature, which was kept at 20 °C.

Incubation Time

Another question was if the incubation time was adequate for the colony formation to be

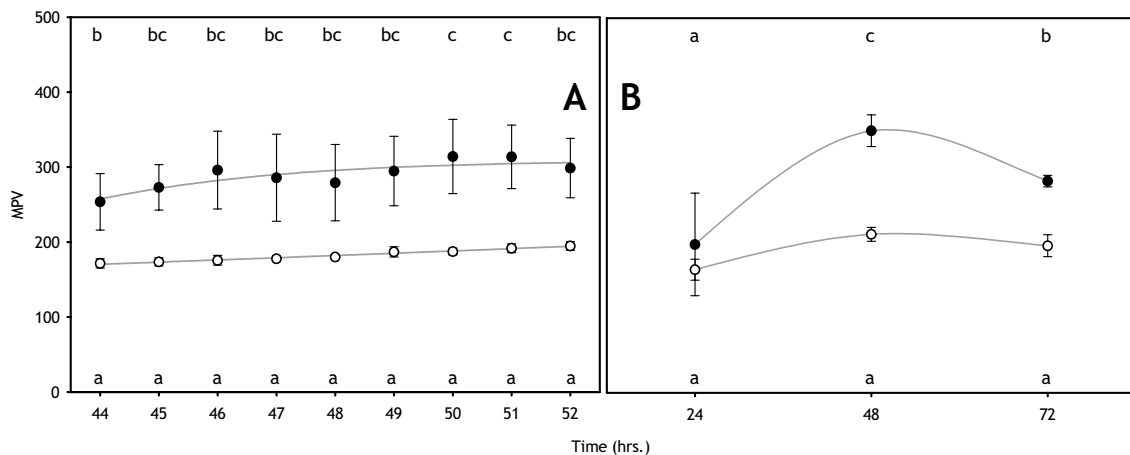


Figure 10. Incubation Time.

Colony formation of active *Daphnia* water (●) and Control water (○) after different incubation times, expressed as mean particle volume (μm^3). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey). A. Fine tuning of incubation time. Error bars represent 1 SD (n=10). Solid (—) lines represent fitted curves (negative control, $R=0.989$ and positive control, $R=0.858$). B. Optimisation of incubation time. Error bars represent 1 SD (n=6).



(fully) expressed. Measurements were taken for several hours around 48 hours, as this was the usual time for the flasks to be incubated (Fig. 10a). Significant differences could be observed from 44 up to 52 hours between positive and negative controls but positive controls were not significantly different from each other. No advantage was gained by extending the bioassay only a few hours. Next the MPV was measured after 24, 48 and 72 hours ($P < 0.0001$, Fig. 10b). The result of a highest MPV at 48 hours and a decline of the MPV after 48 hours concurs with earlier reports^{2,16,20}. So apparently the colony formation ability begins to disappear after 48 hours. This is probably due to the above mentioned biodegradation of the kairomone by bacteria in the cultures^{10,24-26}. Another explanation for the inactivation of *Daphnia* test water is possibly incorporation and absorption of the chemical cue in the algal cells reducing its availability to other cells or the earlier mentioned dilution effect¹⁷. No real advantage could be made by extending the incubation time, which was kept at 48 hours for all subsequent bioassays.

Algae and Medium

All bioassays were performed at the Dutch Institute for Ecological Research (NIOO). To explore the possibility of a problem with the condition of the algae cultures or the stock solutions of the medium at the NIOO, identical experiments were performed with a *Scenedesmus* strain and culture medium obtained from the Aquatic Ecology and Water Quality Management Group (AEW, Dept. Environmental Sciences) of Wageningen University. When looking more closely at the effect of algae strain on the colony formation (Fig. 11), it was clear that although an overall significant difference between positive and negative controls was observed ($P < 0.0001$), this was due to the AEW strain ($P < 0.0001$). For the NIOO strain no significant difference between positive and negative controls was found, ($P = 0.06$). Furthermore growth rates for unicells and colonies were

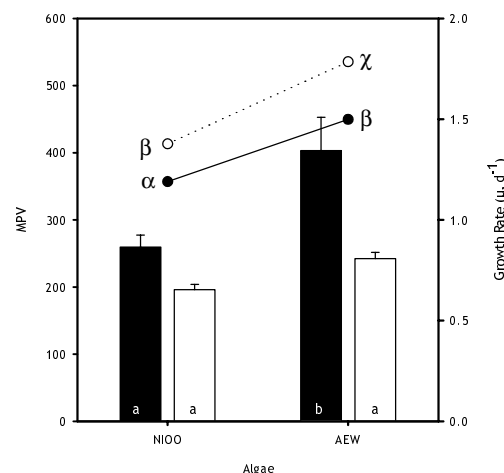


Figure 11. Influence of strain on bioassay performance.

Colony formation (■, □) and Growth Rate (●, ○) of positive control (closed symbol, $n=6$) and negative control (open symbol, $n=3$) at the NIOO, performed with respectively NIOO and AEW algal strain, expressed as mean particle volume (μm^3) and Growth Rate (μ, d^{-1}). Error bars represent 1 SD. Similar symbols (a-b, α - χ) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

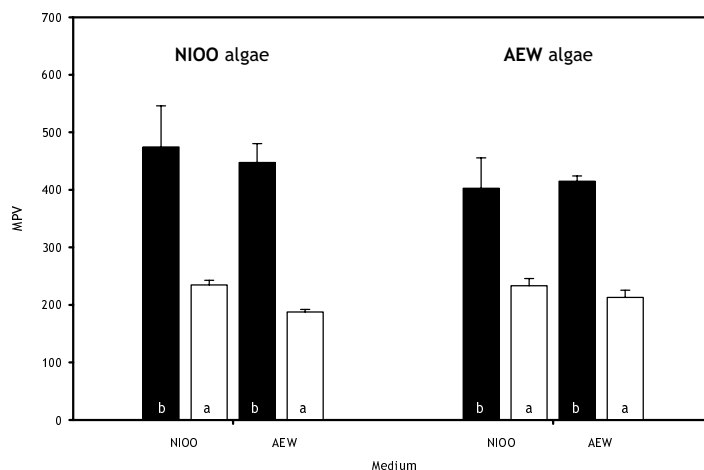


Figure 12. Influence of medium and algae strain on bioassay performance. Colony formation of positive control (■) and negative control (□) at the NIOO and the AEW, performed with respectively NIOO and AEW algal strain and medium, expressed as mean particle volume (μm^3), Error bars represent 1 SD ($n=6$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

significantly different for the NIOO strain ($P=0.0313$) and the AEW strain ($P=0.0014$). Not only that but growth rate was also significantly higher for the AEW strain when comparing with the NIOO strain ($P<0.0001$). Apparently, some factor is causing the unexpected difference in growth between the two strains, which in fact originate from the same mother culture. This means that clonal variation can be excluded as the organisms are genetically identical and that the causal factor has to be sought in the environment of the algae. The difference between media made at the NIOO and at the AEW was not significant ($P=0.138$). Wiltshire et al. claim the culture medium plays a role in colony

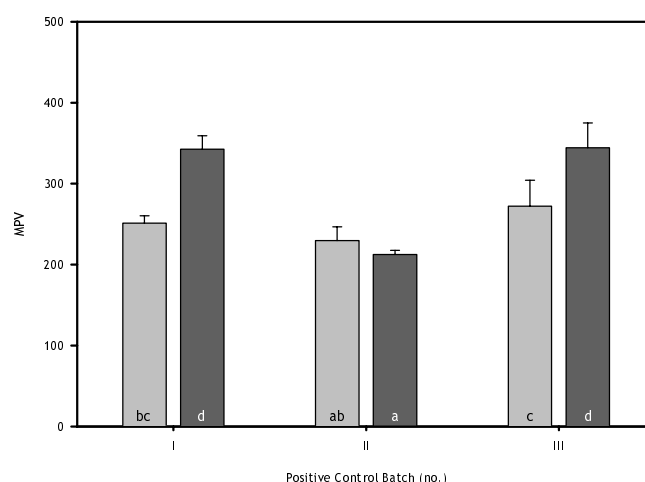


Figure 13. Influence of algae strain on bioassay performance. Colony formation of three positive control batches with algae cultured at the NIOO (■) and the AEW (■) expressed as mean particle volume (μm^3), Error bars represent 1 SD ($n=6$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



formation, however the difference in nutrients they observed between the culture media they used and the media used by other groups is not as great as they claim³³. For example Lüring and Von Elert use WC medium (Appendix II), which contains approximately 14 mg N L⁻¹ and 2 mg P L⁻¹, while Wiltshire uses a combination of CHU 12 and Z4 which contains approximately 12 mg N L⁻¹ and 14 mg P L⁻¹. This is in plain contrast to a truly nutrient limited culture medium such as medium 7 (0.33 mg N L⁻¹ and 0.005 mg P L⁻¹)³. Furthermore, when bioassays were run with WC medium and CHU medium no significant differences were observed¹⁹, showing that differences between the media with the above mentioned compositions are minor and can therefore be excluded as a possible cause for the decline in colony formation.

Two weeks later the experiment was repeated and expanded (Fig. 12). Now a slight significant difference was observed between both algal strains ($P=0.042$), and no significant difference observed for the culture medium used in this experiment ($P=0.1347$). Some 8 months later, *Daphnia* test water from three batches was incubated again with both algae strains and culture media. The result can be seen in Figure 13. Again the AEW *Scenedesmus* strain performed slightly better than the NIOO strain. Furthermore not all *Daphnia* test waters have the same quality ($P<0.0001$).

In this experiment positive control batch I and III are significantly better than II ($P<0.0001$). Since then it has been observed that problems also existed with the AEW bioassay (but these have been identified and are mainly due to time constraints and thus a lack of control over all conditions, Pers. Comm. Lüring), so no real conclusions can be drawn on the quality and condition of the algae. However the condition of the algae used for bioassay measurements has to be watched and recorded carefully, because otherwise too much variation will cause a bioassay to become untrustworthy.

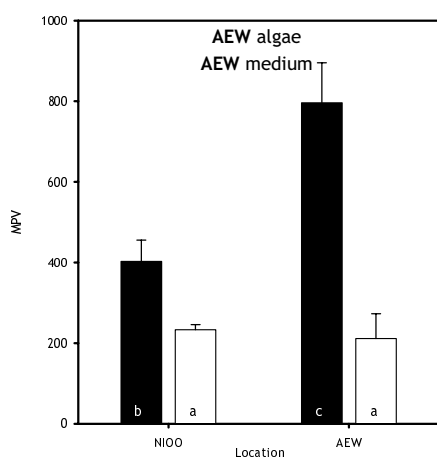


Figure 14. Influence of location on bioassay performance.

Colony formation of positive control (■) and negative control (□) at the NIOO and the AEW, performed with AEW algal strain and medium, expressed as mean particle volume (μm^3), Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

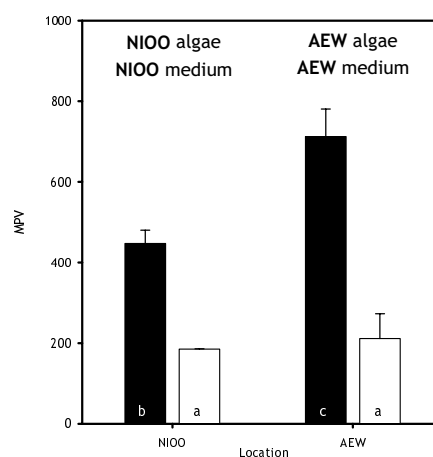


Figure 15. Influence of location, medium and strain on bioassay performance.

Colony formation of positive control (■, $n=6$) and negative control (□, $n=3$) at the NIOO and the AEW, performed with respectively NIOO and AEW algal strain and medium, expressed as mean particle volume (μm^3), Error bars represent 1 SD. Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



Location

All bioassays at the NIOO were routinely performed in an illuminated and climate-controlled orbital incubator (i.e. incubator III). To exclude problems with this incubator due to a broken thermostat, power failure or some other misadventure, identical experiments were performed at the NIOO and the AEW. Figure 14 shows positive and negative controls incubated with the AEW *Scenedesmus* strain and AEW culture medium at the two locations. Clear is the significant difference between the positive control incubated at the NIOO and at the AEW ($P=0.0013$). For some reason colony formation is greater at the AEW. Figure 15 shows the same negative and positive controls incubated at the two locations with strains and culture media obtained at that location. This experiment gives an identical picture to that in the previous experiment. Again the positive controls are significantly different ($P<0.0001$).

Inter Incubator

The illuminated and climate-controlled orbital incubator used at the NIOO to develop the bioassay can keep light intensity, temperature and rotation speed constant. To determine if something was wrong with the incubator that was used for all the previous bioassays (i.e. incubator III). *Daphnia* test water and controls were incubated simultaneously in two incubators (II and III) of the same type (Fig. 16). Interaction effects were not significantly different, however algae ($P=0.0026$), incubator ($P=0.0053$) and kairomone ($P<0.0001$) separately showed significant differences. This experiment was repeated one year later when all three available incubators were compared (Fig. 17) and a more significant incubator effect was found ($P<0.0001$). However this is probably due to light intensity differences, since the lamps from incubator III were replaced a week before this

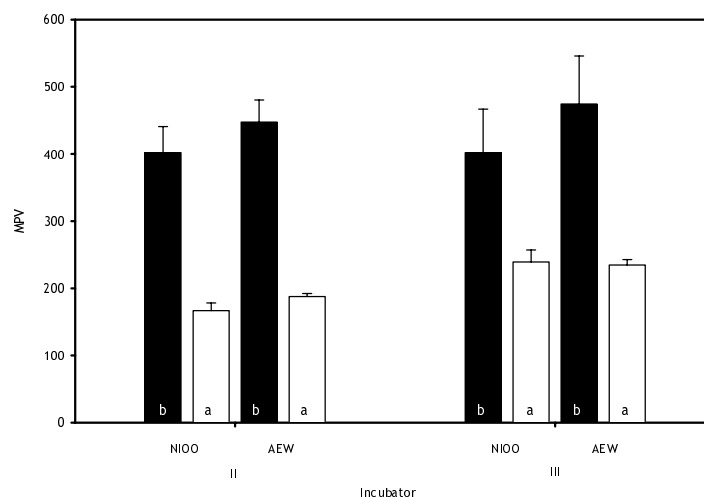


Figure 16. Influence of incubator and algae strain on bioassay performance. Colony formation of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), placed in different incubators using as inoculum algal strains obtained at NIOO and AEW. Error bars represent 1 SD ($n=6$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

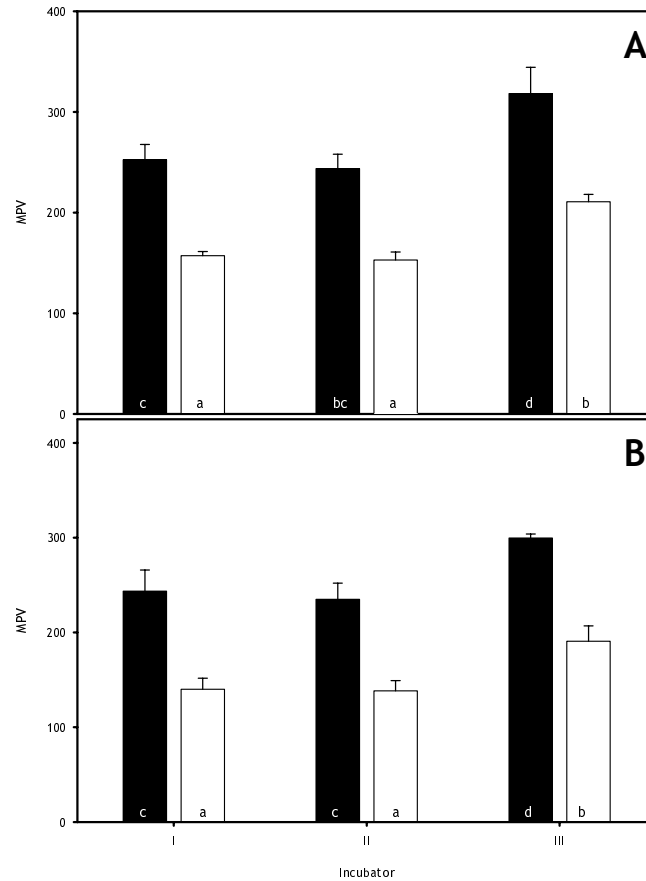


Figure 17. Influence of incubator on bioassay performance. Colony formation of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), placed in different incubators at the NIOO two weeks apart (A: 11-12-2001 and B: 11-21-2001). Error bars represent 1 SD (n=3). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

experiment. After replacement, the light intensity in incubators I and II was $92 \mu\text{mol E m}^{-2} \text{s}^{-1}$, but it was $115 \mu\text{mol E m}^{-2} \text{s}^{-1}$ ($65 \mu\text{mol E m}^{-2} \text{s}^{-1}$ before replacement) in incubator III. Unfortunately no information was obtained regarding the type of lamp or light spectrum

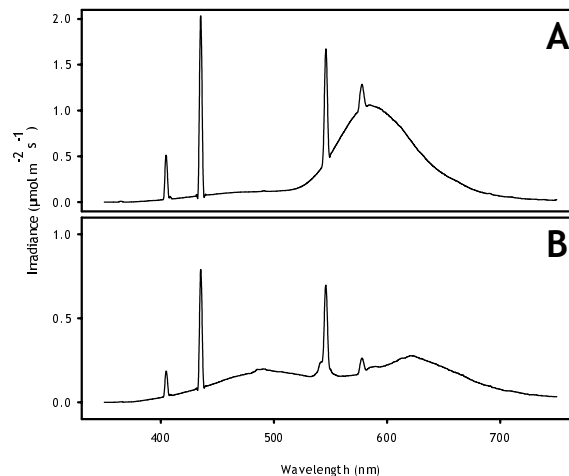


Figure 18. Irradiance of different types incubator lamps (A and B) at $115 \mu\text{mol m}^{-2} \text{s}^{-1}$.

for the three incubators, which is important as every type of lamp has different spectral properties (see Fig. 18) and even old and new lamps can show differences. However it appears that at least incubator II had a lamp with another type of light (i.e. whiter, Pers. Comm. M. Lüring). A certain daily irradiance is needed to enable division. Furthermore cultures can be synchronised by short periods in the dark³⁴⁻³⁶. This will ensure that colonies (or unicells) are released at the same time. The advantage would be less variety in MPV (as all cells will be in the same developmental stage), but a disadvantage would be a more laborious bioassay as cultures would have to be refreshed every single day, including the mother culture.

Intra incubator

When incubator III proved to be significantly better than the other two incubators more specific attention was paid to this incubator. To explore the possibility that there was something wrong with the light intensity distribution. *Daphnia* test water and controls were placed in different parts of the incubator (Fig. 19). Small but significant differences were observed ($P=0.0005$). Apparently the right and middle areas were slightly more favoured than other parts of the incubator.

However when this experiment was repeated roughly one year later no significant differences were found ($P=0.441$, Fig. 20). This could be due to the lower number of replications, but a more likely possibility is the earlier mentioned replacement of the old TL-lamps (performed on 11-12-2001), evidently new lamps give more uniformly distributed light.

Erlenmeyer Flask

The original Lüring bioassay was performed using 100 ml Erlenmeyers that contained 50

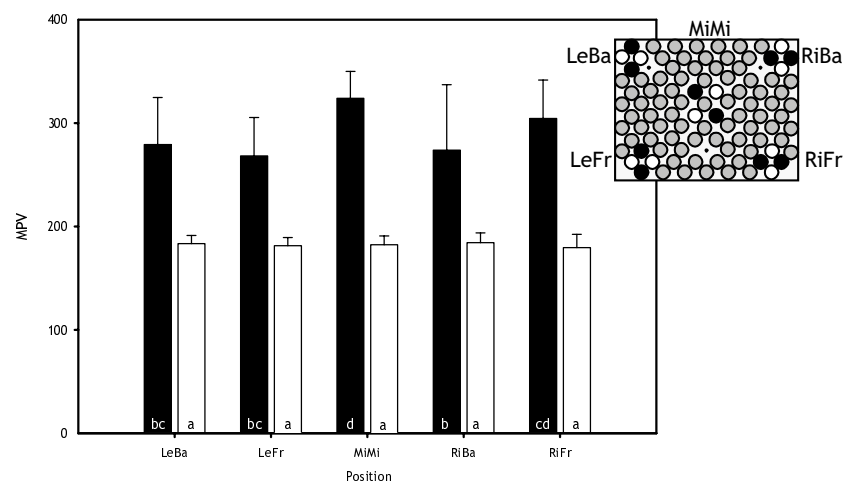


Figure 19. Influence of position inside incubator on bioassay performance. Colony formation of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), placed in different positions in the incubator. Insert displays top view of Erlenmeyer position inside incubator III. Error bars represent 1 SD ($n=16$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

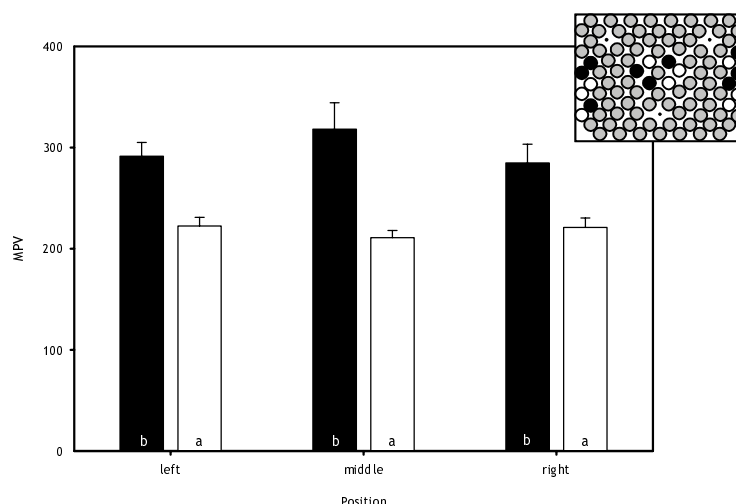


Figure 20. Influence of position inside incubator on bioassay performance. Colony formation of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), placed in different positions in the incubator. Insert displays top view of Erlenmeyer position inside incubator III. Error bars represent 1 SD ($n=3$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

ml test solution. The disadvantage of this system was that only 47 samples could be measured simultaneously under the same conditions. Additionally, significant variation between positive controls in separate incubations has been found, which makes it problematic to compare results between separate incubations¹². By reducing the Erlenmeyer size to 50 ml, not only the amount of samples that could be incubated was doubled but the need to compare between separate incubations was reduced, since most of the time an experiment fitted completely in the incubator. Another advantage was that less material was needed for testing, leaving more for other experiments (e.g. HPLC

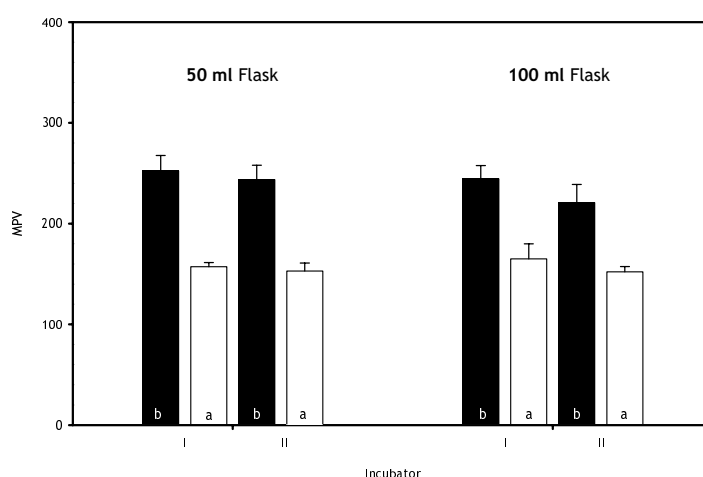


Figure 21. Influence of Erlenmeyer size on bioassay performance. Colony formation of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), placed in 50 and 100 ml Erlenmeyer's (incubators I and II). Error bars represent 1 SD ($n=3$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



analysis). However because the MPV was not as high as expected, the role of the size of the Erlenmeyer was investigated (Fig. 21). Possibly light dispersal was different for the 50 and 100 ml flasks. No significant differences were observed between the two sizes of Erlenmeyers ($P=0.268$) and a slight significant difference between the two incubators ($P=0.028$, see also Fig. 14).

Technician

Bioassays at the NIOO and AEW were performed by two different technicians. Sometimes it has been shown that a human factor can influence measurements. However in this case no significant effect was observed (see Fig. 22, $P=0.463$).

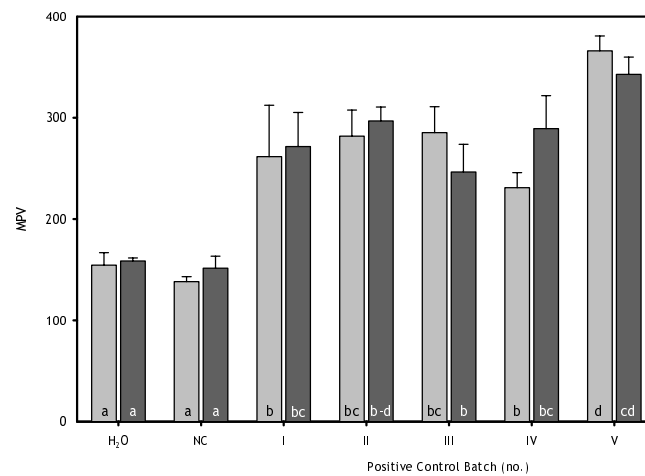


Figure 22. Influence of technician on bioassay performance. Colony formation of five positive control and two negative control batches analysed by technician A (■) and B (■) expressed as mean particle volume (μm^3), Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

Cells per colony

Although several groups have shown that MPV was highly correlated with the amount of cells per colony (CPC)^{2,7,10,13,16} it was investigated if this was indeed the case. After the MPV was obtained, CPC was also determined for every sample. The following regression was attained: $\log(\text{MPV}) = 2.259 + 0.419 \times \log(\text{CPC})$. The regression between MPV and CPC is still significant ($r^2=0.356$, $P<0.0001$, Fig. 23), although it is not as strong as reported by Lüring ($r^2=0.770$) or Lampert ($r^2=0.868$). The regressions obtained by these two groups were respectively $\log(\text{MPV}) = 2.241 + 0.730 \times \log(\text{CPC})$ and $\log(\text{MPV}) = 2.127 + 0.726 \times \log(\text{CPC})$ ^{7,16}. When these three regressions are compared (Fig. 24), the similarity between the regressions found by Lampert and Lüring is obvious with an almost identical slope. However the regression obtained from Figure 23 is different. The intercept is higher and the slope is lower. This means that although colonies are formed the MPV of these colonies is smaller than reported by Lüring and Lampert and therefore less distinguishable from unicells. In these cases regular microscopic analysis remains vital.

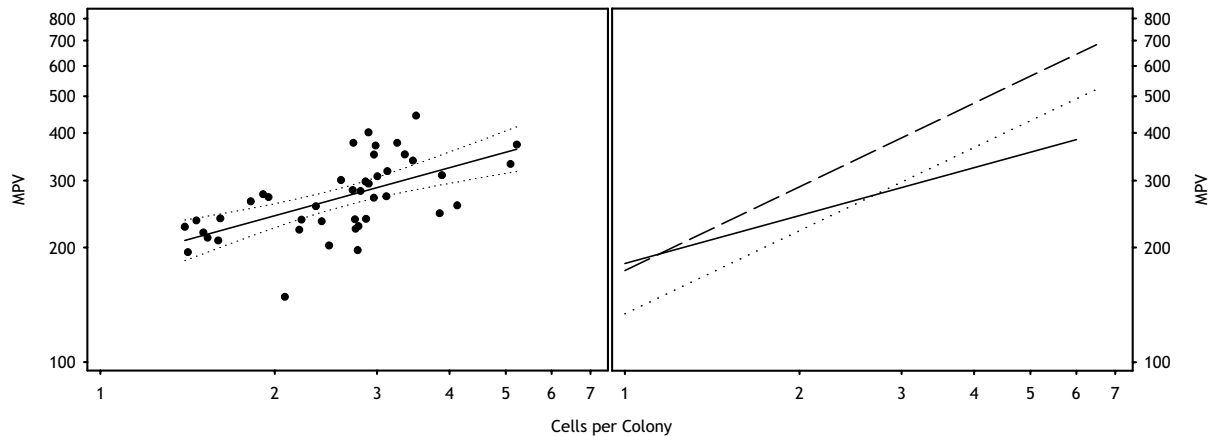


Figure 23. Correlation between MPV and CPC. Colony formation of treatments (●, $r^2=0.356$, $P<0.0001$), expressed as mean particle volume (μm^3), as function of cells per colony. The solid (—) line represents linear regression. The dotted lines (...) represent the 95% confidence intervals.

Figure 24. Comparisons between MPV-CPC regressions found by different groups. Correlation between MPV and CPC reported by Lürling (---, $r^2=0.770$)¹⁶, Lampert (... , $r^2=0.868$)⁷ and this thesis (—, $r^2=0.356$). The lines represent linear regression.

Smaller colonies could be due to a limiting resource. Unfortunately it has not been possible to positively identify this limiting resource. Nonetheless the next graph (Fig. 25) gives an indication what a complicating factor could be, namely bacterial growth.

Bacteria

Shown in Figure 25 is the influence of bacteria on the bioassay performance. Colony formation in algae cultures, which had been laboriously sterilised (i.e. axenic cultures), was significantly higher ($P<0.0001$) than algae cultures containing bacteria (i.e. xenic cultures). This is probably due to biodegradation of the kairomone^{10,24-26}. Algae growth is

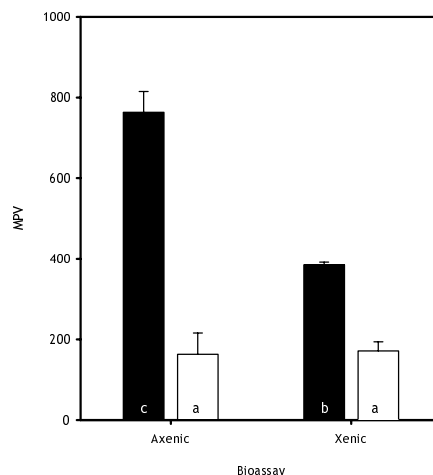


Figure 25. Influence of bacteria on bioassay performance (courtesy of M. Lürling). Colony formation, in *Scenedesmus obliquus* SAG 276/3a, of positive control (■) and negative control (□), expressed as mean particle volume (μm^3), in the presence (xenic) and absence (axenic) of bacteria. Error bars represent 1 SD (n=3). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



slow and limited and kairomone is being degraded before the algal cells have a chance to come in contact with the kairomone and respond by forming colonies. However, unpublished results by Lüring and Van Donk indicate that bacteria (from the digestive tract) may be involved in the production of the chemical cue, as the ability to produce the cue can be blocked by exposing *Daphnia* to antibiotics³⁷. Another explanation for the inactivation of *Daphnia* test water is possibly the earlier mentioned incorporation and absorption of the chemical cue in the algal cells¹⁷.

Microevolution

The *Scenedesmus* strain used in these experiments was originally obtained from the Max Planck Institute and has been cultured in chemostats and batch cultures at the NIOO for the past four years. To investigate whether some kind of evolution or selection had occurred, the original suspended strain was obtained and reconstituted after which an experiment was performed to examine and compare several parameters with the 4-year old cultures. Interestingly, some differences were observed (Fig. 26). Significant differences between the three strains were observed for CPC, MPV, Mean Cell Volume (MCV) and Growth Rate (all: $P < 0.0001$), however no significant interaction effect was found (respectively $P = 0.0796$, $P = 0.2164$, $P = 0.6964$, $P = 0.2730$). Table 3 shows the result of multiple comparisons between the three strains. It then becomes clear that although the differences are small, MPI does seem to function slightly better than NIOO-B and NIOO-B functions better than NIOO-C.

Furthermore the correlation between CPC and MPV has further flattened, compared to the correlation obtained in Figure 23, with the following regression: $\log(\text{MPV}) = 2.297 + 0.323 \times \log(\text{CPC})$ which is now no longer significant ($r^2 = 0.114$, $P = 0.172$), however this could also be due to the relative small size of the experiment.

This could be an indication of microevolution and it will have to be taken into account

Table 3. Results of post hoc Tukey multiple comparisons test for algae (no interaction effect).

Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey). MPI = Max Planck Institute; NIOO-C = chemostats at NIOO; NIOO-B = batch culture at NIOO

	Growth Rate	CPC	MPV	MCV
MPI	b	a	b	b
NIOO-C	b	a	a	a
NIOO-B	a	b	b	a

when working with bioassays of this kind. However, the results shown in Figures 11 and 12 speak against a possible involvement of some kind of microevolution. The MPV of the NIOO strain fluctuates from an MPV smaller than 300 to an MPV higher than 450 within two weeks. This is a remarkable variation in just two weeks, which cannot be explained from microevolution.

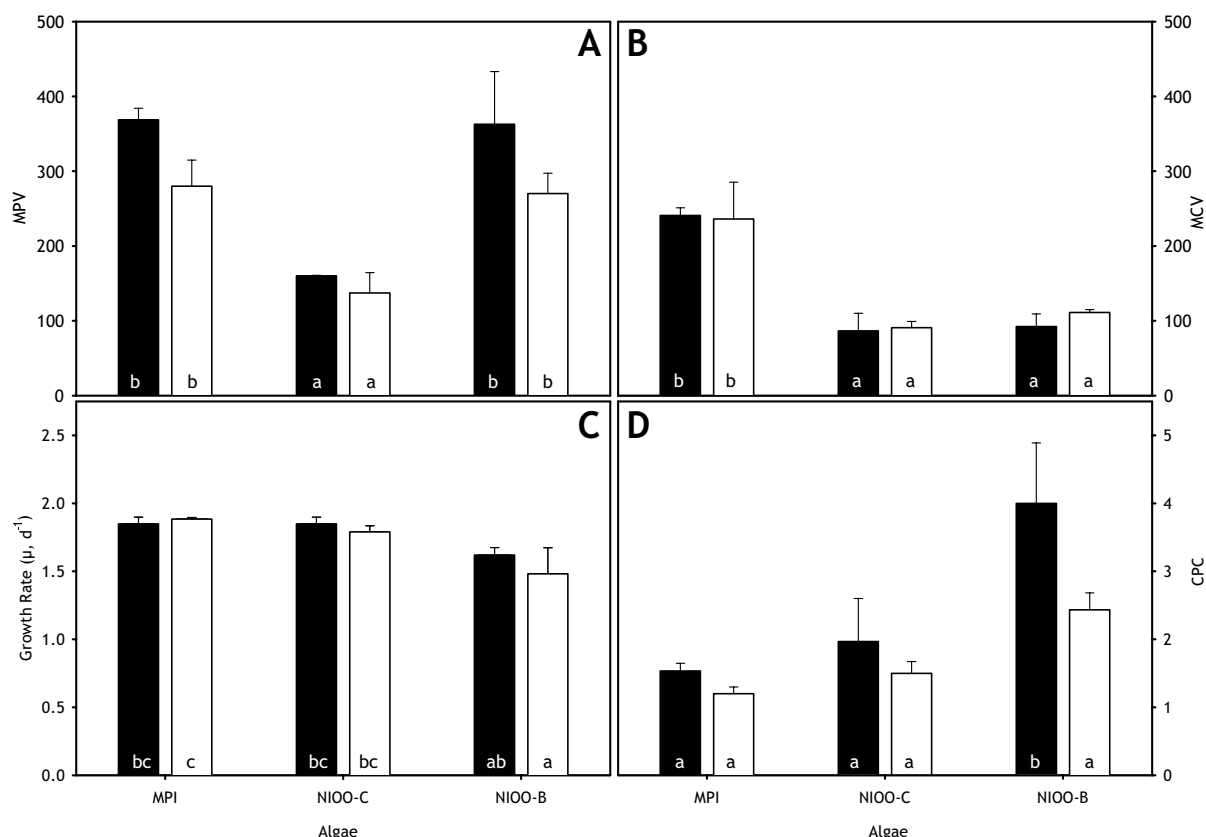


Figure 26. Influence of algae culture (courtesy of I. van der Stap).

Colony formation of positive control (■) and negative control (□), as a function of algae (obtained from the Max Planck Institute = MPI, chemostats at NIOO = NIOO-C and batch culture = NIOO-B), expressed as mean particle volume (μm^3 , A), mean cell volume (μm^3 , B), growth rate (μ, d^{-1} , C) and cells per colony (D). Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

Discussion

Despite the slow decline of the differences between positive and negative controls over time (see Figures 7 and 8), the bioassay remains the best option to solve scientific questions through controlled experiments as long as observed differences are statistically significant. With the aid of the bioassay the optimal dose was determined (Fig. 3, between 1.67 and 3.33 % v/v). Evidence for biodegradation of the biological active compound was obtained from Figure 4, in which it was shown that *Daphnia* test water loses biological activity within one day. Thermal decomposition during the drying (removal of organic solvents) of samples did not occur, it was more likely that biodegradation would become a factor at lower temperatures because drying would take longer. Therefore drying should be done at high temperature (60 °C). The optimal incubation temperature was observed to be 20 °C (*post hoc*). The incubation time was found to be optimal at 48 hours and there was no significant difference between measurements after 44 and 52 hours.

The origin of the culture medium (AEW or NIOO) played no significant role in the bioassay. The influence of the algal strain on the MPV is more variable. In Figure 11 the effect of



the algae strain played a significant role on the performance of the bioassay. The AEW strain was better than the NIOO strain, however in Figure 12 no significant difference was observed between both strains, which was confirmed by the result shown in Figure 13. Possibly, the result shown in Figure 11 was a coincidence, although when location was entered into the equation a similar result was obtained in Figures 14 and 15, where once again the AEW strain outperforms the NIOO strain, but then again this could be due to the different location. Initially no significant differences were observed between the incubators, but when the lamps were replaced in incubator III, it performed significantly better (but it probably had a different type of lamp). Similarly significant differences between the different positions within incubator III disappeared when the lamps were replaced. The size of Erlenmeyer flasks or the technician did not influence the outcome of a bioassay performance. More importantly though the correlation between MPV and CPC did change. Compared to earlier results the slope of regression had decreased, meaning that the MPV of colonies had decreased. Either colonies were smaller or less 4-celled and 8-celled colonies were formed. Additional analysis by microscope should give an answer to this question in the future. The performance of a bioassay is susceptible to bacteria in the cultures. Axenic cultures performed significantly better than xenic cultures.

Given that the bioassay was performed under such variable and not strictly controlled circumstances, this particular bioassay seems to be rather robust. However this does not defer from the fact that the quality of the bioassay did decline over time. To halt the further decline of MPVs, it is advisable to control conditions more strictly in the future, at least until the variable is identified that is responsible for the observed decline in difference between positive and negative controls. There are many variables and for correct and reliable results all of the protocols and parameters (e.g. type of lamp, light intensity measurements, incubator, technician, chemostat, temperature) will have to be more accurately described not just pertaining directly to the bioassay, but also the treatment and culture of the organisms prior to the bioassay (e.g. chemostat, dilution rate, food type, food concentration, animals L^{-1} , the removal of bacteria from the cultures at standardised times). It is important to record everything. For example Gellert and Stommel have found out that even the container material can influence the outcome of a bioassay. Common plastic microplate material led to an underestimation of the bioassay results³⁸. The necessity to record everything can be most clearly observed by the fact that separate bioassay experiments cannot be compared, because of variation in MPV of positive controls¹². However this is a problem that is irrelevant to the experiments described in this thesis since only an answer to the absence/presence question is required. The variation between positive controls of different bioassays could however have been reduced with good laboratory practice. For certain more ecologically orientated experiments this no longer suffices and could possibly be solved by a standardisation of as many variables as possible, improvement of replication, experimental design, establishment of proper controls and possibly the use of high flow through systems³⁹. However the fact remains that bioassays work with living organisms and remain therefore prone to biological variation.



Another variable factor is the *Daphnia* test water. The quality of *Daphnia* test water clearly varies between different production days (Fig. 5). Production of secondary metabolites (i.e. kairomone) often varies with age, density and nutritional status of the zooplankton^{11,37,39}. If the animals are not in good shape or have not fed enough they will not or hardly produce kairomone. However compounds released in nature are subject to flow, and as flow and turnover are minimal in culture chambers this can potentially lead to artificially high concentrations of exudates³⁹. Should this water be used in the bioassay, the performance of the bioassay will be falsely given the blame. The solution could be to use a substance that will always give colony formation. It has been shown that the detergent FFD-6 gives good colony formation. If samples containing this compound were added to every bioassay being run, it would be possible to evaluate the performance of the bioassay and the quality of *Daphnia* test water at the same time. A typical bioassay experiment should then consist of the FFD-6 samples (= positive controls), *Daphnia* test water (= additional positive control), negative controls and the experimental samples.

The most well known example of microevolution is *Linum usitatissimum* L. This plant is grown not only for its fibres (flax) but also for the oil containing seeds. Since the start of cultivation flax and linseed have developed differently. Flax has grown taller, because of benefits for its longer fibres and linseed has grown more compact to divert all available energy away from growth but into seed production. Phenotypically these plants look different, however genotypically they are still the same species. This process has occurred over thousands of years. Although unicellular algae in culture are not thousands of years old, they do multiply very fast. Therefore the same type of phenotypic differentiation can also occur in cultures. The culture medium is not ideal for the growth of algae and although media have been developed to maximise growth of the organism in culture, the fact remains that these media are artificial. The chance that some kind of selection will occur for survival in these artificial media is present. Experiments by Franzot showed that a *Cryptococcus* isolate after continuous in vitro culture became attenuated for several characteristics (e.g. colony morphology, virulence and growth rate). This seems to be a well-known phenomenon for pathogenic bacteria. Presumably the loss of virulence is an adaptation to in vitro culture, due to physical characteristics and nutrient deficiencies associated with in vitro culture⁴⁰. Furthermore Hihara and Ikeuchi have shown a similar example of microevolution in a cyanobacteria, namely *Synechocystis*. Here the WL-type (large colonies) expelled the WS-type (small colonies) from a photoautotrophic culture of wild type (WS)⁴¹. Although it has not been proven, it is not unthinkable that a similar mechanism is at work in cultures of *Scenedesmus* and that over time the *Scenedesmus* cells in culture lose the ability to form colonies by turning essential genes off. However, the *Scenedesmus* strain was obtained from the collection at the MPI where it has been grown for many years in chemostats as a food source for *Daphnia*, prior to being subjected to investigation of its phenotypic plasticity. The strain responded after being unicellular for many years to the *Daphnia* water by forming colonies. Furthermore results obtained from Figures 11 and 12 also seem to discredit this hypothesis.

Finally, a lot of time goes into the development of a suitable and reliable bioassay but



then the maintenance can be overlooked. It is too easily ignored that these bioassays are not machines and therefore they should not be treated as such. That way problems will irrevocably occur. A bioassay is meant to be a controlled experiment designed to improve the understanding of underlying mechanisms but reality is usually sacrificed for statistical power. Generally, the problem is not the bioassay, but the scientist that not always follows the exact protocol of the scientific bioassay or does not understand what certain type of experiments (such as microcosms, mesocosms, enclosure, or whole lake experiments) are best suited for.

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Chapter 3. Sample pre-treatment methods



Introduction

Although the presence of chemical communication cues has been confirmed in many systems, the chemical structures of the compounds involved remain predominantly elusive. Especially in fresh water environments few structures have been elucidated. The isolation and identification of these compounds has proven to be difficult for several reasons. First, these infochemicals occur only in very small concentrations and second, the matrix contains many other disturbing substances. Since knowledge about the nature of a particular infochemical is not known beforehand, a targeted search is almost impossible, necessitating a more universal extraction method. Over the years many different approaches for the extraction of compounds from water have been developed as shown in a review by Pawliszyn¹. These techniques all have different characteristics. However much of the research concerning sample pre-treatment and extraction methods for aqueous samples deals with compounds of one specific group (e.g. pesticides²⁻⁴, phenols^{5,6}, polyphenols⁷ or microcystins⁸⁻¹⁰) instead of a structurally diverse range of compounds. It is relatively simple to develop an optimal extraction method for one known or several chemically similar compounds. This changes when bioactive natural products, potentially with different and unknown structures, have to be isolated from aqueous samples. An isolation method that extracts a wide range of compounds in one procedure is needed in such situations. The purpose of this chapter is to look at several extraction methods for aqueous samples and determine which one offers the best perspective as a universal sample pre-treatment method for the extraction of a diversity of organic compounds from complex aqueous samples of natural origin.

Traditionally, the extraction and isolation of natural products was performed by liquid-liquid extraction (LLE). LLE is still used but has several disadvantages. Large volumes of organic solvents are used, which is not only environmentally unsound but also costly. In the extraction process emulsions that are difficult to separate can be formed and automation of LLE proved difficult¹¹. The introduction of impurities and the loss of volatile compounds further reduce the usefulness of LLE.

During the mid-1970s an alternative approach was developed, which came to be known as solid-phase extraction (SPE). Advantages of this technique are the high recoveries, its simplicity, ease of automation and a reduction in use of organic solvents. Several formats were developed over time, of which small columns and disks (solid-phase disk extraction, SPDE) became the most used. The advantages of the latter are the ability to use higher flow rates and an even greater reduction of organic solvents, however extraction costs (disks) are higher compared to normal SPE¹². The ease of automation and the development of disposable columns aided the swift development of automated forms of SPE, including the appearance of hyphenated techniques such as SPE-LC-DAD or SPE-GC-MS^{13,14}. The advantage of these techniques is that SPE is directly connected to liquid or gas chromatography, which not only eliminates contamination of the sample and the loss of analytes during drying steps but also increases accuracy and reproducibility.

Another widely used technique is solid-phase microextraction (SPME), first developed by the group of Pawliszyn in the 1990s¹⁵. Although it is possible to interface SPME with HPLC^{16,17} or capillary electrophoresis (CE)¹⁸, most applications still rely on the simple and



sensitive interface with GC-MS for volatile and semivolatile compounds, because of the identification power of the MS. However with the emergence of LC-MS the SPME-HPLC interface will become more common¹⁹. SPME is simple, fast, easily automated, portable, inexpensive and environmentally friendly^{20,21}. While faster and simpler, SPME is not as sensitive as SPE²². Furthermore, the fragility of the fibres and the limited volume of the stationary phase (ca. 0.5 μ l) of the polydimethylsiloxane (PDMS) coatings can negatively affect the sensitivity and the sample capacity. This is one of the major drawbacks of SPME. Another drawback is a lack of fibres that are stable in organic solvents for SPME-HPLC²³. Recently sol-gel technology has not only been applied to SPME to solve some of these problems²³ but also to the development of new types of SPME coatings^{24,25}. This technology (usually in capillaries) allows for the creation of chemically bonded coatings with high thermal and solvent stabilities, creating SPME fibres/capillaries with high sample capacity for polar and non-polar analytes. An added advantage is that these capillaries are disposable^{26,27}.

Stir bar sorptive extraction (SBSE) uses a PDMS (24-250 μ l) fibre coated stir bar²⁸ which is comparable to SPME. While SPME is especially well suited for the analysis of volatile compounds, SBSE shows promise for the analysis of non-volatiles. Popp et al. were among the first to describe the liquid desorption of stir bars¹⁹. The stir bars can be desorbed using small amounts of organic solvents (150 μ l^{19,29}- 1000 μ l^{30,31}). It is as yet unclear what role the sample matrix plays, as contradictory claims are made^{30,32-34}. Advantages of SBSE are lower detection limits than SPME, higher enrichment factors (up to 250x³⁵), parallel extraction of several samples, and the speed (as the throughput is only dependent on the instrument run time³⁶). Furthermore because this is a pure sorptive process no secondary adsorptive interactions take place. The extraction can be improved by introducing salt into the sample. Especially some polar compounds³⁰ (with a medium to low log $K_{o/w}$ ³⁵) will show higher recoveries in the presence of salt (15% m/v³⁷). The lack of automation however can be considered a disadvantage³⁶.

Liquid-phase microextraction (LPME) is the most recent development. Small amounts of organic solvent (< 25 μ l) are used to extract analytes from moderate amounts of aqueous samples (< 4 ml). Originally performed by suspending a drop of organic solvent in the sample at the tip of a syringe³⁸, this system proved to be unstable and a hollow polypropylene fibre to protect the extracting solvent was introduced^{22,39-41}. LPME is more precise than SPME with on the whole higher recoveries. Another advantage is that it can be directly used for dirty samples (such as food samples, biological samples and soil samples)³⁹. The preconcentration factors are high which makes this technique very attractive for water analysis²².

This chapter focuses on a comparison between LLE, SPE, SPDE and SBSE (both on-line and off-line). From a larger group of plant-derived natural products with a wide range of different polarities, ten compounds were chosen (Fig. 1). A volatile compound (methyl salicylate **88**) and a compound without a good UV chromophore (sinigrin **89**) are included. Some are glycosides (amygdalin **90**, rutin **91**, and sinigrin **89**); others are not (caffeine **92**, santonin **93**, quercetin **94**, alizarin **95**). Additionally two compounds are carboxylic acids (geranic acid **96** and gallic acid **20**). These compounds cover a wide range of

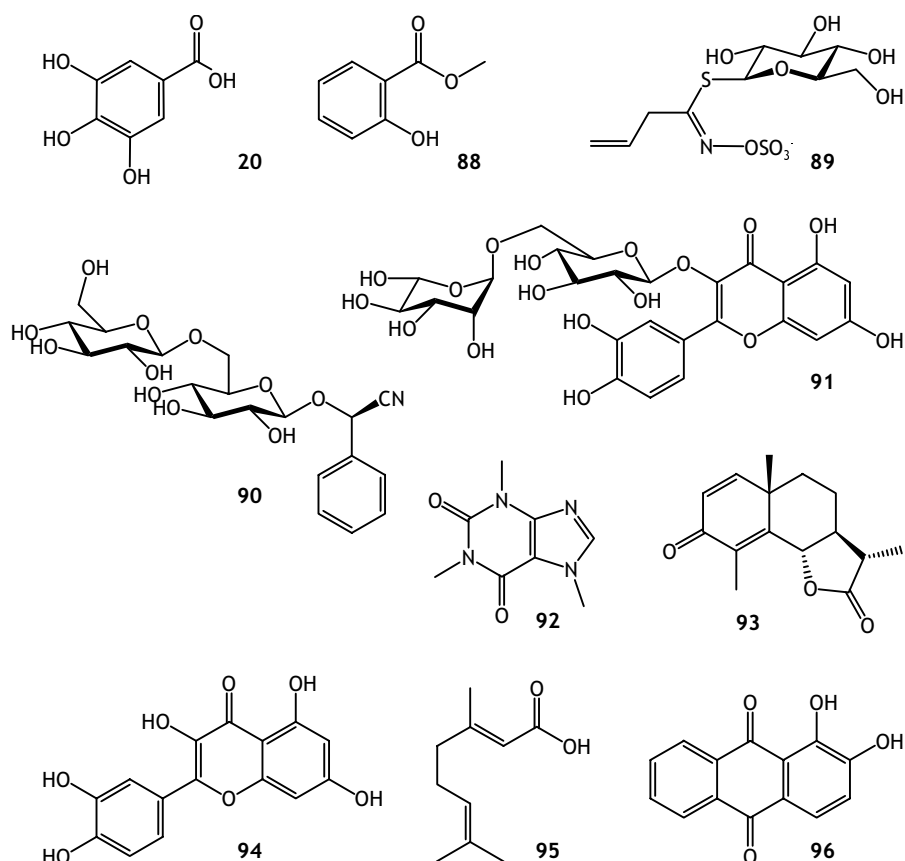


Figure 1. Composition of the reference solution.

20. Gallic Acid; 88. Methyl salicylate; 89. Sinigrin; 90. Amygdalin; 91. Rutin; 92. Caffeine; 93. Santonin; 94. Quercetin; 95. Geranic Acid; 96. Alizarin

polarity. This mixture was then extracted from water using the above-mentioned techniques to determine the effect of the extraction methods on the recovery of the ten compounds in the mixture.

Experimental

Chemicals

Gallic acid **20**, Caffeine **92** and Propyl paraben (PPP) were obtained from the general chemical store at the Laboratory for Organic Chemistry in Wageningen. Santonin **93** was obtained from ICN Biomedicals (Aurora, USA). Methyl salicylate **88** was supplied by Roth (Karlsruhe, Germany). Rutin **91** and quercetin **94** were supplied by Sigma (Deisenhofen, Germany) and geranic acid **95** and tetrahydrofuran (THF) by Fluka (Deisenhofen, Germany). Janssen Chimica (Beerse, Belgium) supplied D(-)-amygdalin **90**, sinigrin monohydrate **89** and trifluoroacetic acid (TFA). Butan-1-ol (BuOH) and methanol (MeOH) were purchased from Labscan (Dublin, Ireland). Acros (Geel, Belgium) supplied alizarin **96**, formic acid (FA) and *tert*-butyl methyl ether (t-BuOMe). Sodium sulphate and phosphoric acid were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.



Standard solutions

A stock solution containing the ten reference compounds (**20**, **89-96**) was prepared in 90% MeOH/water at a concentration of 500 ppm. Prior to use, this stock solution was diluted to 1 ppm with water containing 0.1% FA (= reference solution). A separate internal standard stock solution containing 121.8 ppm PPP in MeOH was made. Depending on the experiment this internal standard stock solution was diluted with MeOH for on-line samples (1:99) and other samples (1:9). These dilutions were then used to prepare the HPLC samples.

Liquid-Liquid Extraction

The reference solution (100 ml) was transferred to a 500 ml separatory funnel and 50 ml t-BuOMe or BuOH was added. The mixture was shaken for 1 min and left standing until 2 distinct layers had formed. The aqueous layer was extracted three times (t-BuOMe) or five times (BuOH) with 50 ml. The resulting fractions were combined and dried over sodium sulphate during 1 hour. The pooled fraction was filtered to remove the sodium sulphate and evaporated at 30 °C (t-BuOMe) or 50 °C (BuOH) to dryness with a rotary vacuum evaporator (Rotavapor 461, Büchi, Flawil, Switzerland). The residue was then taken up in 2.00 ml of the internal standard solution. All extractions were performed in triplicate and analysed by HPLC.

Off-line (syringe) Solid-Phase Extraction

IsoluteTM C₁₈ and Oasis[®] HLB cartridges were purchased ready-made from IST (500 mg, 40 µm, 60 Å, Wageningen, the Netherlands) and Waters (60 mg, 30 µm, 80 Å, Milford, USA) respectively. ENV⁺-cartridges (500 mg) were made from material obtained from IST (80 µm, Wageningen, the Netherlands). The cartridges were solvated with 10 ml of MeOH, followed by 5 ml of water. After the passage of 100 ml of the reference solution, the analytes were recovered from the cartridge by elution with 3 ml of MeOH. Internal standard solution (1.00 ml) was added and MeOH was added to the mark (5.00 ml).

Stirred Solid-Phase Extraction

In a separate experiment, 500 mg of the Isolute C₁₈ sorbent, 60 mg Oasis[®] HLB and 500 mg ENV⁺ were each added to 100 ml of the reference solution and stirred for 2 hours. After two hours the solutions containing sorbent were transferred to an empty SPE cartridge and the remaining fluid was removed under vacuum. After washing with 5 ml of water, elution was performed with 3 ml of THF as described above. All extractions were performed in triplicate and analysed by HPLC.

On-line (cartridge) Solid-Phase Extraction

Sorption, desorption and determination were performed on-line with a Spark cartridge holder and external pump (Knauer K120, Berlin, Germany) connected to a 6-way MUST multiport streamswitch (Spark, Emmen, The Netherlands). The on-line system with forward and backward desorption is shown in Figure 2. Small SPE columns (22 x 1.9 mm)



SBSE

SPE

Analytical column

0.023 A

Waste

Sample

Solid-Phase Disk Extraction

Off-line Stir Bar Sorptive Extraction

58



which helium was led at a flow rate of 50 ml/min at 150 °C for 2 hours. Subsequent to conditioning the bars were placed in the reference solution (100 ml) and stirred for 20 hours. After extraction the bars were removed with clean tweezers and dried with lint free paper. The bars were desorbed by stirring in 150 µl of the internal standard solution. Due to the fragility of the ready-made stir bars and high cost, the experiment was repeated with stir bars made at the laboratory for Organic Chemistry. A stir bar was inserted into a piece of PDMS tube (Masterflex®, 1-1.5 cm, o.d. 2.67 mm, i.d. 1.40 mm). The tube was closed by a glass bead at either end (see Fig. 3) . All extractions were performed in triplicate and injected on HPLC.

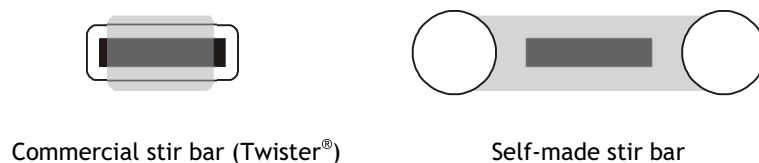


Figure 3. Schematic representation of used SBSE stir bars (1 cm).
Glass (□), PDMS (■), stir bar (■).

On-line Stir Bar Sorptive Extraction

After conditioning as described in the section on off-line SBSE, the bars were placed in a glass tube connected to a pump (Knauer K120, Berlin, Germany) and a HPLC-DAD via a 6-way MUST multiport streamswitch (Spark, Emmen, The Netherlands). The on-line system is shown in Fig. 2. Extraction of the reference solution (500 µl) was carried out at a flow rate of 0.1 ml/min. After 5 minutes the stir-bar was connected to the HPLC by switching the position on the 6-way valve. The gradient was then run over the stir-bar. The internal standard was injected directly onto the column with a loop of 5 µl. Elution and analytical separation were achieved using HPLC. All extractions were performed in triplicate and analysed by HPLC.

Chromatography

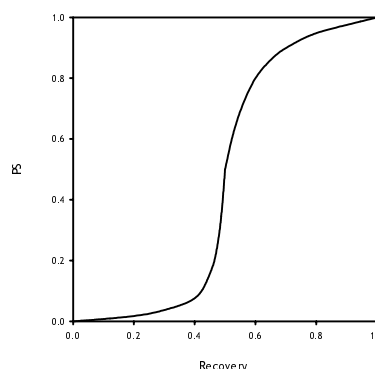
Analysis was performed with an HPLC system consisting of a low-pressure gradient HPLC pump (Gynkotek M480, Dionex, Bavel, The Netherlands). Injections were made by a Basic Marathon autosampler equipped with a 10 µl loop and absorption was monitored using a diode-array detector (Gynkotek UVD340S). The system was connected with CM PCS1 Chromeleon system control (version 4.32, Dionex). The extracts were analysed with a 250 × 4.6 mm reversed-phase HPLC column (Alltima C₁₈, 5 µm, Alltech, Breda, The Netherlands). Prior to use, MeOH and ultrapure water (Seralpur Pro 90 C, Seral, Ransbach, Germany) were filtered over a 0.45 µm, Ø 47 mm membrane filter (RC55, Schleicher & Schuell, Dassel, Germany) and degassed for 10 min in a Retsch Transsonic 570 (Emergo, Landsmeer, The Netherlands). The linear gradient program started at 98% A (water) and 2% B (MeOH), followed by a linear increase to 98% B (0-20 min). The pH of the eluents was adjusted with 0.1% phosphoric acid (pH = 2). The flow rate was 1.0 ml min⁻¹.



Suitability

A method to determine an overall recovery index, suitability index (SI), suitable for comparing methods was described by Decaestecker et al. They introduced a transformation function, which disproportionably penalises recoveries under 50% and rewards recoveries over 80% (see Fig. 4). Thus the impact of a single very high recovery is reduced⁴². For each compound the partial suitability (PS) was calculated for every method. These PS values were then added to obtain the SI for the method (see Eq. 1). This method along with relative standard deviations of the recoveries was used to compare the various methods and sorbents mentioned in this chapter.

$$\begin{aligned} R < 50 \quad PS_n &= 7.5(2R - 1) + 0.5(1 + 224(2R - 1)^2)^{1/2} \\ R \geq 50 \quad PS_n &= 1 + 5(R - 0.5) - 0.5(1 + 96(R - 0.5)^2)^{1/2} \\ R > 100 \quad PS_n &= 1 + 5((2 - R) - 0.5) - 0.5(1 + 96((2 - R) - 0.5)^2)^{1/2} \\ SI &= \sum_{n=10} PS_n \end{aligned}$$



Equation 1. Equations used to determine suitability of extraction methods⁴².

Figure 4. Graphical representation of Eq. 1⁴².

R = Recovery (%), PS = partial suitability, SI = suitability index, n = compound from test mixture

Results and Discussion

Lipophilicity

For caffeine **92**, gallic acid **20** and methyl salicylate **88** experimentally obtained values for the octanol/water partition coefficient $\log K_{o/w}$ were available (respectively -0.07, 0.7 and 2.55). The $\log K_{o/w}$ values of the compounds in the reference mixture were estimated using KOWWIN software⁴³. This program showed the highest correlation between estimated and experimental $\log K_{o/w}$ values ($r = 0.984$)⁴⁴. The $\log K_{o/w}$ values for amygdalin **90**, sinigrin **89**, rutin **91**, quercetin **94**, santonin **93**, alizarin **96** and geranic acid **95** were respectively -4.34, -4.18, -2.02, 1.48, 1.78, 3.16 and 3.70.

Liquid-Liquid Extraction

The reference solution was extracted with *t*-BuOMe and BuOH. BuOH is the most polar organic solvent that is still immiscible with H₂O. This solvent is often used for the extraction of glycosides as is shown by the improved extraction of rutin **91** compared to extraction with *t*-BuOMe. On the other hand *t*-BuOMe is more non-polar and comparable to diethyl ether (Et₂O). Furthermore, it has been proposed as a more environmentally friendly substitute for halogenated solvents (such as dichloromethane)⁴⁵. Both solvents



Table 1. Liquid-liquid extraction.

Recovery (%) of ten standards by LLE with *t*-BuOMe and BuOH, expressed as mean ($n=3$) \pm standard deviation.

		Recovery (%)	
		<i>t</i> -BuOMe	BuOH
90	Amygdalin	27.7 \pm 14.6	33.3 \pm 4.2
89	Sinigrin	0 \pm 0.0	0 \pm 0.0
91	Rutin	0.6 \pm 0.6	87.0 \pm 4.3
92	Caffeine	15.0 \pm 1.1	85.7 \pm 2.8
20	Gallic acid	68.2 \pm 0.2	90.5 \pm 1.3
94	Quercetin	69.3 \pm 14.3	63.9 \pm 6.6
93	Santonin	92.8 \pm 3.4	91.8 \pm 1.7
88	Methyl salicylate	72.3 \pm 25.6	27.8 \pm 18.9
96	Alizarin	86.0 \pm 8.3	77.3 \pm 2.0
95	Geranic acid	97.9 \pm 4.4	71.5 \pm 3.1

t-BuOMe = tert-butylmethylether, BuOH = butanol

showed the same pattern of extraction with two notable differences. When partitioning with *t*-BuOMe rutin **91** and caffeine **92** were not or hardly recovered and methyl salicylate **88** showed poor recovery when BuOH was used, probably much of the compound was lost due to the evaporation at 50 °C. The overall recovery appears higher when BuOH was used to extract the reference solution, only two compounds (sinigrin **89** and caffeine **92**) could not be extracted with this solvent. Especially for *t*-BuOMe, there seemed to be a correlation between compounds with a high log $K_{o/w}$ and high recoveries ($r^2 = 0.826$, $P < 0.00027$). BuOH did not show such a correlation ($r^2 = 0.299$, $P < 0.101$). Although often named as a labour intensive, environmentally unfriendly and expensive method, LLE does have its advantages. Especially in combination with analysis by LC-MS. Extracts obtained by other methods (especially SPE with Oasis[®] HLB and ACN precipitation) tended to suppress ionisation more than LLE extracts⁴⁶.

Stirred Solid-phase Extraction

The sorbent was removed from the cartridge and added to the solution of analytes to be extracted. The sorbent has equal opportunity to interact with all of the analytes in solution at the same time and the whole mixture reaches the same equilibrium at once. This might be considered a single step process. In contrast, analytes passing a packed sorbent go through a whole series of equilibria as the solvent moves the analytes down the packed sorbent to new layers or plates. Although the single step process is less labour intensive than packed SPE, it is dependent on the formation of equilibrium and theoretically will show lower recoveries than the packed methods¹¹. Still recoveries obtained using this single step method are quite good (Table 2), although not as high as



Table 2. Stirred solid-phase extraction.

Recovery (%) of ten standards with three different SPE sorbents, expressed as mean ($n=3$) \pm standard deviation.

	Recovery (%)		
	Oasis®	IST	
	HLB	C ₁₈	ENV ⁺
90 Amygdalin	20.6 \pm 5.0	39.1 \pm 1.9	77.6 \pm 8.3
89 Sinigrin	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
91 Rutin	87.8 \pm 4.0	84.0 \pm 0.5	43.1 \pm 0.9
92 Caffeine	42.0 \pm 0.6	71.5 \pm 0.6	95.1 \pm 4.5
20 Gallic acid	9.2 \pm .5	0.2 \pm 0.2	12.3 \pm 1.3
94 Quercetin	81.2 \pm 6.4	70.4 \pm 19.0	63.3 \pm 0.9
93 Santonin	84.0 \pm 3.2	88.1 \pm 0.7	67.2 \pm 0.2
88 Methyl salicylate	89.5 \pm 1.4	83.5 \pm 0.5	31.1 \pm 1.5
96 Alizarin	97.5 \pm 2.7	107.0 \pm 14.0	85.3 \pm 4.9
95 Geranic acid	87.3 \pm 8.4	93.7 \pm 2.4	74.9 \pm 0.2

the off-line syringe SPE. Furthermore standard deviations are also higher. ENV⁺ and Oasis® HLB are polymeric sorbents and have a capacity that is about twice as high as that of C₁₈. The same amount of these sorbents can therefore extract approximately double the amount that C₁₈ can.

Interestingly though C₁₈ performs as good or even better than the polymeric sorbents. Where recovery usual decreases quickly once a silica-based sorbent has been allowed to run dry, polymeric sorbents are less prone to this problem. Often polymeric sorbents do not have to be conditioned before use while silica based sorbents do need such a step. This is clearly an advantage when using the sorbent in this particular way without its cartridge. However this does not seem to be a problem here. All three sorbents (especially C₁₈) have problems with the extraction of gallic acid **20** and only sinigrin **89** is not extracted at all. Probably these compounds are too polar to be extracted successfully using reversed-phase sorbents. Moreover the extraction of rutin **91** and methyl salicylate **88** by the ENV⁺ sorbent is not as good as by the C₁₈, while the Oasis® HLB sorbent has problems with the extraction of caffeine **92**. This unusual use of sorbent is more common

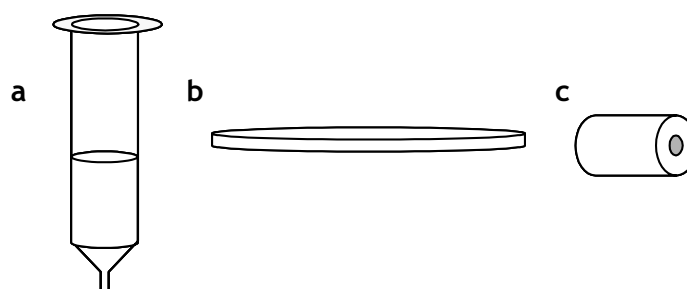


Figure 5. Current SPE formats

a. SPE cartridge, syringe or barrel (off-line); b. SPE disk; c. SPE precolumn or cartridge (on-line)



for resin-based sorbents, such as Amberlite® and Dowex®. The recovery of stirring with the resin was as good as when the resin was used in a bed and the extraction was actually quicker⁴⁷. Furthermore, recently Anastassiades and Lehotay⁴⁸ introduced a new technique (dispersive SPE) that resembles the method described here. Although they use the SPE sorbent as a way to remove interferences, they stated that is also possible to use it in retention/elution applications. They mentioned several advantages for this method, such as its simplicity, low cost, minimal use of organic solvents, lack of need for specialty equipment and glassware, and finally the possibility to make custom mixes of more than one sorbent⁴⁸.

Syringe Solid-phase Extraction

Syringe SPE is one of the most universal extraction methods used today. Some confusion in nomenclature can occur as the used pre-columns in on-line SPE are named cartridges (Fig. 5). Although new sorbents are introduced regularly, C₁₈ is still one of the most used sorbents despite its limitations, such as residual surface silanol groups, a narrow pH stability range and poor selectivity⁴⁹ and C₁₈ still regularly outperforms other sorbents when developing a new analysis method for a specific group of compounds (e.g. estrogens)⁵⁰.

The interactions between functional alkyl chains of the sorbent and the chains of the analyte are due to van der Waals forces. Polymeric sorbents do not contain silanol groups but do have a relatively large number of aromatic sites that allow π - π interaction with unsaturated analytes⁴⁹. The same three sorbents that were used for the stirred SPE experiment were also tested in this more traditional approach. The test mixture with the ten compounds was led over the sorbent in a cartridge and subsequently eluted with MeOH (Table 3). C₁₈ was able to extract all compounds from the test mixture, except for

Table 1. Syringe solid-phase extraction

Recovery (%) of ten standards with three different sorbents, expressed as mean (n=3) \pm standard deviation.

		Recovery (%)		
		Oasis®	IST	
		HLB	C ₁₈	ENV ⁺
90	Amygdalin	74.9 \pm 3.4	91.9 \pm 6.2	21.0 \pm 2.1
89	Sinigrin	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
91	Rutin	93.8 \pm 1.5	95.4 \pm 1.3	39.3 \pm 1.1
92	Caffeine	93.1 \pm 0.9	96.5 \pm 0.6	67.9 \pm 0.2
20	Gallic acid	31.2 \pm 0.7	2.0 \pm 0.6	80.9 \pm 0.8
94	Quercetin	92.3 \pm 2.2	93.6 \pm 0.6	86.3 \pm 0.8
93	Santonin	96.3 \pm 1.0	95.0 \pm 1.6	92.9 \pm 1.0
88	Methyl salicylate	95.3 \pm 1.8	96.0 \pm 5.4	89.6 \pm 0.3
96	Alizarin	76.5 \pm 5.8	75.9 \pm 8.55	82.6 \pm 1.4
95	Geranic acid	98.3 \pm 1.2	95.4 \pm 5.7	93.9 \pm 0.3



sinigrin **89** and gallic acid **20** was extracted only in a minor amount. Bonded silica sorbents are being replaced by polymeric sorbents because silica-based sorbents cannot trap very polar analytes effectively⁴⁹. This can be clearly seen by the extraction of gallic acid **20**. Similarly, ENV⁺ only missed to extract sinigrin **89**, while all other nine compounds were extracted with quite good recoveries, although recoveries of amygdalin **90** and rutin **91** were not as high as for the other compounds. Also standard deviations were small, which indicates why this is such a popular sorbent. Oasis[®] HLB showed a pattern similar to C₁₈, with two notable differences.

Extraction of the polar gallic acid **20** was about 15x times higher than with C₁₈ and the extraction of amygdalin **90** was less good than with C₁₈. Oasis[®] HLB performs better than ENV⁺ due to a lower particle size and pore size, which allows better contact between the analytes and the sorbent⁴⁹. In contrast, Ask Reitzel and Ledin found that ENV⁺ performs better than Oasis[®] HLB for phenols. They found greater retention, less breakthrough and low resistance to flow. Furthermore they regarded the colour change (of the ENV⁺ sorbent) from dry to wet sorbent an additional advantage especially for the optimisation of drying steps⁵¹

Cartridge Solid-phase Extraction (also known as on-line SPE)

All three sorbents that were analysed with syringe and stirred SPE were also coupled on-line with HPLC (see Fig. 2). Additionally two other sorbents were investigated, namely Oasis[®] MAX and Hysphere[®] SH. Due to the absence of evaporating or redissolving steps during the analysis, a decreased limit of detection was expected which is named as one of the biggest advantages of on-line SPE analysis. Other advantages include the ease of operation, possibilities for automation, reduction of operator errors, no loss of sample, no contamination and less sample-to-sample variation^{11,14,49,52}. Much effort was undertaken

*Table 4. Cartridge solid-phase extraction
Recovery (%) of ten standards with two different sorbents, expressed as mean (n=3) ± standard deviation.*

		Recovery (%)	
		Oasis MAX	IST C ₁₈
90	Amygdalin	0.0 ± 0.0	64.0 ± 3.6
89	Sinigrin	0.0 ± 0.0	0.0 ± 0.0
91	Rutin	85.8 ± 3.1	116.8 ± 11.8
92	Caffeine	54.5 ± 2.1	67.3 ± 4.3
20	Gallic acid	29.3 ± 3.9	63.5 ± 2.3
94	Quercetin	61.1 ± 3.0	97.5 ± 5.3
93	Santonin	117.6 ± 3.4	109.9 ± 6.5
88	Methyl salicylate	75.5 ± 4.0	113.6 ± 5.1
96	Alizarin	57.3 ± 2.3	107.8 ± 4.6
95	Geranic acid	77.2 ± 2.9	117.5 ± 6.0



to establish a suitable on-line SPE method. Unfortunately most of these efforts failed and contrary to literature the recoveries were neither reproducible nor accurate. The silica based C₁₈ sorbent performed the best of all sorbents, although recoveries and standard deviations were higher as expected. A comparable result was obtained for Oasis[®] MAX. When Jiménez-Lozano et al. compared sorbents for the off-line extraction of quinolones, they found no significant differences for Oasis[®] HLB, Oasis[®] MAX and ENV⁺ (Hysphere[®] SH was not included in this study), except for the most polar compound (recovery by Oasis[®] MAX was about 50%)⁵³. The Oasis[®] MAX sorbent combines reversed phase and strong anion-exchange characteristics. The recovery of rutin **91** given in Table 4 is comparable to the extraction and isocratic elution of rutin **91** with 16.5% aqueous ACN reported by Ishhi et al.⁵⁴. The other polymeric sorbents displayed all kinds of complicating behaviour (such as peak broadening, distortion and coelution) and eventually the conclusion was drawn that these sorbents are not suitable for the analysis of this reference solution. More time needs to be invested for obtaining a workable on-line SPE system.

Some possible causes for the difficulties have been found in several references. First, the coupling of the SPE to the LC system is important as only with a good transfer between both systems peak broadening can be avoided¹⁴. The choice of the analytical column is significant as the retention has to be comparable or even greater to refocus analytes sufficiently on the column head after elution from the cartridges. This requirement may be difficult to achieve as especially polymeric sorbents often have highly absorptive capacities and are thus prone to peak broadening (e.g. Hysphere[®] SH^{6,55}, ENV⁺⁵³ and Oasis[®] HLB⁵⁰). Therefore 2 mm i.d. cartridges are preferred over 3 mm i.d. cartridges^{6,55} and these are preferably connected to an analytical column with at least a 4 mm i.d. and a length of 15 cm long⁶. Low recoveries that were encountered with Oasis[®] HLB, Hysphere[®] SH and Isolute ENV⁺ could be due to some of these problems, even though other requirements which were mentioned above, such as the i.d. of the cartridges and the dimensions of the analytical column were all met. Not only were peaks broader but several analytes coeluted when on-line extraction was attempted and some analytes appeared to be retained on the cartridge after gradient elution.

Secondly, Patsias and Papadopoulou-Mourkidou observed that peak shape for polar and moderately polar analytes (such as caffeine **92**) extracted with Hysphere[®] SH was dependent on the sample volume⁵⁵. Peak shape was very good up to a sample volume of 10 ml but peak distortion occurred gradually as sample volumes increased up to 150 ml. Although small sample volumes (500 µl) were used for the on-line SPE experiment described in this chapter, the analyte concentration in the reference solution was greater than the concentration used by Patsias and Papadopoulou-Mourkidou (1 mg/L compared to 2 µg/L⁵⁵). Actually they loaded about five times less analyte onto the on-line cartridges than the amount in this investigation. During the on-line SPE experiment with the reference solution a similar effect (peak distortion at higher sample volumes) was possibly observed for Oasis[®] HLB.

The similar absorptive behaviour of Hysphere[®] SH and Oasis[®] HLB sorbent for phenols (log K_{o/w} between 1.51 and 4.74) was also observed by Wissiack et al. and they found that Hysphere[®] SH displayed the strongest retention among the sorbents they investigated⁶. In



my experiments ENV⁺ seemed to be a stronger sorbent than Hysphere[®] SH or Oasis[®] HLB as no compounds could be eluted at all from the ENV⁺ cartridge with MeOH and only the more non-polar compounds were missing from the Oasis[®] HLB and Hysphere[®] SH cartridges. This could be due to the gradient elution with MeOH which may not be sufficient to elute some analytes from these strongly absorptive sorbents.

Thirdly, recoveries above 100% for some analytes were observed for all sorbents when extracting large volumes. According to Wissiack et al. this is not due to experimental error but rather to integration problems due to peak broadening and alteration of chromatographic behaviour when on-line elution was performed⁶. This was also observed in my experiments as several analytes in the test mixture showed different elution behaviour (e.g. coelution of methyl salicylate **88** and quercetin **94** with on-line Oasis[®] HLB and partial coelution of caffeine **90** and rutin **91** with on-line Hysphere[®] SH). Although on-line SPE offers many advantages, off-line SPE will remain useful for the analysis of complex samples, due to its greater flexibility, better scale-up opportunities⁵⁶ and the fact that aliquots of off-line SPE obtained extracts can be analysed using different techniques (e.g. HPLC or GC)⁴⁹. Additionally aliquots of extracts can be taken and tested in a bioassay.

Solid-Phase Disk Extraction

The disk is the second most popular format for performing SPE⁵². It allows higher flow rates without channelling due to their large area surface enabling faster extraction of large amounts of sample⁵². Reported disadvantages include a decrease in breakthrough volume mainly for more polar compounds (confirmed here by the inability to extract sinigrin **89** and gallic acid **20**) and the limited number of commercially available sorbents on disks⁴⁹.

The overall pattern of extraction resembles the off-line SPE with C₁₈ sorbent closely albeit that the recoveries for SPDE are somewhat lower (Table 5). Dachs and Bayona

Table 5. Disk solid-phase extraction.

Recovery (%) of ten standards expressed as mean (n=3) \pm standard deviation.

	Recovery (%)
90 Amygdalin	53.3 \pm 7.9
89 Sinigrin	0.0 \pm 0.0
91 Rutin	78.3 \pm 1.8
92 Caffeine	76.1 \pm 6.0
20 Gallic acid	0.43 \pm 0.2
94 Quercetin	77.8 \pm 2.2
93 Santonin	86.8 \pm 3.1
88 Methyl salicylate	80.9 \pm 5.6
96 Alizarin	75.3 \pm 2.6
95 Geranic acid	82.7 \pm 0.8



found that the extraction efficiency of n-alkanes, PAHs and PCBs decreased with molecular weight leading to a lower breakthrough volume (5-10 L)⁵⁷, but as these are related series of compounds this is not readily transferred to the results described here. The lower breakthrough volume could be due to a size exclusion effect of the sorbent with the larger molecules, however the largest of these only had an MW of 302⁵⁷. A low molecular weight compound performs poorly (gallic acid **20**) and a high molecular weight compound performs very well (rutin **91**).

Stir Bar Sorptive Extraction

With the aid of equations (see Eq. 2) reported by Baltussen, the expected theoretical recovery in SBSE can be calculated for any compound²⁸. Generally complete recovery is possible for analytes with a $K_{o/w}$ larger than 500 ($\log K_{o/w} = 2.7$), but analytes with a $K_{o/w}$ between 10 and 500 ($\log K_{o/w} = 1.0-2.7$) can still be analysed using a calibration method⁴. It became clear even before the experiment was performed that SBSE would have problems extracting polar compounds (see Table 6). Even with total equilibrium only low recoveries would be reached for several compounds in the test mixture. Recovery will be even lower when the extraction would be terminated before equilibrium was achieved. Equilibrium times differ between compounds and can take several hours, although often an hour is more or less sufficient. It is not essential to wait until equilibrium is reached as long as calibration is performed in an identical way^{37,58-60}. Temperature^{37,60,61} and pH^{35,37,60,62} also influence the recovery. The pH has to be adjusted for the improved extraction of acids and bases⁶⁰. Sonication can be applied to accelerate desorption when stirring the stir bar in organic solvents^{19,30}, however Peñalver et al. reported that results obtained by sonication were worse than with stirring⁶¹.

As the stir bars were used to extract compounds from a relatively large amount of water, it is not surprising that low recoveries were found for all compounds (see Table 7). When

$$\beta = \frac{V_w}{V_{PDMS}} \quad \frac{m_{PDMS}}{m_{total}} = \frac{K_{ow}/\beta}{1 + K_{ow}/\beta}$$

Equation 2. Recovery of an analyte by a sorptive process in equilibrium²⁸

V_w , volume of water phase; V_{PDMS} , volume of PDMS phase; m_{total} , total amount of analyte originally in the water sample; m_{PDMS} , amount of analyte in the PDMS phase; $K_{o/w}$, octanol-water partition coefficient; β , phase ratio

extrapolating this result to the ultimate goal, the preparative extraction and often concentration of biologically active compounds from aqueous matrices, it becomes clear that SBSE is not the right technique. The time necessary to reach equilibrium limits complete extraction and thus decreases concentration factors, especially in the large samples, which are necessary for the extraction of biologically active compounds usually only present in small concentrations. Once biologically active compounds have been isolated and identified there is no need for large sample sizes and SBSE might then be a



Table 6. Log $K_{o/w}$ values and calculated theoretical recoveries at equilibrium.
 $\beta_{0.5\text{ ml}} = 20.83$, $\beta_{100\text{ ml}} = 4166.67$

		Log K _{o/w}	K _{o/w}	Theoretical Recovery (%)	
				0.5 ml	100 ml
90	Amygdalin	-4.34*	4.57 × 10 ⁻⁵	2.2 × 10 ⁻⁴	1.1 × 10 ⁻⁶
89	Sinigrin	-4.18*	6.61 × 10 ⁻⁵	3.2 × 10 ⁻⁴	1.6 × 10 ⁻⁶
91	Rutin	-2.02*	9.55 × 10 ⁻³	0.05	2.3 × 10 ⁻⁴
92	Caffeine	-0.07	8.51 × 10 ⁻¹	3.94	0.02
20	Gallic acid	0.70	5.01	19.4	0.12
94	Quercetin	1.48*	3.02 × 10 ¹	59.2	0.72
93	Santonin	1.78*	6.03 × 10 ¹	74.3	1.43
88	Methyl salicylate	2.55	3.55 × 10 ²	94.5	7.85
96	Alizarin	3.16*	1.45 × 10 ³	98.6	25.8
95	Geranic acid	3.70*	5.01 × 10 ³	99.6	54.6

* calculated with KOWWIN software

suitable analytical technique to quickly screen for these compounds, possibly even in the field. On-line SBSE shows a marked improvement, as equilibrium is repeatedly formed and broken, which leads to higher recoveries and keeping in mind that the total volume was much smaller but again this will only be well suited for analytical purposes. In contrast to SPME, SBSE can as yet not be fully automated which can be a disadvantage. For semi-volatiles there is the possibility of using a thermodesorption unit. After manual transfer into thermodesorption tubes up to 20 stir bars can be desorbed and analysed automatically. On the other hand SBSE does not need specialty equipment, has a low cost, higher recoveries and losses to adsorption on the Teflon or glass-coated stirrer are avoided with SBSE as the stir bar is the active element²⁹.

As expected the more non-polar compounds with higher log $K_{o/w}$ values (log $K_{o/w} > 2.5$)

Table 7. Stir-bar sorptive extraction

Recovery (%) of ten standards expressed as mean (n=3) \pm standard deviation.

	Recovery (%)			
	Off-line	On-line		
	Twister®	Self-made		
90	Amygdalin	0.20 ± 0.07	0.12 ± 0.07	25.5 ± 0.23
89	Sinigrin	0.0 ± 0.0	0.0 ± 0.0	1.43 ± 0.31
91	Rutin	0.61 ± 0.11	0.65 ± 0.45	52.5 ± 10.8
92	Caffeine	0.01 ± 0.02	0.0 ± 0.0	28.0 ± 0.88
20	Gallic acid	0.0 ± 0.0	0.0 ± 0.0	27.0 ± 0.48
94	Quercetin	0.06 ± 0.02	0.03 ± 0.04	80.5 ± 26.9
93	Santonin	0.03 ± 0.01	0.09 ± 0.04	51.3 ± 11.4
88	Methyl salicylate	0.52 ± 0.16	1.21 ± 0.51	49.3 ± 21.3
96	Alizarin	0.19 ± 0.04	0.47 ± 0.26	83.6 ± 31.1
95	Geranic acid	0.29 ± 0.04	0.83 ± 0.35	67.1 ± 15.6



were extracted somewhat better from the test sample, which can be explained by the non-polar nature of PDMS. PDMS is the most commonly used fibre to date, however six other SPME coatings are available, namely PDMS-divinylbenzene (DVB), polyacrylate (PA), Carboxen-PDMS, Carbowax-DVB, Carbowax templated resin and Stableflex DVB-Carboxen-PDMS²⁵. New techniques have aided the development of novel SPME fibres and especially sol-gel technology shows promise. As yet SBSE has only been performed using PDMS stir bars, however when these new SPME phases would also become available for SBSE stir bars, this would extend the usefulness of the technique.

Conclusion

Conclusions about the suitability of the various extraction methods are made separately for analytical and preparative use.

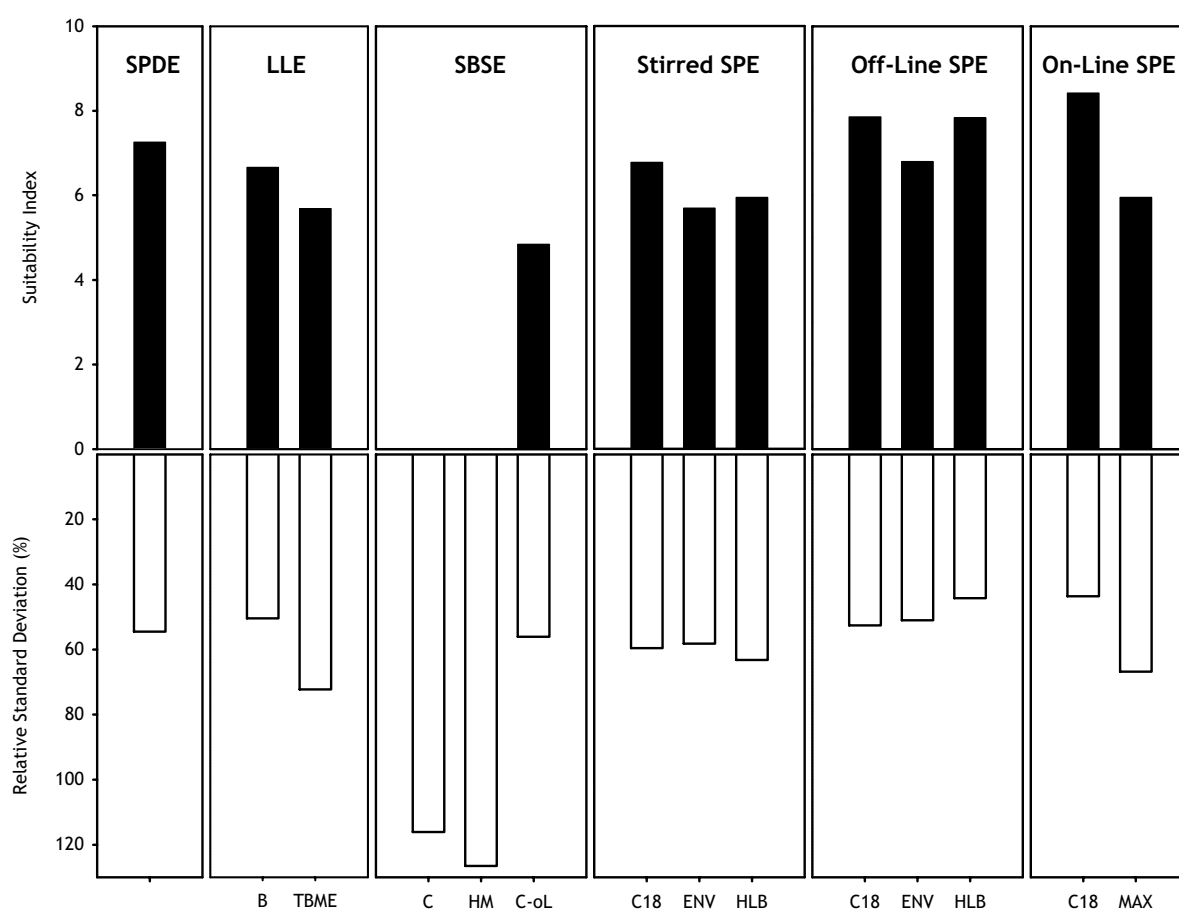


Figure 7. Calculated suitability indices (SI) and relative standard deviation (RSD, %) for different extraction methods.

B = BuOH; TBME = *t*-BuOMe; C = commercial stir bar; HM = home-made stir bar; C-oL = commercial stir bar (on-line); C18 = Isolute C₁₈, ENV = Isolute ENV⁺; HLB = Oasis[®] HLB

Suitability for analytical use

For analytical extraction, high recoveries, low standard deviations and preferably little manual participation (especially if larger amounts of samples have to be analysed) are



Table 8. Advantages and disadvantages of examined methods for analytical analysis of bioactive compounds

	+	-
LLE	<ul style="list-style-type: none">• Less suppression of LC-MS ionisation• Simple, no expensive equipment• No irreversible absorption	<ul style="list-style-type: none">• Loss of volatile compounds• Environmentally unfriendly• Introduction of impurities• Labour intensive
SPDE	<ul style="list-style-type: none">• High flow rates• Quick	<ul style="list-style-type: none">• Limited available sorbents
Syringe SPE	<ul style="list-style-type: none">• Flexible• Many available sorbents	<ul style="list-style-type: none">• Slow
Stirred SPE	<ul style="list-style-type: none">• Custom made sorbent mixtures• Flexible	<ul style="list-style-type: none">• Single Step Process
Cartridge SPE	<ul style="list-style-type: none">• Complete transfer samples to HPLC• No contamination• Automation• Quick• Sensitivity	<ul style="list-style-type: none">• Technically demanding• Peak broadening• Cost
SBSE	<ul style="list-style-type: none">• Ease of use• Quick	<ul style="list-style-type: none">• Fragile• No automation

needed. The relative standard deviations (RSD) given in Figure 7 are on the high side, but this is mostly due to the more polar compounds (sinigrin **89**, gallic acid **20** and amygdalin **90**) that are often poorly extracted. When the RSD is recalculated without any of these three polar compounds the RSD could be up to 4 times smaller. As the aim was to develop a more universal extraction method, the earlier mentioned polar compounds are kept in the calculations of the RSD. The traditional methods of extraction perform very well. LLE has suitability indices comparable to stirred SPE, but the high cost, use of larger amounts of organic solvents and the introduction of contaminants caused the rejection of this method (see Fig. 7 and Table 7). It has to be kept in mind though that due to evaporation of the samples, methyl salicylate **88** was lost due to its volatile nature, so when methyl salicylate **88** is excluded the SI for both LLE methods would have been higher. When Suitability Indices were recalculated without methyl salicylate **88** to eliminate the influence of this volatile compound, a pattern for LLE similar to the one in Figure 7 emerged. The pattern of extraction for SPDE was similar to stirred and syringe SPE. In theory SPDE could be more valuable than syringe SPE as the capacity of the disk is higher than the beds of the syringes. This is partly due to the smaller particle sizes that enable faster contact between sorbent and sample. Furthermore the lower bed height of the disks allows higher flow rates, reduces channeling and less organic solvents are needed¹¹. Furthermore obstruction of the disk by particulate matter is less likely to occur. However



SPDE performs similarly to syringe or stirred SPE (see Fig. 7), but the latter extraction methods have the added advantage that more sorbents were available. Unexpectedly stirred SPE did quite well, despite being a single step method and thus dependent on the formation of equilibria unlike syringe SPE, which was expected to do much better. Some of the compounds showed higher standard deviations (**94** and **96**) though. The Suitability Indices for the more traditional syringe SPE are high for all three sorbents tested with quite reasonable standard deviations. On-line SPE seemed an interesting option for analytical purposes, as all of the extracted material is transferred from the on-line cartridge to the HPLC column and a better result is reached with an equivalent amount of extracted solution, but only two sorbents (C_{18} and Oasis[®] MAX) yielded satisfactory results. Similarly much was expected of the application of SBSE on-line. Indeed this extraction method performed better than the off-line application of SBSE but the RSD was very high and only a small sample volume was applied. The size of the sample does not limit its use for analytical analysis of natural products but the large standard deviations do. Furthermore the application of SBSE in the more traditional way (stirring in a sample for a fixed amount of time) yielded very low Suitability Indices and huge RSD's. Due to the large volume of the sample (100 ml), the highest theoretical recovery was only around 55% for the most non-polar compound (geranic acid) but even this was not reached. The SBSE methods were disregarded for the abovementioned reasons.

Concluding, SPE remains the best method for the analytical extraction of a diverse group of natural products. It does not seem to matter whether stirred, syringe SPE or even SPDE is chosen as long as equilibrium time is reckoned with during stirred SPE experiments and a suitable sorbent is available in disk format. Stirred SPE has the same advantages as syringe SPE, but the added option of combining different sorbents to solve specific extraction problems, whereas SPDE is limited by the amount of available sorbents.

Suitability for preparative isolation

For preparative extraction the most important factors are reasonable recoveries, the avoidance of sample degradation and sufficient capacity. The aim is to extract as much of a bioactive compound as possible. The occurrence of an occasional high standard deviation is not problematic. Therefore other considerations play a role in the choice of a suitable scale-up method. For LLE the large amounts of needed organic solvents would not only be costly but also hazardous (risk of explosions and health issues). Still scale-up may theoretically be possible, but the handling of large amounts of samples (> 20 L) will be difficult and labour intensive in a standard laboratory. SPDE has several advantages that would enable it to extract large volumes. It is fast and is less likely to become obstructed by particulate matter, which can become a problem when larger volumes are concentrated. Large volume analysis by SPDE was reported in 1993 for the analysis of PCBs and dioxins⁶³. This group found that the disks (\varnothing 90 mm, styrene-divinylbenzene) were able to extract up to 100 L of water but could be susceptible to clogging with sample volumes over 30 L. In contrast, a much lower breakthrough volume of 5-10 L for PAHs, PCBs and n-alkanes was reported by another group, but they used a disk with C_{18} sorbent (\varnothing 90 mm, C_{18})⁵⁷ which has a lower capacity than SDB. However there are doubts



Table 9. Advantages and disadvantages of examined methods for preparative isolation of bioactive compounds

	+	-
LLE	<ul style="list-style-type: none">• Very large scale possible• Simple, no expensive equipment• No irreversible absorption	<ul style="list-style-type: none">• Labour intensive• Formation of emulsions• Unsuitable for highly polar compounds• Risk of fire/explosion• Loss of volatile compounds• Environmentally unfriendly, unhealthy• Introduction of impurities
SPDE	<ul style="list-style-type: none">• Less likely obstructed• High flow rates	<ul style="list-style-type: none">• Low breakthrough volume for polar compounds• Scale-up ?
Syringe SPE	<ul style="list-style-type: none">• Suitable scale-up• Flexible	<ul style="list-style-type: none">• Low flow rate• Easily obstructed
Stirred SPE	<ul style="list-style-type: none">• Custom made sorbent mixtures	<ul style="list-style-type: none">• Limited scale-up• Difficult removal of solvent
Cartridge SPE*	<ul style="list-style-type: none">• No contamination• Automation	<ul style="list-style-type: none">• Very limited scale-up• Low capacity• Technically demanding• Excessive peak broadening
SBSE	<ul style="list-style-type: none">• Ease of use	<ul style="list-style-type: none">• Low capacity

* = not usually used in combination with HPLC for preparative isolation

on about the ability of SPDE to extract 50 L of *Daphnia* test water, especially for the C₁₈ sorbent. Although the use of stir bars (SBSE) is straightforward, the absorption is driven by an equilibrium between the PDMS and water phase. This inhibits sufficient extraction of analytes from large volumes of sample (see Eq. 2 and Table 6). Even the development of other phases in the future will not change this dependency on the equilibrium between water and adsorbent phase. The off-line methods were therefore not able to successfully extract relatively large amounts of water. In contrast the on-line method, reached quite a reasonable SI, but this was mostly due to the small sample volume, which swayed the equilibrium in the direction of the PDMS phase. On-line SBSE of a sample volume (100 ml) similar to the off-line SBSE method was not tested, but it is expected to do better than the off-line method as equilibria will be formed continuously. However this should be tested before definite conclusions can be reached.

Although on-line SPE seemed an interesting option for analytical purposes, as all of the extracted material was transferred from the on-line cartridge to the HPLC column, it excludes the possibility of performing a bioassay simultaneously, which is essential for preparative isolation of bioactive compounds. A preparative use of on-line SPE is not



normally performed with the HPLC attached, so after elution samples for bioassay testing could be taken. More importantly the technique has a limited capacity and is technically demanding and not easily set-up.

As stated before stirred SPE did quite well but the scale-up (up to 20 L) of this method may be problematic because suitable glassware is not readily available and removal of large volumes of *Daphnia* water and solvents is not easy.

Ultimately syringe SPE was really the only method that could be scaled-up in a relatively inexpensive way. The only disadvantage was the limited flow rate, which is not high to begin with but also decreases over time as the sorbent slowly becomes more clogged during the extraction. Extraction can then take up to 12 hours. However all other methods had more disadvantages, which excluded them from being used. The different sorbents did not differ much so all three were further tested with *Daphnia* test water (see Chapter 4). Furthermore scale-up of this method is more feasible as only a glass sorbent holder is needed to hold a larger amount of the chosen sorbent^{56,64}.

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Chapter 4. Extraction and fractionation of Daphnia activated water

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van Holthoon, F.L., T.A. van Beek, M. Lüring, E. van Donk and Ae. de Groot, 2003. *Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone*. *Hydrobiologia*. **491**: 241-254.



Introduction

Often isolation and identification of a biologically active compound do not start with chemical experiments. First the biological activity is studied and it is determined whether it is due to a mechanical or chemical cue. Once chemically mediated induction has been demonstrated in an ecologically relevant way, fractionation or other chemical experiments can progress to identify the compound in question. However first a bioassay has to be developed, which consists of identifying a consistent set of reactions (i.e. colony formation in *Scenedesmus*) stimulated by the release of the supposed chemical cue under controlled conditions (Chapter 2). Only then a start can be made with the isolation and identification of the kairomone(s) involved. Over the years several approaches to bioassay-assay directed fractionation have been developed. Binary-splitting is the most commonly used. It consists of splitting an extract into two (sometimes more) fractions and then testing them separately for biological activity. This method relies heavily on the assumption of only one chemical cue or co-elution of several compounds that induce the biological activity¹. As early as the 1960s, Silverstein et al. have shown that often chemical cues work together to induce biological activity (synergism)²⁻⁴. They developed the additive-combination method, which has shown success at identifying synergistic compounds but it is a very labour-intensive method, because an apparently inactive fraction cannot be discarded until it has been tested in combination with all other fractions or subfractions⁴. Small fractions are collected using chromatography and then tested individually followed by recombination, first two-way combinations, then three-way combination and so forth, in all possible combinations to test for biological activity. Later Byers et al. developed the subtractive-combination method. Small fractions are collected using chromatography and tested individually and subsequently individual fractions are removed from the blend in sequence until biological activity disappears^{8,9}.

Table 1. Advantages and disadvantages of bioassay directed fractionation methods.

	+	—
Binary Splitting ¹	<ul style="list-style-type: none">• Few bioassays	<ul style="list-style-type: none">• Multiple chromatographic passes required• Difficult detection of synergists
Additive Combination ²⁻⁴	<ul style="list-style-type: none">• Few chromatographic passes• Detection of synergists	<ul style="list-style-type: none">• High number of Bioassays• Labour-intensive
Subtractive Combination ⁵	<ul style="list-style-type: none">• Few chromatographic passes• Detection of synergists	<ul style="list-style-type: none">• High number of Bioassays
Differential Diagnosis ⁶	<ul style="list-style-type: none">• No bioassay necessary• No extensive use of organic solvents	<ul style="list-style-type: none">• Compound co-elutes• Compound synergistic
Chemical Screening ⁷	<ul style="list-style-type: none">• Quick	<ul style="list-style-type: none">• Least rigorous• Compounds have to be known• Ratio has to be known



This method is also suited for the identification of synergistic compounds.

Another technique commonly used for the isolation and identification of biological active compounds is differential-diagnosis, albeit without the aid of a bioassay. Chromatogram profiles are compared for unique differences (e.g. male versus female GC traces)^{6,10}. Finally there is the possibility of screening for known chemicals found in closely related species⁷. All these methods have advantages and disadvantages (Table 1). Except for differential-diagnosis the bioassay is crucial for all the described methods of bioassay guided fractionation (Chapter 2). Without it, no knowledge would be gained about location and nature of the chemical cue. In this chapter chemical experiments are described using the binary-splitting method. An assumption is made that this chemical cue works without synergistic effects, until experiments prove otherwise. *Daphnia* test water will be extracted with LLE, SPE and fractionated with an HPLC.

Experimental

Algae and Daphnia

Growth conditions and culture of *Scenedesmus* and *Daphnia* were as described in Chapter 2.

Bioassay

The bioassay was performed as described in Chapter 2.

Liquid-Liquid Extraction (pH dependent)

The pH of *Daphnia* water was adjusted to 2.0 (HCl), 7.0 (NaOH) and 12.0 (NaOH). *Daphnia* water (6 ml) was then transferred to a 100 ml separatory funnel and extracted twice with ethyl acetate (10 ml, EtOAc). The mixture was shaken for 1 min and left standing until 2 distinct layers were formed. The resulting organic layers were combined. The pooled layer (= EtOAc) was evaporated to dryness with a rotary vacuum evaporator (Rotavapor 461, Büchi, Flawil, Switzerland) and afterwards taken up in 15 ml of ultrapure water. The aqueous layer was desalted by passing over a C₁₈ solid-phase cartridge (see Solid-phase extraction), evaporated and reconstituted. Both layers were then tested in the bioassay in duplicate.

Solid-Phase Extraction

The colony-inducing activity was enriched from *Daphnia* incubation water by solid-phase extraction (SPE). End-capped C₁₈-SPE cartridges (500 mg, IST) were preconditioned with methanol and ammonium formate buffer (0.01 M, pH=3) prior to loading the sample. The pH of the sample was adjusted to 3.0 with ammonium formate buffer (1 M). Methanol (MeOH, 1%) was added and the resultant solution was passed through the cartridge. The unretained solution was collected (= Unretained). The loaded cartridge was washed with ammonium formate buffer (0.01 M) prior to elution with an increasing percentage of MeOH and/or tetrahydrofuran (THF). All fractions were evaporated separately to dryness



and resuspended in ultrapure water. This procedure was used on several different types of cartridges, specifically MFC C₁₈ (500 mg, IST), non-endcapped C₁₈ (500 mg, IST), C₈ (500 mg, IST), C₂ (500 mg, IST), CN (500 mg, IST), ENV⁺ (150 mg, IST) and Oasis[®] HLB (60 mg, Waters). To determine the reproducibility of the C₁₈-SPE experiment the procedure was repeated on 5 consecutive days. Finally, the preferred method was used to scale up the SPE experiment. A cartridge was made containing 100 g C₁₈ sorbent, which was used to extract 30 L *Daphnia* water.

Ion exchanger

Strongly basic silica-based anion-exchange cartridges (SAX, 500 mg, Cl⁻-counterion, IST) were washed with ammonia (0.1 M) and methanol, 10 ml each (method A). The methanol was removed by washing with 20 ml of ultrapure water. The pH of the *Daphnia* incubation water was adjusted to 8.0 with 0.1 M NaOH or phosphate buffer (0.25 M) or to pH 2.5 with HCl and the incubation water was passed through the ion exchanger. The unretained fraction was collected. The retained fraction was eluted using 10 ml 0.5 M HCl. Both fractions were evaporated to dryness and redissolved in ultrapure water. This experiment was repeated without solvating the cartridge with ammonia and methanol (method B). The entire ion exchange experiment was repeated with incubation and control water that had been desalted by passing through a C₁₈ cartridge (see Solid-phase extraction) prior to application on ion-exchange cartridges. Additionally the silica-based cartridges were replaced with strongly basic resin-based anion-exchange cartridges (Amberlite[®] IRA-400,

Table 2. Experiments performed with SAX sorbent.

	Sorbent	Method	Test water	pH control	pH	Eluate	Result
1	SAX	A		HCl, NaOH	2.5, 7.0	NaCl	Figure 11
2	SAX	A		Phosphate Buffer	8.0	HCl	Figure 12a
3	SAX	A		NaOH	8.0	HCl	Figure 12b
4	SAX	A, B		NaOH	8.0	HCl	Figure 13
5	SAX, Amberlite	A, B	Desalted	NaOH	8.0	HCl	Figure 14
6	SCX	A	Desalted	NaOH	8.0	HCl	Figure 15

86 mg, Cl⁻-counterion, BDH Chemicals). Finally a small experiment was performed using strongly acidic silica-based cation-exchange cartridges (SCX, 500 mg, H⁺-counterion, IST).

HPLC Fractionation

Some SPE extracts were analysed with reversed-phase HPLC on a 250 × 4.6 mm column (Alltima C₁₈ endcapped, 5 µm, Alltech) by a linear gradient of 0.01 M ammonium formate buffer (A, pH = 3) and acetonitrile (B): 0-2 min. 2% B; 2-27 min. linear increase to 98% B; 27-32 min. hold on 98% B; 32-33 min. linear decrease to 2% B; 33-43 min. hold on 2% B. The flow rate was 1.0 ml min⁻¹. Absorption was monitored using a diode-array detector (Gynkotek UVD340S). Fractions were collected, evaporated, reconstituted and tested for colony formation activity in the bioassay.



Natural Compounds

Small amounts of natural compounds specifically urea **24** (ca. 0.2 mg ml⁻¹), geranic acid **94** (ca. 0.2 mg ml⁻¹), juvenile hormone III **96** (ca. 0.27 mg ml⁻¹) and 20-hydroxyecdysterone **47** (ca. 1.3 µg ml⁻¹) were dissolved in water and assayed in triplicate for colony formation activity in the bioassay.

Statistical analysis

Statistical analyses were performed with SAS software (SAS system for Windows, version 8.0, SAS institute Inc.) using the PROC GLM procedure, followed by a Tukey *post hoc* comparison test to distinguish mean differences that are significantly different at $P = 0.05$.

Results and Discussion

Possible causes for the large variation in the positive controls have been described in Chapter 2.

Liquid-Liquid Extraction (pH dependent)

Daphnia test water was extracted with EtOAc at pH 2, 7 and 12 (Fig. 1). The biological activity is significantly found in the organic solvent layer at pH 2 and 7 (respectively $P=0.0001$ and $P=0.0095$), although slightly less obvious at pH 7. However at pH 12 no

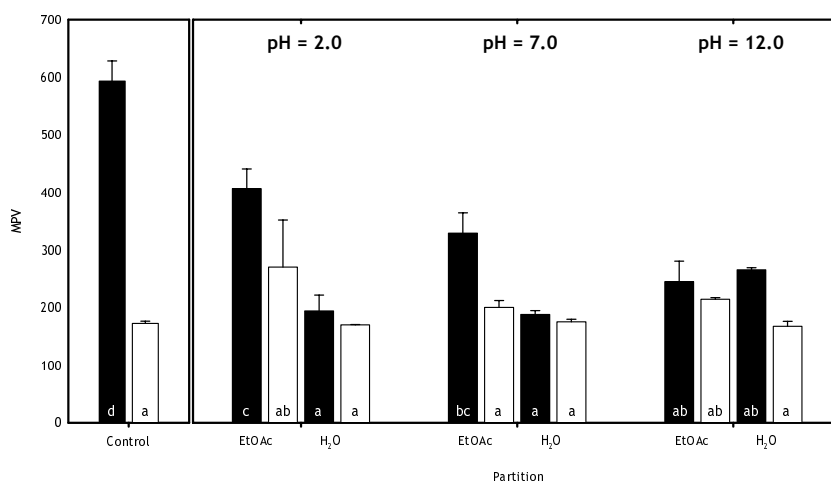
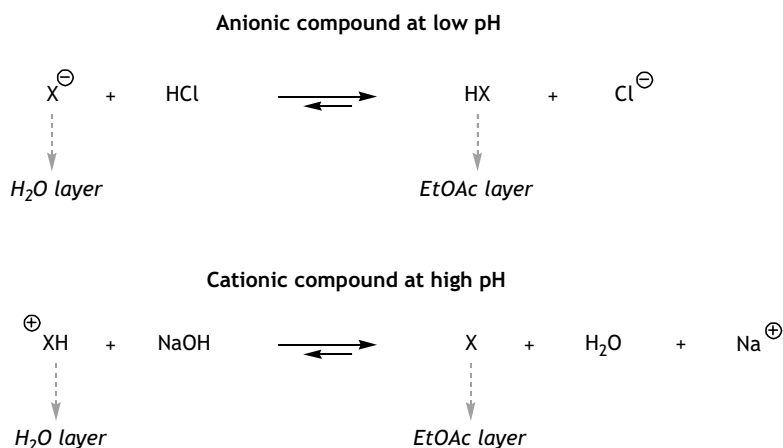


Figure 1. pH-dependent Liquid-Liquid Extraction Biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □) were extracted with ethyl acetate (EtOAc) at pH 2.0, 7.0 and 12.0. Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

significant difference is found between the organic and aqueous layer ($P=0.999$) or between the *Daphnia* test water and the corresponding control treatment ($P=0.998$). This is quite unexpected because the biological activity has to be in one of the two layers. Possible causes could either be inactivation of the kairomone by the basic conditions in



the aqueous layer or possibly the presence of more than one compound with synergistic or additive effects.



Scheme 1. Protonation

In general, ionic compounds (e.g. salts) are water soluble and insoluble in organic solvents (except MeOH). Extraction of these compounds from the aqueous to the organic layer would then occur only when the solubility in water is reduced so that EtOAc layer becomes more attractive (Scheme 1). For anionic compounds this would be at low pH (complete protonation), but at high pH for cationic compounds. This experiment shows that the biological activity clearly shifts at low pH to the EtOAc layer and this could be an indication that the active compound contains an anionic group. This concurs roughly with the findings of Von Elert and Franck¹¹.

Solid-Phase Extraction

As one of the most commonly used techniques, solid-phase extraction was extensively tested. The aim was to develop a method that could elute the active compound in one fraction. To reach this goal several different sorbents, elution profiles and the reproducibility were studied.

Endcapped C_{18}

The biological activity could be extracted from the *Daphnia* water with trifunctional silane end-capped C_{18} cartridges (Fig. 2a and Fig 2b). Figure 2a shows the experiment performed with 30%, 70% aqueous MeOH and pure MeOH (100%) as eluent, whereas Figure 2b shows a similar experiment using 50%, 85% and 100% MeOH. However both the 70% and 100% fraction differ significantly from the corresponding extract obtained from a control experiment (both $P < 0.0001$). In this case it was therefore not successful to move all of the biological activity into one fraction. Possibly the 70% MeOH was not strong enough to remove all the active compound, to resolve this the experiment was repeated with slightly stronger solvents (50%, 85% and 100% MeOH).

Most of the biological activity was recovered from the 85% fraction ($P = 0.0005$), however some activity still remained in the 50% ($P = 0.0176$). The other treatments were not significantly different from the corresponding controls. Again biological activity is divided

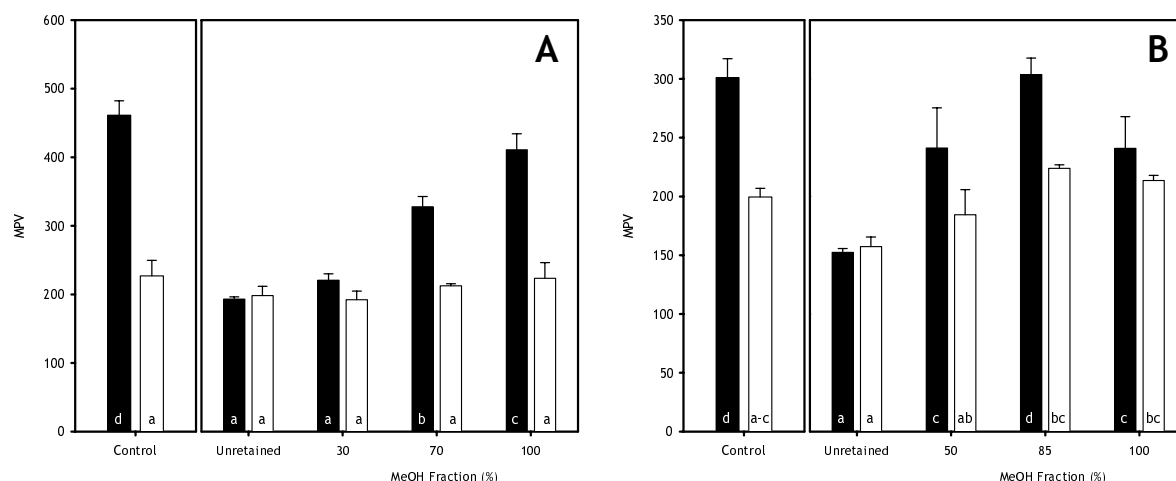


Figure 2. Solid-phase extraction with endcapped C_{18} sorbent

A. Eluted with 30%, 70% and pure methanol (100%); **B.** Eluted with 50%, 85% and pure methanol (100%). Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

over two extracts.

To determine whether the elution volume was sufficient, the cartridges were eluted twice with 50%, then twice with 85% and finally twice with 100% MeOH (Fig. 3). Biological

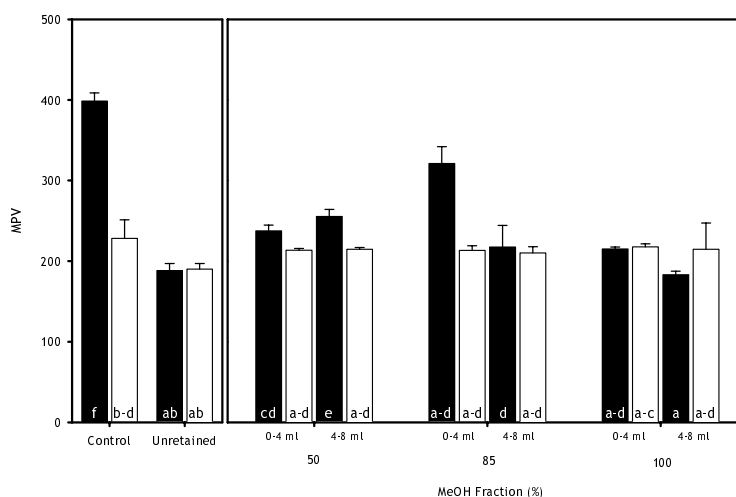


Figure 3. Solid-phase extraction

Activity of Solid-Phase Extraction fractions, extracted with endcapped C_{18} sorbent, in the *Scenedesmus* bioassay expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted twice (2 x 4 ml) with aqueous methanol of increasing strength. Each fraction was treated separately. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a-f) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



activity was only found in the first elution with 85% MeOH, no activity was recovered after elution with a second aliquot of 85%. It can be concluded therefore that the elution volume (= 4 ml) in these experiments is sufficient. In this experiment no significant difference was observed between the 50% ($P=1.000$) and 100% fraction ($P=0.786$) and their corresponding control treatments, although an active 50% fraction was observed in the previous experiment (Fig. 2b). Apparently there is quite some variability in biological activity between bioassays of different SPE experiments. This could be due to a slightly different composition of *Daphnia* test waters, but also to small differences in the performance of the SPE experiment or even the bioassay itself (see Chapter 2).

To investigate this further, five equal portions of the same batch were extracted on five consecutive days. As expected significant differences were found for *Daphnia* test water extracts with the equivalent control treatments. Unfortunately a significant difference was also observed between the five experiments. However this significance seems to be mostly due to the 100% fraction, which clearly showed the most variation. When this fraction was omitted from the statistical analysis not only was the significance of the day effect removed ($P=0.057$), but also the interaction effects were eliminated, except for the fraction \times kairomone interaction ($P < 0.0001$). Apparently this final elution step (100%) is not very reproducible. Figure 4 shows clearly that it was not possible to

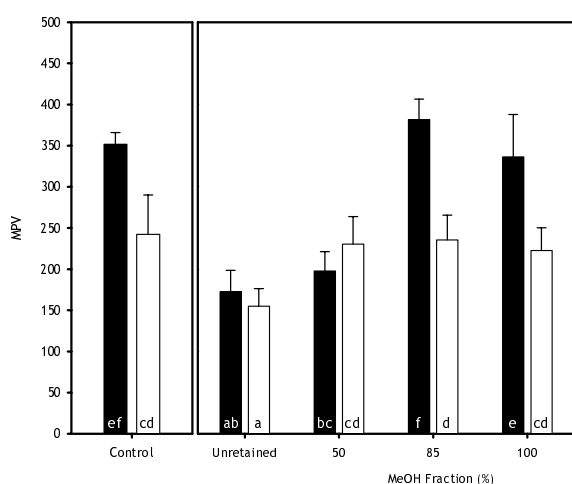


Figure 4. Reproducibility of solid-phase extraction with endcapped C_{18} sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=15$). Similar symbols (a-f) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

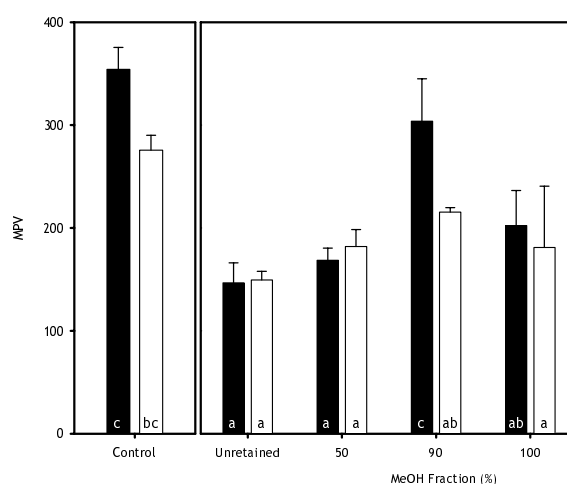


Figure 5. Solid-phase extraction with endcapped C_{18} sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



concentrate all of the biological activity into one fraction. Both the 85% and 100% fraction differ significantly from the corresponding controls (both $P < 0.0001$). The presence of activity in the 100% fraction could also indicate the existence of more than one active compound. Unlike the previous experiment the 50% extract showed no biological activity ($P = 0.1333$), but the 85% and 100% extract did (both $P < 0.0001$).

One last attempt to recover the activity in one fraction on a C_{18} cartridge was to increase the solvent strength to 90% MeOH. The result is shown in Figure 5. This combination seems to give only one biological active fraction (90%), which differs significantly from the corresponding control ($P = 0.0269$). However no reproducibility experiment was done with this elution protocol and the colony formation activity of this entire bioassay batch is poor (no significant difference was found between the positive and negative controls, $P = 0.0657$).

Although Lampert et al. were able to extract the cue from *Daphnia* water with a C_{18} cartridge, they were not able to remove the cue from the cartridge with methanol¹². Results reported in this thesis concur with Von Elert and Franck that the cue could be eluted from the cartridge with ethanol or methanol¹¹. When the SPE methods used by these two groups were compared with the results in this thesis (Table 3), it is clear that there are some differences.

Table 3. SPE methods used to extract the compound that induces *Scenedesmus* to form colonies.

Type	Sorbent		Manufacturer	Solvation	Equilibration	Elution
	End-capped	Amount				
C_{18}	?	?	?	10 ml	50 ml	methanol ¹²
C_{18}	?	500 mg	Analytichem	20 ml	20 ml	ethanol ¹¹
C_{18}	yes	500 mg	IST	10 ml	10 ml	methanol ¹³

The importance of knowing the source of a C_{18} sorbent is obvious, since C_{18} sorbents may vary from manufacturer to manufacturer and switching to a different brand can be a way to enhance isolation^{14,15}. As it is unknown which manufacturer or what type of sorbent the group of Lampert used, it is difficult to compare their results with those of Von Elert and Franck. It makes it also hard to explain why the compound could not be eluted with methanol by Lampert et al.^{11,12}. C_{18} sorbent can only retain isolates when the sorbent is correctly solvated with an organic solvent. The cartridge is subsequently rinsed (equilibrated) with water or buffer to remove the excess solvent. Lampert et al. used an excess amount of water to rinse the cartridge after solvation. Possibly this had an effect on the retention and elution with methanol by undoing the solvation process. IST (International Sorbent Technology) recommends adding small amounts of organic solvents (0.5-2%) to samples to reduce the danger of inactivation of the sorbent surface. A benefit from this practice can be expected from sample sizes as small as 100 ml. However, typically this would only be expected to be important for the prevention of break-through and recovery loss¹⁶.



Other Silica based Sorbents

The previous experiments were all performed on C₁₈, which is the most universally used sorbent at present. Most of these are end-capped to reduce polar secondary interactions associated with surface silanol groups. Often this will lead to cleaner extracts, because the stationary phase is more uniformly non-polar.

Table 4. Physical properties of several silica sorbents

Sorbent	End-capped	Size μm	Porosity \AA	Retention Mechanism
Silica C ₁₈ (ec)	Yes	50	60	Non-polar
Silica C ₁₈	No	50	60	Non-polar, Polar, Cation Exchange
Silica MF C ₁₈	No	50	125	Non-polar, Polar, Cation Exchange
Silica C ₈ (ec)	Yes	50	60	Non-polar
Silica C ₂ (ec)	Yes	50	60	Non-polar
Silica CN (ec)	Yes	50	60	Non-polar, Polar

However since knowledge on the active compound is limited it was considered worthwhile to repeat some of the experiments using other silica based sorbents (Table 4). Figure 6a shows the experiment performed with MF C₁₈ material. This material is manufactured using monofunctional silane and is not end-capped. It has polar secondary interactions and therefore has improved retention of basic and polar compounds compared to endcapped C₁₈ and has more capacity for polar compounds than trifunctional sorbents¹⁵, however it is more susceptible to adverse acidic conditions.

For example, heterocyclic amines show better recovery on monofunctional C₁₈ than on trifunctional C₁₈¹⁴. Biological activity is recovered in the 50% and 85% MeOH fractions (both $P < 0.0001$). Figure 6b shows the experiment performed with non-endcapped C₁₈

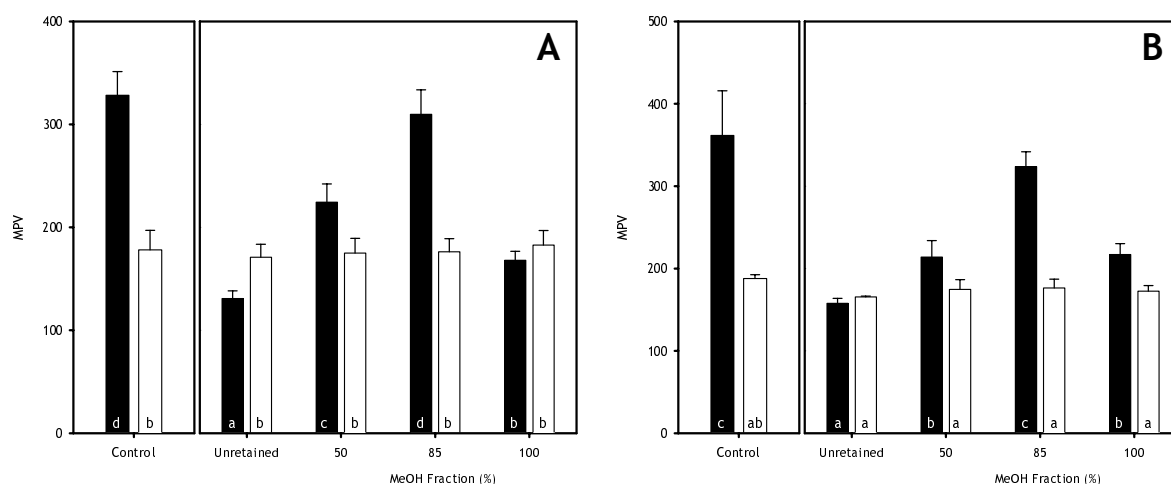


Figure 6. Solid-phase extraction

A. MF C₁₈ sorbent; B. non-endcapped C₁₈ sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD (n=6). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



material. This material is manufactured using trifunctional silane. It has polar secondary interactions and therefore has improved retention of basic compounds compared to endcapped C_{18} . Biological activity is basically recovered in the 85% MeOH fractions ($P < 0.0001$), although 50% ($P=0.0101$) and 100% ($P=0.0021$) also have some biological activity. The C_8 sorbent contains shorter chains than the traditional C_{18} . Slightly weaker interactions with the C_8 sorbent can facilitate the elution of analytes that are strongly retained on C_{18} . The C_8 used here is endcapped so secondary polar and basic interactions are reduced. As can be expected the active compound is less retained on C_8 than on C_{18} , it is recovered in the 50% ($P=0.0002$) and 85% ($P=0.0001$) MeOH fractions, however still in two extracts (Fig. 7a).

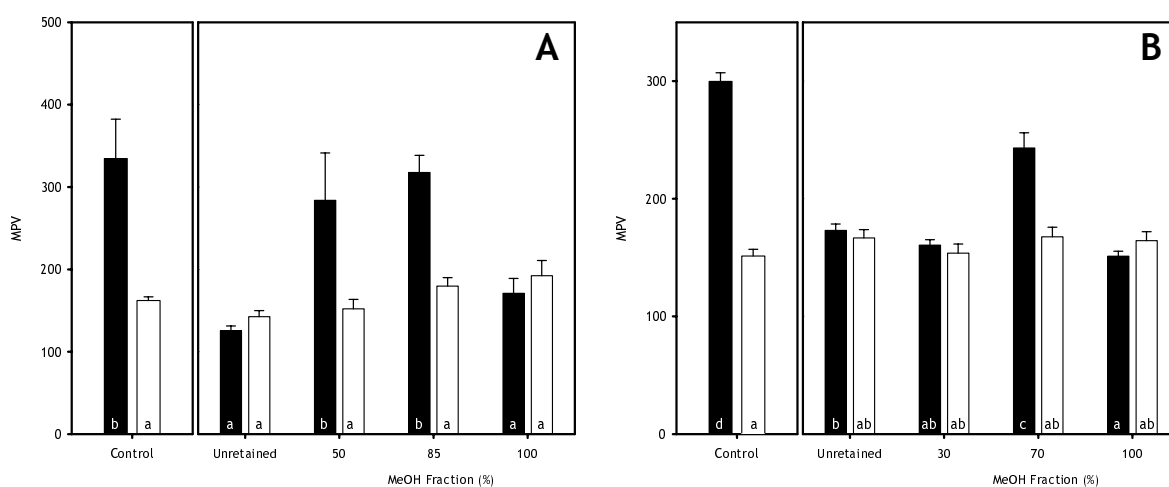


Figure 7. Solid-phase extraction

A. C_8 sorbent; B. C_2 sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

The C_2 sorbent is the shortest of the commonly used sorbents with aliphatic chains. It is used when analytes are too strongly retained on C_8 and C_{18} . It is primarily used to extract non-polar molecules from aqueous samples, but it can be difficult to obtain a stable and reproducible C_2 sorbent. It reduces the need for large volumes of elution solvent, because retention is more easily broken. The C_2 used here is endcapped so secondary polar and basic interactions are reduced (Fig. 7b). As can be expected the active compound is less retained on C_2 than on C_{18} , and it seems to be recovered in one fraction (70% MeOH, $P < 0.0001$). Unfortunately the quality of the bioassay was poor and no replicates were done to further investigate this result. Finally, a similar experiment was performed with a sorbent which is normally used in normal-phase experiments (CN, cyanopropyl). However it can also be used in certain reversed-phase applications as a less hydrophobic alternative to C_{18} or C_8 . The CN sorbent is especially useful when non-polar molecules are too strongly retained on C_{18} or C_8 . Additionally the cyano group gives this sorbent a unique

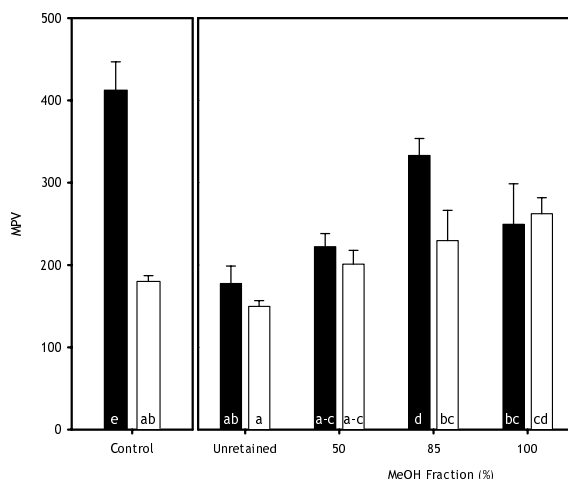


Figure 8. Solid-phase extraction with CN sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with aqueous methanol of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD (n=3). Similar symbols (a-e) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

selectivity and the polarity is slightly less than C_2 ¹⁷. The endcapped sorbent was used to reduce secondary interaction (Fig. 8). This sorbent gives a very similar result to the C_2 sorbent. The biological activity was recovered from the 85% fraction (P=0.003).

Polymeric Sorbents

These types of sorbents have several advantages over traditional silica sorbents. First most of them make the need to solvate the material before use unnecessary. This reduces also the risk of low recoveries should the material run dry during an experiment. Secondly the capacity is usually higher than that of the silica sorbents, which decreases bed size and consequently cost and solvent use. Hydrophobic interactions cause a decrease of retention as polarity of the analyte increases, however this is compensated by the

Table 5. Physical properties of polymeric sorbents

Sorbent			Surface Area m ² /g	Size μm	Porosity Å
ENV ⁺	Polymeric	PS-DVB	1000	90	100
Oasis [®] HLB	Polymeric	PS-DVB-NVP	800	30-60	55

PS-DVB = Poly(styrene-divinylbenzene), NVP = N-vinylpyrrolidone

polymeric matrix containing aromatic rings. The π - π interactions give stronger retention with analytes with available π -electrons^{15,18}. Table 5 shows some characteristics of polymeric sorbents used for experiments. Figure 9a shows the results of solid-phase extraction with the ENV⁺ sorbent. Colony formation is found in two fractions, namely 85%

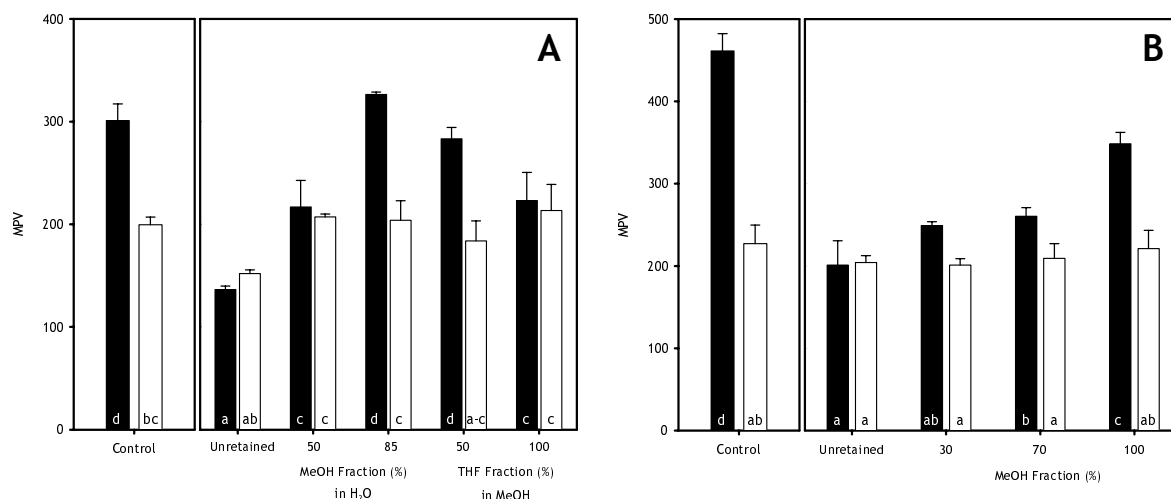


Figure 9. Solid-phase extraction with polymeric sorbents

A. ENV⁺ sorbent; B. Oasis[®] HLB sorbent

Activity of Solid-Phase Extraction fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with organic solvents of increasing strength. Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD (n=3). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

MeOH and 50% MeOH in THF (both $P < 0.0001$). Especially Oasis[®] HLB has been reported as a very robust sorbent which can extract a wide variety of compounds at once¹⁹. The use of this sorbent (Fig. 9b) did not seem to offer any particular benefit over C₁₈ because the biological activity was reported in two fractions, namely the 70% and 100% MeOH fraction (respectively $P=0.023$ and $P < 0.0001$). A possible explanation for the poor performance of the polymeric sorbents is given by Millar, who states that aqueous MeOH

Table 6. Summary of statistically active SPE extracts.

Figure	Sorbent	Aqueous MeOH (%)					Methanolic THF (%)	
		30	50	70	85	90	100	50
2a	C ₁₈	-		***			***	
2b	C ₁₈		*		***		-	
3	C ₁₈		-		***		-	
4	C ₁₈		-		***		***	
5	C ₁₈		-			*	-	
6a	MF C ₁₈		***		***			
6b	C ₁₈ nec		*		***		**	
7a	C ₈		***		***		-	
7b	C ₂	-		***			-	
8	CN	-		**			-	
9a	ENV ⁺		-		***			***
9b	Oasis [®] HLB	-		*			***	
10	C ₁₈		-		***		***	

*** = $P < 0.001$; ** = $P < 0.01$, * = $P < 0.05$, - = not significant



is not the best solvent system for use with polymers because it does not wet the surface very well. Aqueous ACN would be a better alternative²⁰.

In summary, Table 6 gives the most important results from the SPE experiments. A real statistical comparison is not possible since all experiments were performed at different times and controls between experiments showed great variation. However a general clue can be gained from looking at Table 6. The overall score of the 85% extracts is the best, followed by the 100% extracts and the 50% extracts, which shows less frequently biological activity. Based on these results the C₁₈ material was used for further experiments with 50%, 85% and 100% elution.

It can be concluded that the biological active compound is relatively hydrophilic, but that by manipulating the pH, it is still possible to extract the compound from *Daphnia* water with lipophilic sorbents such as C₁₈, which was also reported by Von Elert and Franck¹¹.

Scale-up

Figure 10 shows the result of scaling up the enrichment process to 30 L on SPE material. A one-way ANOVA showed significant differences between the fractions ($P < 0.001$) but no significant difference between the 85% and 100% fraction was observed ($P = 0.704$). For dilute aqueous solutions where solvent extraction is impractical and C₁₈ SPE might be too time-consuming a suitable alternative might be the use of hydrophobic resin beads such as the Amberlite® XAD (and other related beads) or the Bio-Beads® SM resins to absorb and concentrate organic compounds. The resins have a high capacity for lipophilic compounds, so hundreds of litres can be extracted with small volumes of resin²¹.

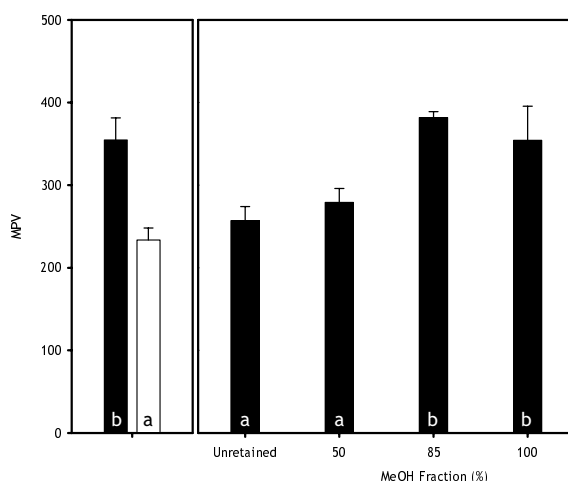


Figure 10. Solid-phase extraction Scale-up

Activity of Solid-Phase Extraction (C₁₈) scale-up fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The C₁₈ material (100 g) was eluted successively with aqueous methanol of increasing strength. The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a,b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



Ion Exchanger

Experiments were performed with three ion exchange materials and focussed initially on anion exchangers, but when results were not as expected, a cation exchanger was tested. Furthermore to exclude problems due to pH sensitive silica based sorbents, experiments were repeated on a resin based sorbent. Properties of the sorbents are given in Table 7. Earlier research¹¹ and the LLE experiment pointed to the presence of an anionic group. To further test this hypothesis, the *Daphnia* test water was applied at pH 2.5 and 7.0 on a silica based anion-exchange sorbent (SAX), which is shown in Figure 11. It is clear that at low pH the compound is not retained on the ion exchanger and all of the biological activity is found in the unretained fraction ($P < 0.0001$). This could mean that indeed protonated anionic groups hinder retention on the ion exchange sorbent and corroborate the finding of the LLE experiment that an anionic moiety is present. At first glance it seems that more biological activity is found in the first retained fraction (0.1 M) at the higher pH of 7.0, however this fraction did not differ significantly ($P=0.0572$) from its

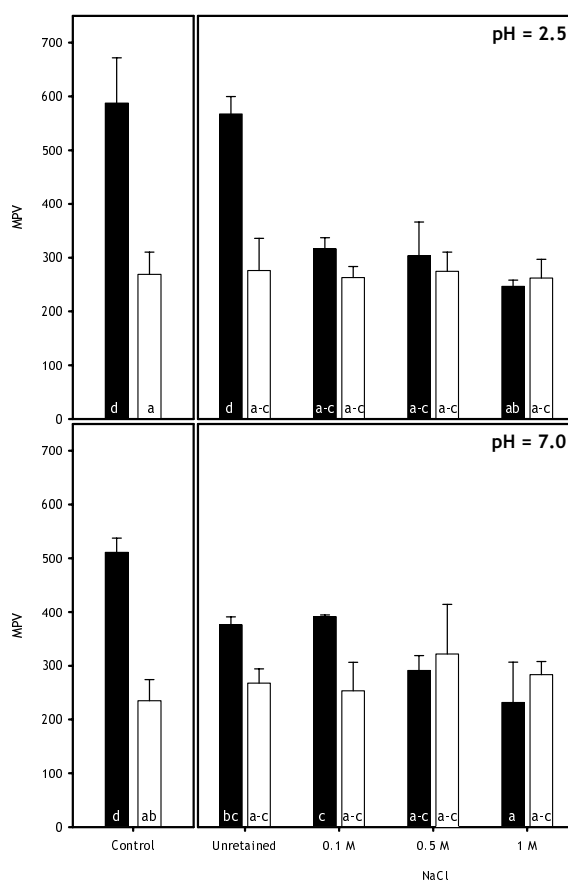


Figure 11. pH-dependent Solid-phase extraction with SAX sorbent

Activity of Ion Exchanger (SAX) fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). NaOH and HCl were used to adjust the pH to respectively 2.5 and 7.0 prior to the experiment. The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The sorbent was eluted successively with sodium chloride of increasing strength (0.1 M, 0.5 M and 1 M). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD ($n=3$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



Table 7. Physical properties of ion exchanger sorbents

Sorbent	Size	Porosity Å	Retention	Capacity meq/g
Silica SAX	50 µm	60	Anion Exchange, Non-polar	0.6
Silica SCX	50 µm	60	Cation Exchange, Non-polar	0.3
Resin Amberlite® IRA-400	16-50 mesh		Anion Exchange	3.8

corresponding control, maybe the effect was just too weak to be analysed statistically. Possibly pH 7.0 was still too low for sufficient deprotonation and the experiment was repeated with the higher pH of 8.0.

Figure 12a shows the experiment repeated at pH 8.0. A phosphate buffer (0.25 M) was used to reach the desired pH of the test waters. Furthermore the cartridges were eluted with HCl (0.5 M). Unexpectedly the biological activity was found in the unretained fraction and not in the retained fraction. Furthermore this was a significant result ($P < 0.0001$). Possibly the buffer salts and ions interfered too much with retention mechanism and protonation.

The experiment was repeated again (Fig. 12b) but pH 8.0 was fixed with NaOH (0.01 M). However the same result was obtained as the previous experiment. A previous study by Von Elert and Franck showed that they were able to retain the active compounds on a anion-exchange cartridge. They performed their experiments without solvating the cartridge with ammonia and MeOH prior to use (Method B). Their experimental set-up was used for the next experiment and compared with Method A (solvation of the cartridge with ammonia and MeOH). When the cartridge was solvated with ammonia (Method A), the active compound could not be retained on the cartridge (Fig. 12 and 13). Even

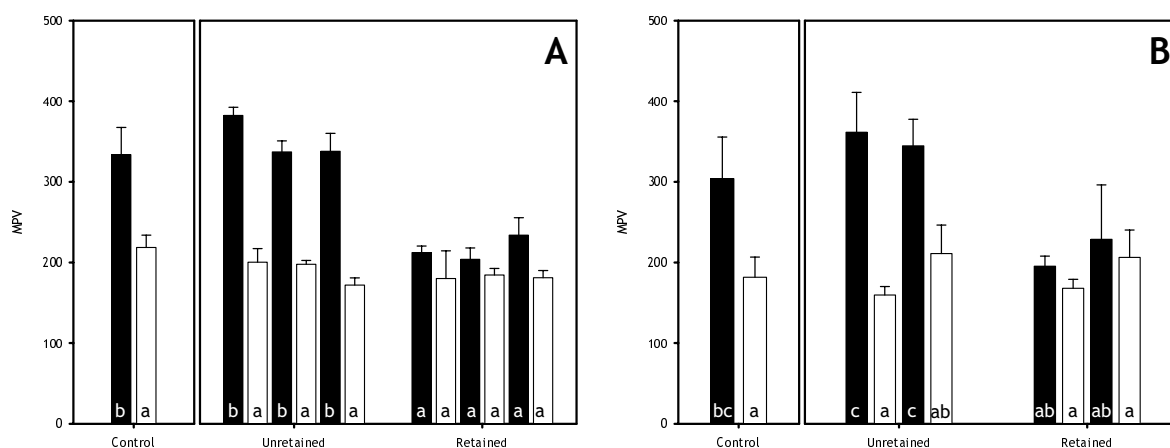


Figure 12. Solid-phase extraction with SAX sorbent

Adjustment to pH 8.0 of the test water prior to the experiment with Phosphate Buffer (0.25 M, A) or Sodium hydroxide (0.01 M, B).

Activity of Ion Exchanger (SAX) fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Retained is the fraction that was eluted with hydrochloric acid (0.5 M). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD (n=3). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



equilibration with a large amount of ultrapure water (180 ml, results not shown) after solvation but before application of the sample could not retain the cue on the SAX cartridge. However, the cue was partially retained on the cartridge if the above-mentioned solvation step was omitted (Method B, Fig. 13). Furthermore the biological activity was now divided over the two fractions. This did not occur when the cartridge was solvated, then only one active unretained fraction was recovered.

Below pH 2 the silyl ether linkage becomes unstable and the functional groups on the surface begin to cleave, changing the sorptive characteristics irreversibly and above pH 7.5 the silica is subject to dissolution in aqueous solutions. However Van Horne claims that in practice silica can be used in a pH range from 1 to 14 as sorbents are generally exposed to solvents for only short periods of time¹⁷, however in our laboratory obvious breakdown of silica sorbent at pH 14 was observed. The maximum pH attainable is usually

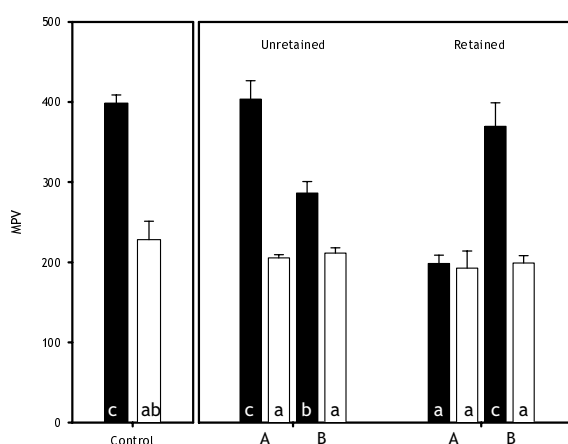


Figure 13. Solid-phase extraction with SAX sorbent

Activity of Ion Exchanger (SAX) fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The results of extraction on SAX with (A) and without (B) solvating the cartridge with ammonia (0.1 M) prior to use are shown. The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Sodium hydroxide (0.01 M) was used to adjust test water to pH 8.0 prior to the experiments. Retained is the fraction that was eluted with hydrochloric acid (0.5 M). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD. Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

near 12. To exclude partial decomposition of the stationary phase due to high and low pH's, the silica-based ion exchanger was replaced by Amberlite[®] (resin). This resin has a workable range from pH 1 to 14 in contrast to the SAX sorbent which is stable within a pH range of 2 to 7.5. However this offered no improvement, again the active compound could not be retained on the cartridge (Fig. 14). The composition of the medium²² was another potential cause of the poor extractability, because it contains many exchangeable anions. These ions could possibly compete with the active compounds for the available exchange sites on the sorbent¹⁹. When the experiment was repeated with desalted *Daphnia* test and control water the extractability of the active compound did not improve (compare Fig 13 and 14). Given that Von Elert and Franck (1999) used a

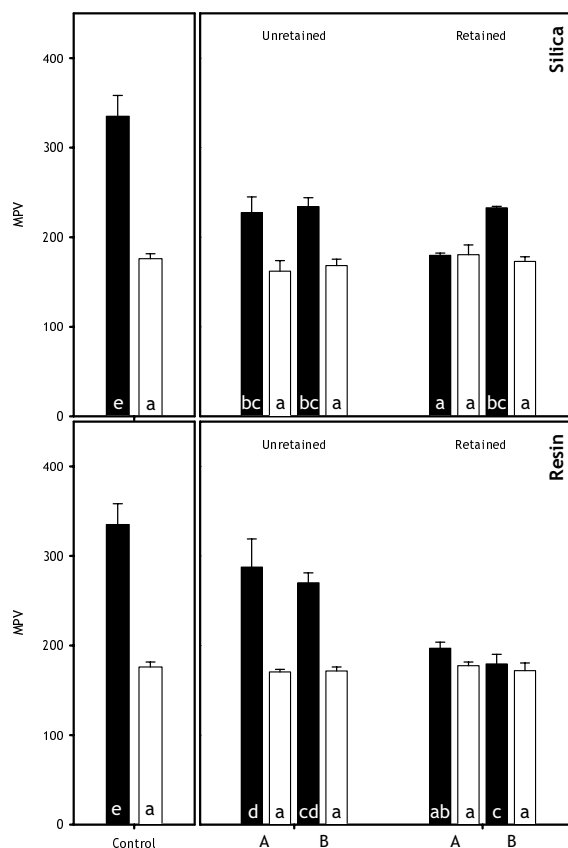


Figure 14. Solid-phase extraction with SAX sorbent and resin based Amberlite Activity of Ion Exchanger (SAX) fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The results of extraction on SAX (silica, upper panel) and Amberlite® (resin, lower panel) with (A) and without (B) solvating the cartridge with ammonia (0.1 M) prior to use are shown. The experiment was performed with desalted biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Sodium hydroxide (0.01 M) was used to adjust test water to pH 8.0 prior to the experiments. Retained is the fraction that was eluted with hydrochloric acid (0.5 M). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD. Similar symbols (a-e) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

similar medium¹¹ and did not desalt prior to ion exchange, no differences should have been found between these and their results. Nonetheless, the biological activity was recovered in the unretained fraction.

Several possible causes for the poor extractability with an anion exchanger and contrasting results were investigated, ranging from the type of sorbent or the elution procedure to the composition of the growth medium but they could not provide a satisfying answer. When environmental samples are handled, often the high amounts of inorganic ions overload the capacity of the sorbent²³. At present the results, reported in this thesis pointed more towards a cationic than an anionic moiety. Possibly a quaternary ammonium moiety is present, however a strongly acidic cation exchanger (PRS) was tested by Von Elert and Franck and the biological activity could not be retained on such a cartridge¹¹. They clearly showed that a strongly basic anion exchanger could extract the



cue with a buffer of pH = 7 (Cl^- , pH of eluate unknown). Although our study concurs that the cue could be extracted on an anion exchanger using Method B (Cl^- , buffer pH = 8, eluate pH = 4.0), the extraction was not as good as reported by Von Elert and Franck¹¹. Method A (OH^- , buffer pH = 8, eluate pH = 9.6) showed no retention at all. Several factors affect retention: pH, ionic strength and counter-ion. Adjusting the pH to 8.0 ensures complete ionisation (assuming the compound has an anionic moiety). Secondly ionic strength is important because increased ionic strength will increase competition between anions. This could be solved by dilution of the sample or an extra aqueous wash step. Finally the choice of counterion has proved to be very important. Lower recovery of the analyte indicates that the counterion is more difficult to displace from the surface²⁴. Counterion selectivity tables can be found in many books on SPE^{15,17}. However, they do show slight differences^{15,17,24}. Apparently, the selectivity of a counterion depends also on the analytes to be extracted. Other factors that influence the selectivity are Van der Waals interactions of the counterion with the sorbent and polarisation of the counterion may increase binding more than can be expected by the hydrated radius comparison¹⁵. During production SPE cartridges are loaded with Cl^- counterions and these ions are replaced during solvation by OH^- counterions (method A). Although an OH^- counterion is weaker than a Cl^- counterion and thus more easily replaced, it can react as a base and the ion exchange mechanism will work in a different way.

Concluding, the differences between the results of Von Elert and Franck and this study could be due to the initial ion present on the ion exchanger and subtle pH and concentration differences of the used buffers. As yet, no clear explanation can be forwarded for the lack of retention when OH^- is present. Possibly, pH dependent

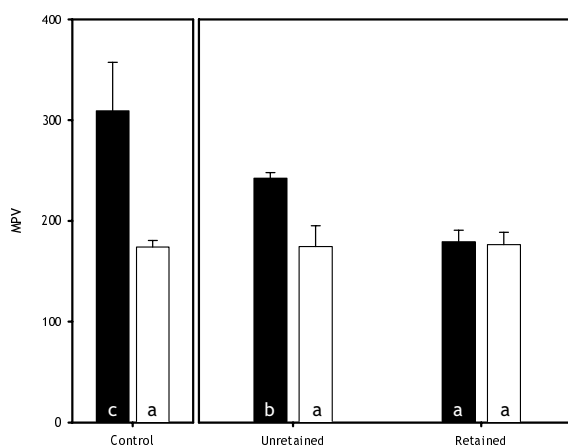


Figure 15. Solid-Phase Extraction with SCX sorbent

Activity of Ion Exchanger (SCX) fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The results of extraction on SCX without solvating the cartridge with ammonia (0.1 M) prior to use are shown. The experiment was performed with desalted biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Sodium hydroxide (0.01 M) was used to adjust test water to pH 8.0 prior to the experiments. Retained is the fraction that was eluted with hydrochloric acid (0.5 M). Unretained is the fraction that passed the cartridge without retention. Error bars represent 1 SD. Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



secondary interactions, like van der Waals interactions play a role. SAX has been shown to display polar and non-polar interactions under certain conditions¹⁷. For larger molecules size exclusion becomes an important secondary interaction. The pore diameter of normal cartridges (60 Å) is small and does not allow the entry of larger molecules. It will exclude molecules as small as 2 kDa¹⁵. However, as mentioned before, the colony-inducing compound is probably smaller than 0.5 kDa and should therefore not encounter this problem. Another explanation could be the presence of a cationic group. A small experiment was performed with a cation exchanger (SCX, Fig. 15) to exclude the possibility that a cationic moiety was present at pH 8.0. Again the biological activity was recovered from the unretained fraction (P = 0.0298).

Chromatography

The enriched extract obtained by solid-phase extraction (C₁₈) was fractionated by HPLC. First, extracts obtained in the reproducibility experiment were injected on HPLC and fractionated. The five collected fractions showed a lot of variation in biological activity between the injected five extracts, however the individual extracts did not differ significantly (P=0.331) from each other. When they were averaged (Fig. 16), one fraction showed a significantly higher biological activity relative to a control (fraction C, 42-69% ACN, P < 0.0001). Although reproducibility was quite high in this experiment, it was greatly reduced in the next one.

The fractions that were collected unfortunately all showed significant biological activity

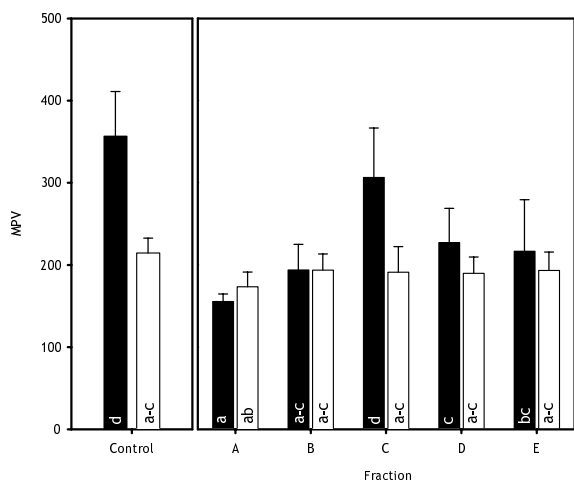


Figure 16. Averaged results of HPLC fractionation
Activity of HPLC fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was repeated five times with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The eluate was collected in fractions A-E (A: 0-14.5; B: 14-18.5; C: 18.5-23.5; D: 23.5-28; E: 28-36 min.). Error bars represent 1 SD (n=15). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level

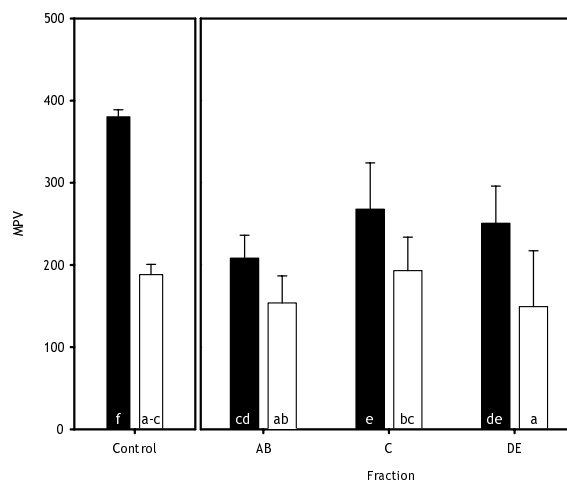


Figure 17. Averaged results of HPLC fractionation
Activity of HPLC fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was repeated five times with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The eluate was collected in fractions AB-DE (AB: 0-18.5; C: 18.5-23.5; DE: 23.5-36 min.). Error bars represent 1 SD (n=15). Similar symbols (a-f) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

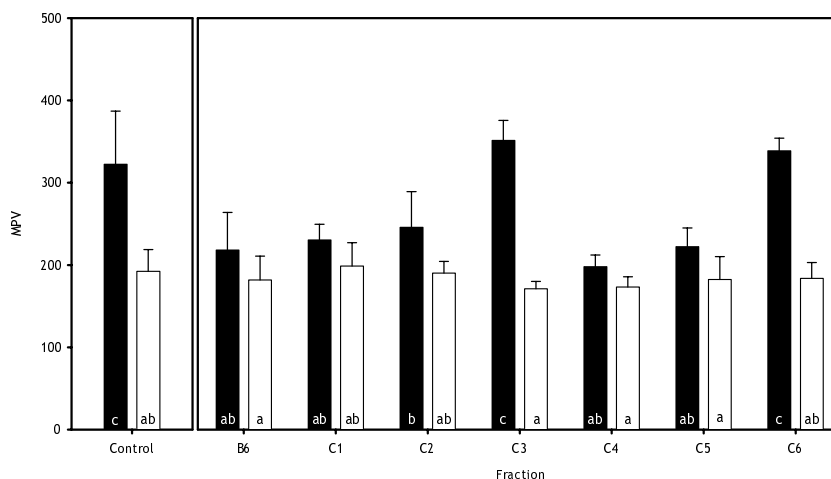


Figure 18. Averaged result of HPLC fractionation

Activity of HPLC fractions in the *Scenedesmus* bioassay, expressed as mean particle volume (MPV). The experiment was repeated twice with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). The eluate was collected in fractions B6-C6 (B6: 17-18; C1: 18-19; C2: 19-20; C3: 20-21; C4: 21-22; C5: 22-23; C6: 23-24 min.). Error bars represent 1 SD ($n=6$). Similar symbols (a-d) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

when compared to the corresponding control (Fig. 17). (Fraction AB: $P = 0.0094$; Fraction C: $P < 0.0001$; DE: $P < 0.0001$). A similar problem was identified by Fink when biological activity was found in almost all fractions when the HPLC gradient was made less steep²⁵. This could be another indication that more than one compound is responsible for the activity. The hypothesis of the presence of more than one active compound was further strengthened when the next fractionation was performed. Here smaller fractions were

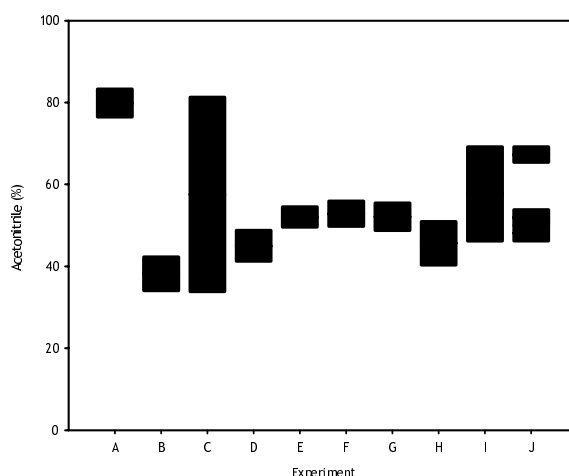


Figure 19. Observed active fractions

Active fractions reported by different research groups expressed as percentage acetonitrile in gradient elution.

A. Lichrospher[®] C₁₈¹¹; B-D. KromasilTM C₁₈²⁵; E. Spherisorb[®] C₁₈²⁵; F-H. Lichrospher[®] C₁₈²⁵; I-J. Alltima[®] C₁₈ (this thesis)



collected within the earlier identified region called 'Fraction C' (Fig. 18). Surprisingly three fractions (C2, 46-50% ACN : $P = 0.0264$; C3, 50-54% ACN: $P < 0.0001$; C6, 65-69% ACN: $P < 0.0001$) were significantly different from the control, albeit one less strong (C2). Finally, the HPLC results presented in this thesis confirm the results of Von Elert and Franck¹¹. Both groups found one active fraction after fractionation. Over the past years other groups have also performed HPLC fractionations^{11,25}. Figure 19 shows the results of known HPLC fractions by several groups. The results were standardised to ACN (%). It then becomes clear that the active compound elutes most likely in the region between 40-55% aqueous ACN.

Natural Compounds

Several compounds, which have been mentioned in recent research as being candidates for the kairomone, were dissolved in water and screened in the bioassay (Fig. 20). Not only were these compounds not biologically active, but they also fell outside the area where the biologically active compounds were found to elute from the HPLC column ('Fraction C') when analysed with the same gradient.

Several groups^{11,12,26} have reported on the isolation of the cue that induces *Scenedesmus* to colonise. Based on their results combined with the results described here, it can be concluded that urea²⁷ is not the chemical cue that induces colonisation.

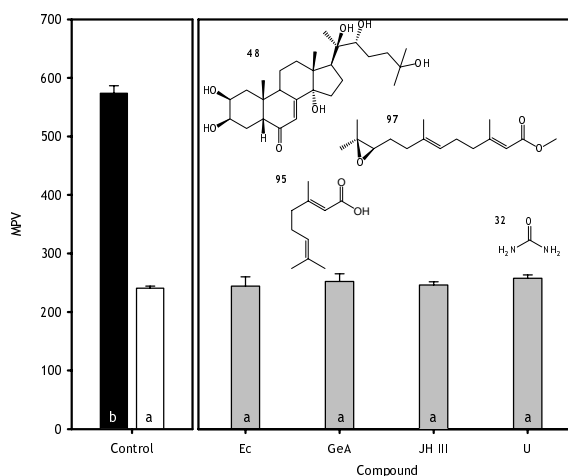


Figure 20. Natural Compounds

Colony formation activity of several hormones (■, Ec = 20-hydroxyecdysterone **48** and JH III = Juvenile hormone III **97**) and natural compounds (■, GeA = Geranic acid **95** and U = Urea **32**), expressed as mean particle volume (μm^3). Positive control (■) and negative control (□). Error bars represent 1 SD ($n=3$). Similar symbols (a-b) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

Recommendation

Although an assumption was made to ignore possible synergistic effects in these experiments, it might be worthwhile to repeat these experiments using for example the



subtractive-combination method, given that more than one active fraction was found. Since this method generates a lot of samples to be tested in the bioassay, a functional and reliable bioassay is essential. Unfortunately the bioassay was no longer reliable (see Chapter 2). The occurrence of synergism is only one of many possible explanations for the loss of biological activity. Among these are the problems associated with repeated fractionation, metabolite instability (due to the used extraction techniques), separation over several fractions (decreased concentration in each fraction) or the loss of volatile compounds when solvents are evaporated. Some of these problems can be avoided by developing more appropriate procedures, which can only be acquired with a high probability of success with the aid of increased chemical expertise and experience. It is therefore highly recommended that biologists seek input from experienced natural product chemists, particularly phytochemists and analytical chemists^{10,28}.

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Chapter 5. Chromatographic analysis of Daphnia water extracts



Introduction

In the previous chapter, several different approaches to identify compounds responsible for biological activity (i.e. colony formation in *Scenedesmus*) were described. Among these were binary splitting¹ and differential diagnosis². Inherent to binary splitting is its dependence on a bioassay, which is fine when a suitable bioassay is available but less so in the absence of such a bioassay. Differential diagnosis though can be performed independent of a bioassay. It can be a viable option if for some reason no feedback from a bioassay can be obtained. In some cases differential diagnosis can be very straightforward when one active peak is present solely in chromatograms of an active extract. At other times extracts are highly complex mixtures with a whole range of peaks. Numerous possible candidates can then be identified or no unique peaks are found at all³. Furthermore in some cases compounds are not active individually, but only in combination or in mixtures. These mixtures can also contain synergistic compounds or are only active in certain specific ratios.

Before each analysis several choices have to be made. Nowadays several chromatographic methods are available for the analysis of samples. HPLC and GC are used universally, but other techniques such as capillary electrophoresis (CE), supercritical fluid chromatography (SFC), and gel-permeation chromatography (GPC) can also aid in the analysis of unknown samples. A wise choice of analytical column can further optimise GC and HPLC. Especially for HPLC a lot of different packings have been developed. Even packings that are nominally the same can show different selectivities⁴⁻⁷, so switching to a different supplier can be advantageous. Secondly, a suitable detection method has to be selected. The choice of detection depends on the nature of the compounds to be investigated. UV detection is most universally used for HPLC and GC is increasingly combined with an MS detector. Other possibilities for HPLC include differential refraction, fluorescence, electrochemical, radioactivity, conductivity and evaporative light scattering (ELS) detectors. UV and ELS detectors are often termed complementary techniques as they have different ways of detection. Furthermore new spectroscopic combinations with HPLC are becoming popular, such as MS and NMR. Finally, solvent composition in HPLC can influence separation drastically. The chosen solvent (HPLC) has to be compatible with the selected detection method (e.g. volatile buffers for MS and ELS detection). Combining all these different elements of a separation method can be quite difficult, especially if the nature of the active compound(s) is not known. In that case no literature can be used to base a method upon.

The approach described in this chapter tries to maximise the chance that at least in one of the tried techniques some unique peaks in active *Daphnia* water extracts will be recognised and possibly identified. Results will be described of the analysis of *Daphnia* test water extracts with gas and liquid chromatography. All *Daphnia* test water extracts were compared against extracts of control water. A combination of UV, ELS and MS detection was chosen for the LC experiments. Furthermore the extracts were analysed with liquid chromatography on four different columns (C₁₈, C₈, another C₈ and Phenyl). However for the GC analysis, an MS detector was chosen because this detector had the additional benefit of providing structural information and is universal for organic



compounds. Extracts were analysed with GC-MS after silylation.

Experimental

Algae and Daphnia

Growth conditions and culture of *Scenedesmus* and *Daphnia* were as described in Chapter 2. After filtration (GF52, Schleicher & Schuell, Dassel, Germany) the incubation water was put directly into the freezer, to which new batches of incubation water were added during two weeks until a volume of 20 L was reached.

Table 1. *Daphnia* test water extracts

	Produced	Extracted
I <i>Daphnia</i> test water	3 June 2002	7 June 2002
II <i>Daphnia</i> test water	11 June 2002	12 June 2002
III <i>Daphnia</i> test water	17 June 2002	18 June 2002
IV HPLC Waste I, II, III re-extracted	8 July 2002	22 August 2002
V <i>Daphnia</i> test water	8 October 2002	9 October 2002
VI Control	22 November 2002	25 November 2002
VII <i>Daphnia</i> test water	22 November 2002	2 December 2002
VIII Control	16 December 2002	17 December 2002
IX <i>Daphnia</i> test water	16 December 2002	18 December 2002

The incubation water was thawed by moving it from the freezer to the fridge the day before extraction. Similarly untreated (without *D. magna*) WC medium was prepared (= control). Prior to the experiment, the pH of incubation water and WC medium was adjusted to 3.0 with ammonium formate (10 mM) and formic acid (= ammonium formate buffer). Methanol (2%) was added to incubation water and WC medium to keep the SPE material solvated during extraction.

Chemicals

Methanol (p.a.) was purchased from Labscan (Dublin, Ireland). Methanol (MeOH), ammonium formate (AF), dimethyl formamide (DMF), N,O-Bis (trimethyl) trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) and formic acid (FA) were obtained from Fluka (all HPLC or GC grade, Zwijndrecht, the Netherlands). Acetonitrile (HPLC grade) and ammonia (NH₃) were supplied by Riedel-de Haën (Zwijndrecht, the Netherlands). Ammonium bicarbonate (NH₄HCO₃) and deuterated methanol (99.8%) were supplied by Aldrich (Gillingham, Dorset, United Kingdom). Buffers were made with HPLC grade nanopure water (Seralpur Pro 90 C, Seral, Ransbach, Germany). All other chemicals were of analytical grade.



Chromatography

HPLC-DAD-ELSD Instrumentation

Analysis was performed with an HPLC system consisting of a low-pressure gradient HPLC pump (M480, GynkoteK). Injections were made by a Basic Marathon autosampler equipped with a 10 μ l loop and the effluent was monitored using a diode-array detector (DAD, UVD340S, GynkoteK, Germering, Germany) and an evaporative light scattering detector (ELSD, Sedex 55, Sedex, Alfortville, France). The effluent of the DAD was evaporated at 42 °C and transported with a stream of pressurised air (2.0 bar, room temperature). The DAD was connected with CM PCS1 Chromeleon system control (Separations, H.I.-Ambacht, The Netherlands). The ELSD (gain 10) was interfaced with a C-R6A chromatopac integrator (Shimadzu, Kyoto, Japan) for data handling and chromatogram generation.

HPLC-DAD-ELSD Analysis

The extracts were analysed with several reversed-phase HPLC columns. From Alltech (Deerfield, USA) a C₁₈ (Alltima, 250×2.1 mm, 5 μ m), a C₈ (Alltima, 250×2.1 mm, 5 μ m), and a Phenyl-2 (Hypersil, 250×2.1 mm, 5 μ m) column were used. An additional C₈ column (Xterra® RP 8, 150×3.0 mm, 3.5 μ m) was obtained from Waters (Milford, USA). Solvent A consisted of 5 mM ammonium formate buffer (pH 3.0), solvent B consisted of ammonium formate buffer in ACN (20:80, 5mM) and solvent C of ammonium formate buffer in MeOH (2:98, 5 mM).

Table 2. Gradient conditions (% v/v)

A (ammonium formate buffer, 5 mM, pH 3.0), with either B (ammonium formate buffer in ACN, 20:80, 5mM) or C (ammonium formate buffer in MeOH, 2:98, 5 mM).

%A	Time (min)		
	Alltima C ₈ Alltima C ₁₈ Hypersil Phenyl (2.1 mm)	Xterra C ₈ (3.0 mm)	Alltima C ₁₈ (4.6 mm)
100	0	0	0
100	2	5	2
2	40	34	27
2	46	38	32
100	47	39	33
100	75	55	46

The LC gradients are shown in Table 2. Prior to use, the solvents were filtered over a 0.45 μ m, \varnothing 47 mm membrane filter (RC55, Schleicher & Schuell, Dassel, Germany) and degassed for 10 min in a Retsch Transsonic 570 (Emergo, Landsmeer, The Netherlands) ultrasonic bath. The flow rate was 0.2 ml min⁻¹ in all cases except for the Xterra® C₈ column where the flow rate was 0.3 ml min⁻¹. The test mixture described in Chapter 3 (containing sinigrin **89**, amygdalin **90**, gallic acid **20**, geranic acid **95**, alizarin **96**,



caffeine **92**, santonin **93**, quercetin **94**, rutin **91** and methyl salicylate **88**) was used to obtain information on differences in selectivity of the four above mentioned columns.

HPLC-MS Instrumentation

The HPLC system consisted of TSP SpectraSYSTEM including a SN4000 controller, an HPLC quaternary pump P4000, an autosampler AS3000 and a UV2000 detector. After UV detection the effluent was introduced into the MS system. A Finnigan LCQ ion trap mass spectrometer was equipped with a Finnigan electrospray ionisation (ESI) interface. Data were processed by Finnigan Xcalibur software on a Gateway computer (Thermoquest, Breda, The Netherlands).

Prior to experiments the MS was tuned using a standard solution of alizarin (25 mg/50 ml in 90% aqueous MeOH). To promote formation of ions, the standard solution was diluted once with 5% NH₃ for a negative mode (NI) tune-file or 2.5mM NH₄HCO₃ for a positive mode (PI) tune-file. The syringe (containing the alizarin with modifier) and the outlet of the HPLC were coupled to the MS interface by a T-piece. The syringe was operated at a continuous flow of 3 µl min⁻¹. During tuning, the eluent, ammonium formate buffer in ACN (20:80, 5mM) that will be used during the HPLC analysis was simultaneously added at a flow-rate of 0.2 ml min⁻¹ to simulate conditions during HPLC analysis.

HPLC-ESI-MS analysis

Separations were performed on a C₁₈ (Alltima, end-capped, 250×2.1 mm, 5µm), and a Phenyl-2 (Hypersil, end-capped, 250×2.1 mm, 5µm). The same experimental conditions used for previous experiments were used for LC-MS analysis. After HPLC separation, 5% NH₃ in water (NI) or 2.5 mM NH₄HCO₃ (PI) was added at a flow rate of 3 µl min⁻¹ by a

Table 3. MS parameters.

NI = negative ionisation mode; PI = positive ionisation mode

	NI	PI
Capillary Temperature (°C)	200	
Sheath gas	96	
Auxiliary gas	51	
Source voltage (kV)	2.5	4.5
Capillary voltage (V)	-22	31
Tube Lens offset (V)	-10	25
1 st Multipole offset (V)	7.5	-3
2 nd Multipole offset (V)	8.5	-6.5
Inter Multipole Lens Voltage (V)	14	-24

syringe which was connected by a T-piece. All extracts were analysed in positive and negative mode. The MS parameters are given in Table 3. ESI spectra were acquired in the range of *m/z* 50-1000.

MS-MS experiments

During MS² experiments helium was used as collision gas. Only a single parent ion (base peak) was kept in resonance (isolation width 2.00 *m/z*), all other ions were ejected from the trap without mass analysis. The parent ion was agitated and allowed to fragment by collision-induced dissociation (CID). The collision energy was 35%.

LC-NMR Instrumentation

The HPLC consisted of a Bruker LC22 solvent delivery pump and a Bruker DAD UV detector (Bruker BioSpin, Rheinstetten, Germany). The sample was injected using a Rheodyne model 7725i injection valve equipped with a 100 µL injection loop (Cotati, USA). A Bruker/Spark Prospekt 2 Solid Phase Extraction Unit (Bruker BioSpin, Rheinstetten, Germany & Spark, Emmen, the Netherlands) was used to manually trap peak **B** on a Hysphere[®] SH cartridge (ca. 13 mg, 10 × 2 mm i.d., 15-25 µm, Spark, Emmen, The Netherlands). Post-column addition of aqueous formic acid solution (0.1% v/v) using a HPLC pump (1.0 ml min⁻¹, D-14162, Knauer, Berlin, Germany) ensured retention of peak **B** by lowering the elution strength of the effluent. The trapped peaks were dried with dry nitrogen gas and eluted with deuterated methanol into a Bruker DPX-400 NMR spectrometer equipped with a 4 mm LC SEI ¹³C ¹H probe head with an active volume of 120 µL from Bruker BioSpin (Rheinstetten, Germany).

LC-NMR Analysis

Separation of *Daphnia* water extracts (20.000x, 100 µl, V,VII,IX,) was performed on a C₁₈ column (Alltima, end-capped, 250×4.6 mm, 5µm). The flow rate was 1.0 ml min⁻¹. The same experimental conditions used for previous experiments were used for LC-NMR analysis, only the gradient differed (see Table 2). Peak **B**, from multiple HPLC runs (8x), was trapped on one cartridge using the multi-trap method. After residual solvents were removed by drying with nitrogen gas the analyte was transferred to the NMR probe head with CD₃OD (540 µl) and 1-D ¹H NMR was performed.

GC-MS Instrumentation

The extracts were analysed using a Hewlett-Packard (HP, Waldbronn, Germany) Series 6890 gas chromatograph combined with a HP MSD Series 5973 mass spectrometer and HP Chemstation Series G1701C version A03.00 software. The MSD fitted with a quadrupole mass filter was used in full scan mode (Electron impact) mode and operated under 70 eV with a scan range *m/z* 40-550.

GC-MS Analysis

Chromatographic separation was performed using a DB-5 capillary column (5%-Phenyl-methylpolysiloxane, 30 m × 0.25 mm, 0.25 µm film thickness, J&W Scientific, Folsom, California). Helium was used as the carrier gas with an initial inlet pressure of 1.4 bar, constant flow of 1 ml min⁻¹ and linear velocity of 36 cm s⁻¹. The initial column temperature was 50°C. The temperature was raised at a rate of 10°C min⁻¹ to 300°C and held for 5 min. The operating temperature of the injector was 250°C. The injection



volume was 1 μL with a split ratio of 50:1, 10:1 or splitless. All data was not only analysed manually, but also with metAlign-software (β -version, developed by Dr. A. Lommen, RIKILT, Wageningen, the Netherlands). Identification of interesting peaks was performed by searching the combined Wiley/NIST library (7th edition, 360.000 entries).

HRMS

Interesting trapped peaks were analysed with a Finnagan Mat 95 spectrometer. EI mode was operated under 70 eV with a scan range m/z 24-900. A temperature program was run from 50 °C to 360 °C.

Sample pre-treatment

The colony-inducing activity was enriched from *Daphnia* incubation water (20 L) and WC medium (20 L) by solid-phase extraction (SPE) using an extraction unit. This extraction unit was manufactured by Glasinstrumentmakerij (Wageningen) from a ready-made glass fibre filter (\varnothing 10 cm, pore size 10-16 μm , Schott). A glass lid (fastened with a metal clamp) was made with a screw thread on top to add a piece of PTFE tubing. A commercially available SPE sorbent (100 g, Isolute[®] C₁₈, end-capped, , 60 Å, 40-70 μm , IST, Wageningen, the Netherlands) was added to the unit (bed height was approximately 1 cm).

First, the unit was conditioned by passing through MeOH (1 L) and ammonium formate buffer (10 mM, pH=3, 1 L), then the *Daphnia* test water (20 L) or WC medium (20 L) was applied. Vacuum was obtained from a vacuum-pumping unit (700 mbar, PC 5, Vacuubrand, Wertheim, Germany). Approximately 30 ml *Daphnia* test water was led over the sorbent bed per minute. Afterwards, the loaded cartridge was washed with the above mentioned ammonium formate buffer (500 ml) followed by elution with an increasing percentage of aqueous methanol, subsequently 50% (300 ml), 90% (300 ml) and 100% MeOH (300 ml). All fractions were evaporated separately to dryness and resuspended in elution solvent, (1.0 ml of 50%, 90% or 100% MeOH, 20.000x concentrated), followed by filtration (PVDF, 13 mm, 0.2 μm , Alltech, Deerfield, USA). After drying the 90% fraction of extract V weighed approximately 26 mg. Prior to analysis by HPLC the samples were diluted once with the appropriate elution solvent (10.000x concentrated) and the extracts were diluted 1:4 for LC-MS analysis (4.000 x concentrated). However for analysis by GC-MS the original 20.000x concentrated extracts were dried and derivatised. For derivatisation 100 μL DMF and 100 μL of BSTFA/TMCS were added to the dried extract in a 1.2 ml vial. The vial was kept at room temperature for 1 hour (20 °C) and subsequently 1 μL was injected into the GC-MS. Next the same vial was heated at 120 °C for 45 minutes in an oven, cooled down, after which again 1 μL was injected into the GC-MS.

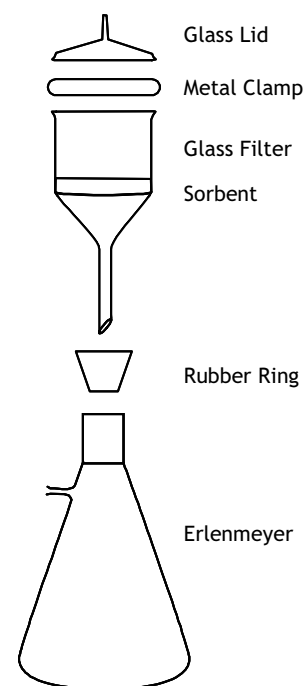


Figure 1. Experimental design of the extraction unit

Results and Discussion

Bioassay

Fortunately after the performance of all HPLC experiments, the bioassay became once more available and the 90% aqueous MeOH extracts (IV-IX) were tested for activity in this bioassay (see Fig. 2). The detergent (see Chapter 1), FFD-6, was used as a positive control. Although the extracts did not reach the same level of activity as FFD-6, they were significantly different from all controls. Only extract IX did not differ significantly from one control (VI). This bioassay was verification that all experiments described in this chapter were indeed performed with active *Daphnia* water.

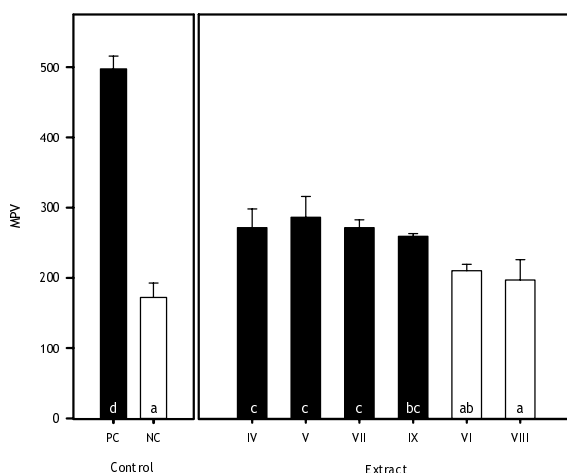


Figure 2. *Daphnia* water extracts

Activity of 90% aqueous MeOH extracts in the *Scenedesmus* bioassay obtained by solid-phase extraction with C_{18} , expressed as mean particle volume (MPV) and cells per colony (CPC). The experiment was performed with biologically active (water incubated with *Daphnia*, ■) and inactive water (water incubated without *Daphnia*, □). Similar symbols (a-d) represent homogenous groups at the 95% level (Tukey test). Error bars indicate standard deviation ($n=3$).

Characterisation of HPLC columns

From the behaviour of the compounds in the test mixture (see Chapter 3) it is obvious that both Alltima columns (C_{18} and C_8) differ the least and show comparable patterns (Table 4). The Alltima C_{18} shows a slightly better resolution as no compounds coelute. The Phenyl column shows a similar picture, however more peaks coelute, and some peak shapes are not as good as on the C_{18} column (especially for rutin) and quercetin was not detected at all. The characteristics of phenyl bonded columns are similar to those of a C_8 stationary phase, however the selectivity is different. Hypersil Phenyl columns offer 'alternative' selectivity compared to alkyl chain phases due to π - π interactions from the benzene ring, which makes them a good choice for compounds typically considered difficult to analyse⁸. Most different from the other columns is the Xterra[®] C_8 . Xterra columns are based on a different particle that combines features of silica and polymeric



chromatographic supports applicable over a mobile phase pH range of 2-12. All compounds were separated (except for quercetin and methyl salicylate) and detected, albeit in a different order from the other columns.

The differences between Xterra C₈ and Hypersil Phenyl on the one hand and C₈ and C₁₈ on the other hand are more extreme when a MeOH-H₂O gradient is used. When the same gradient program was used that was used for ACN-H₂O gradient, geranic acid **95** and alizarin **96** could not be eluted from the C₁₈ and C₈ columns. The compounds were then eluted in the next run. This problem could be solved by increasing the isocratic hold of 98% MeOH at the end of the program. However, on the basis of a generally better peak shape the ACN-H₂O gradient was chosen for analysis of *Daphnia* water and control water extracts.

Table 4. Elution order of compounds present in the test mixture used in Chapter 3 on four different columns with an ACN-H₂O or MeOH-H₂O gradient.

C₁₈ = Alltima C₁₈; C₈ = Alltima C₈; Ph = Hypersil Phenyl-2, XC₈ = Xterra® C₈

		ACN				MeOH			
		C ₁₈	C ₈	Ph	XC ₈	C ₁₈	C ₈	Ph	XC ₈
89	Sinigrin	1	1	1	1	1	1	1	1
20	Gallic acid	2	2	2	2	2	2	2	2
90	Amygdalin	3	3	3	4	3	3	3	3
92	Caffeine	4	3	3	3	4	4	4	3
91	Rutin	5	5	5	5	5	5	5	6
94	Quercetin	6	6	nd	7	7	6	6	8
93	Santonin	7	7	7	6	6	6	8	5
96	Alizarin	8	8	8	9	nd	nd	10	10
88	Methyl salicylate	9	9	8	7	8	8	7	7
95	Geranic acid	10	10	10	10	nd	nd	10	8

nd = not detected

HPLC-DAD

First all extracts were analysed on the abovementioned four different HPLC columns. When the chromatographic profile obtained with C₁₈ (Fig. 3) was compared with the profiles obtained on C₁₈ by Von Elert and Fink^{9,10}, they looked very different. Their traces contained almost no peaks, whereas here quite a few peaks can be observed. This could be due to the smaller enrichment factor of the extracts in the experiment of von Elert and Fink (respectively unknown and 1000x)^{9,10}. For each column UV spectra were obtained for all peaks in the traces of all extracts. These spectra were then compared for each column to identify peaks that were present in more than one extract. Next peaks were disregarded if they were also present in the control extracts (VI and VIII) or were present in less than three *Daphnia* test water extracts. Finally results were pooled to obtain peaks that could be recognised in chromatograms of all four columns. Peaks were recognised

and labelled foremost on the basis of their UV spectrum. Additionally peaks were identified on the basis of retention (especially while comparing C_{18} and C_8 chromatograms). This was less useful for the phenyl and Xterra C_8 as they showed a different selectivity (see Table 4). For instance, phenyl bonded columns are known to be more retentive for aromatic compounds. Thirdly, the areas of the compounds showed a repeatable pattern between columns (Fig. 3). The UV spectrum, the presence and quantity per column of these candidate peaks are given in Figure 3. Due to the different dimension of the Xterra column, the obtained areas were smaller than for the other three columns. Peaks (A-C, E-H, J-L) that satisfy all these criteria are shown in Figure 4. Since the phenyl and Xterra C_8 columns were more different than the more traditional C_{18} and C_8 , some peaks could not be identified from the UV traces of these columns, as retention

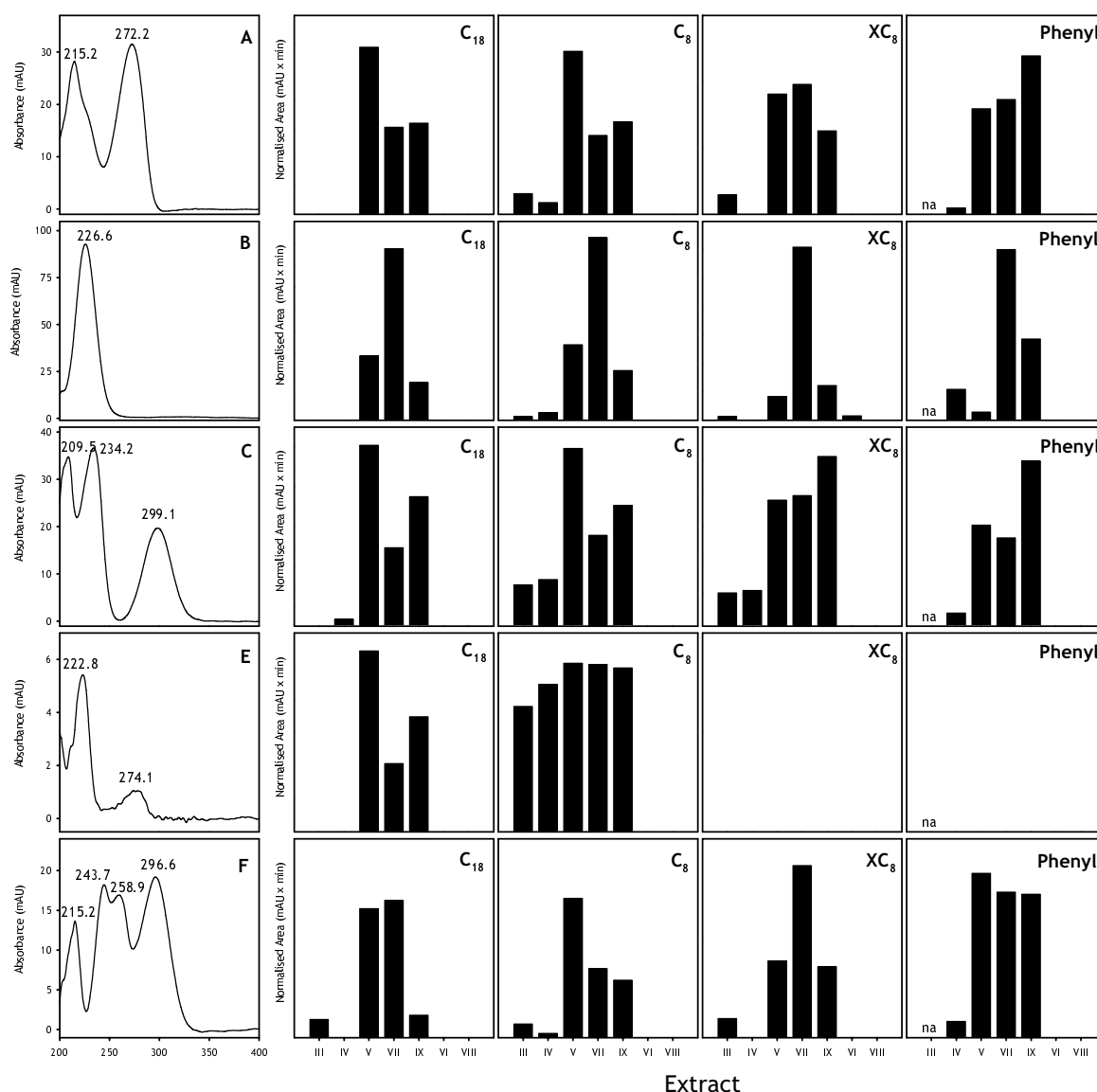


Figure 3. Spectra, presence and quantity of possible candidates responsible for the colony formation activity. Empty places in the bar diagrams indicate peaks that were not detected in HPLC profiles of a certain column.

na = not analysed; III-V, VII, IX = *Daphnia* test water extracts; VI, VIII = Control water Extracts; C_{18} = Alltima C_{18} ; C_8 = Alltima C_8 ; Phenyl = Hypersil Phenyl-2, XC_8 = Xterra® C_8

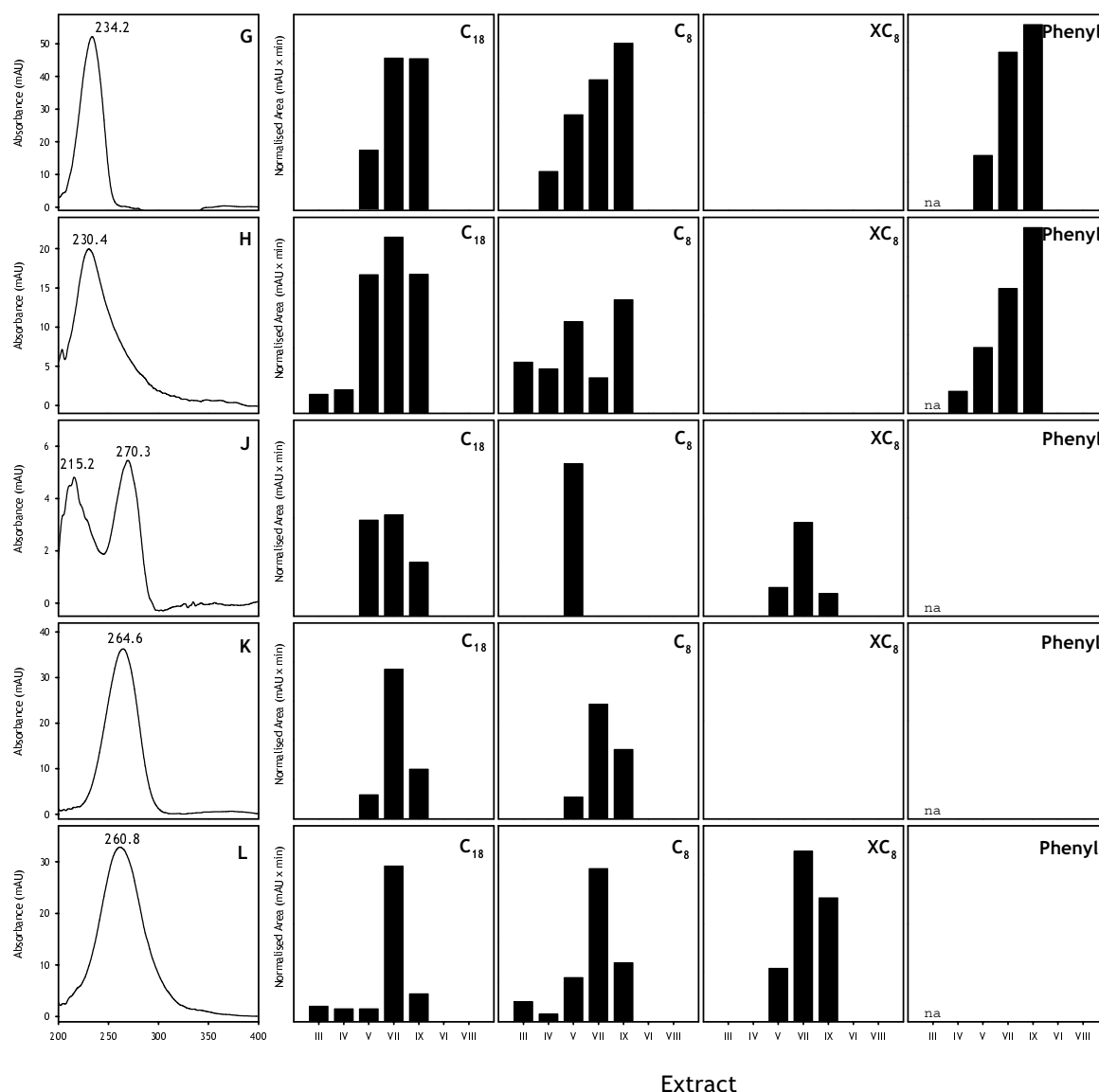


Figure 3. Continued

behaviour could not be predicted as easily as between C_8 and C_{18} . Additionally, some peaks coelute, which can sometimes be observed in the UV spectra. As mentioned before all extracts were also analysed with a MeOH- H_2O gradient. The resulting chromatograms are given in Figure 5. Especially larger peaks with clear UV spectra were identifiable. On the whole, peaks were less sharp and smaller, though the same amount of sample was injected every time. Therefore less peaks could be positively recognised and linked to peaks in chromatograms obtained with the other columns. Peak **B** that was recognised as interesting in the traces obtained with an ACN- H_2O gradient, is also identified in the MeOH- H_2O gradient traces of all four columns. Clearly a gradient with ACN and H_2O is better for the analysis of extracts of *Daphnia* water.

In Chapter 4, a region from HPLC fractionation was identified that showed significant colony formation activity in the bioassay. This fraction, 'fraction C', was collected from 42 to 69% of an ACN- H_2O gradient. This fraction roughly corresponds with the region of

33.7 to 46.8 minutes. Interestingly most identified peaks fall outside this region except peaks **B**, **G** and **K**. Not only that but peak **G** corresponds with another significantly biologically active region, namely, 'C2'. Peak **K** is found in region 'C3' and peak **B** in 'C6' (see Chapter 4). Region 'C2' corresponds with the ACN-H₂O gradient of 46 to 50% ACN (35.6 to 37.5 minutes), region 'C3' with the ACN-H₂O gradient of 50 to 54% ACN (37.5 to 39.3 minutes) and region 'C6' with the ACN-H₂O gradient of 65 to 69% ACN (44.9 to 46.8 minutes). It has to be kept in mind though, that 'fraction C' was determined and identified in a different experiment with a different batch of *Daphnia* test water. The presence of some biological variation can therefore not be excluded.

HPLC-ELSD

The information that has been collected over recent years on the nature of the compound which causes *Scenedesmus* to colonise is very limited and does not include specific knowledge on UV absorbance⁹⁻¹². Therefore, the fact that the compound possibly does not contain a UV chromophore and can consequently not be detected by a UV detector cannot be disregarded. Experiments from Chapter 4 rejected that the possibility that the compound responsible for colony formation had a volatile nature, thus analysis by ELSD became an option. All extracts were therefore analysed with a DAD and an ELSD. These detectors were placed in line where the waste from the non-destructive DAD was directly pumped into the ELSD¹³. The chromatograms obtained with the ELSD and four different columns are shown in Figure 6. Although some peaks can be assigned by comparison with the UV trace (Fig. 4) many smaller peaks are lost in the noise of the ELSD trace (i.e. **A**, **E**, **F**, **L** and **K**). The peak, labelled **H**, shows the added value of additional detection with an ELSD, because it was hardly detected with the DAD, however shows up large in the ELSD trace of all four columns. Possibly this compound has a chromophore with a low extinction coefficient, comparable to amygdalin. The ELSD traces obtained with the MeOH-H₂O gradient were of less quality than those obtained with the ACN-H₂O gradient. No extra information could be gained from these chromatograms. Even though the sensitivity of the ELSD was set very high, few peaks were detected. Generally sensitivity of an ELSD is lower than for a UV detector¹⁴.

LC-MS

By comparison with the chromatogram profiles obtained on the HPLC-DAD an attempt was made to identify corresponding peaks in the NI and PI modes of the LC-MS (Fig. 7). This approach was only partly successful. Generally speaking PI showed higher abundances, however peak patterns were not readily comparable with HPLC-DAD chromatograms. In contrast, NI chromatograms had a lower abundance but showed more detail and peaks in the profiles. Peaks **B**, **F** and **H** could only be identified in the NI profiles of the C₁₈ and phenyl columns, although peak **B** coeluted with another peak on the phenyl column. Peak **B** gave a main ion at *m/z* 752.8, peak **F** at *m/z* 933.7 and peak **H** at *m/z* 223.2. A natural products database (Dictionary of Natural Products, version 11:2, Chapman & Hall, CRC Press) was used to attempt identification of these peaks but possible candidates with the correct mass and UV spectrum were not found. Parent and daughter ion spectra for peaks

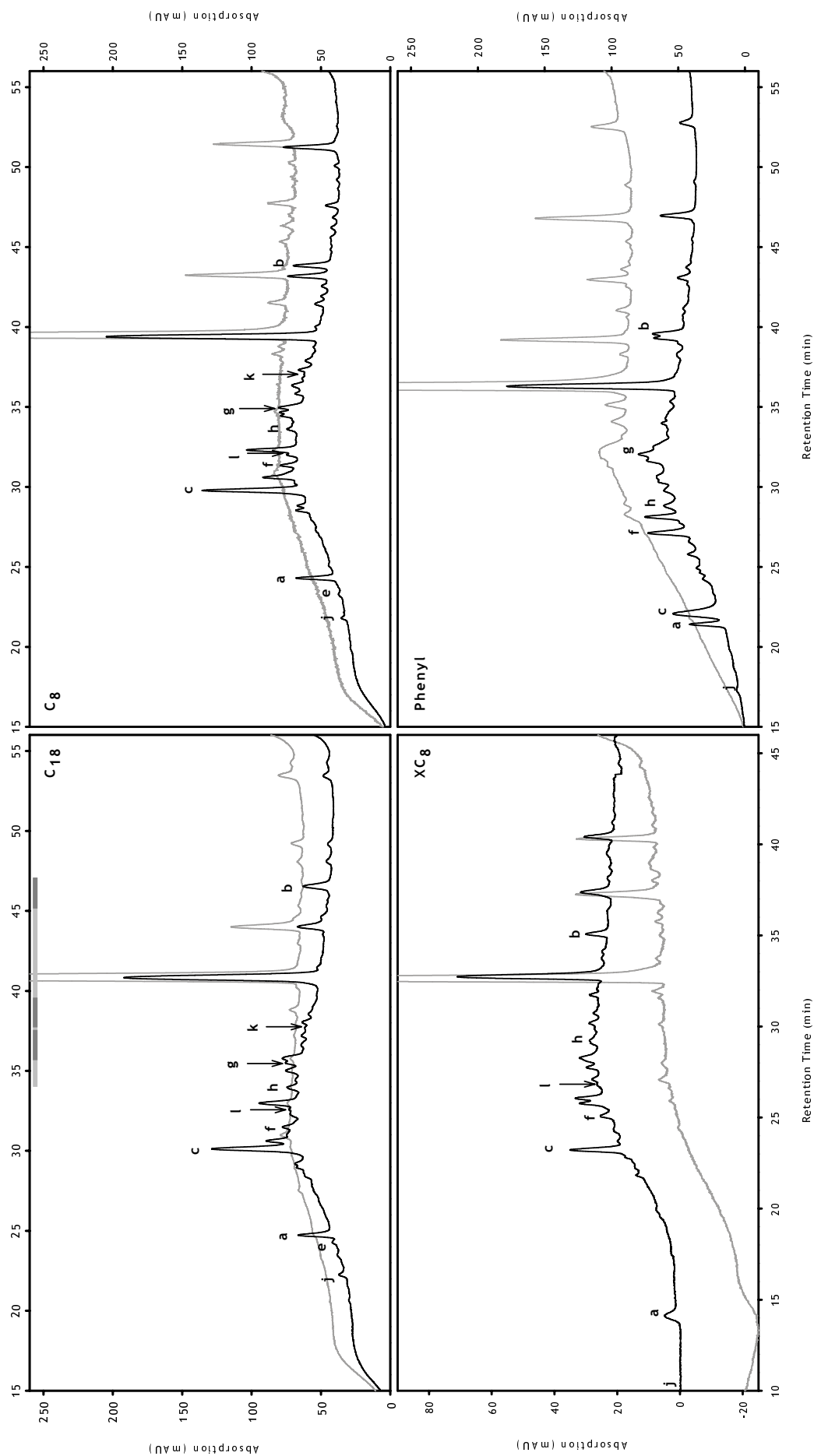


Figure 4. HPLC profiles of *Daphnia* test water extract V (—) and control water extract VI (—, trace is in another scale) analysed on four different columns at 210 nm with an ACN-H₂O gradient. In the profile of the C₁₈ column the approximate location of 'Fraction C' (■) and Fractions C2, C3 and C6 (■) are indicated. C₁₈ = Alltima C₁₈; C₈ = Alltima C₈; Phenyl = Hypersil Phenyl-2, XC₈ = Xterra® C₈

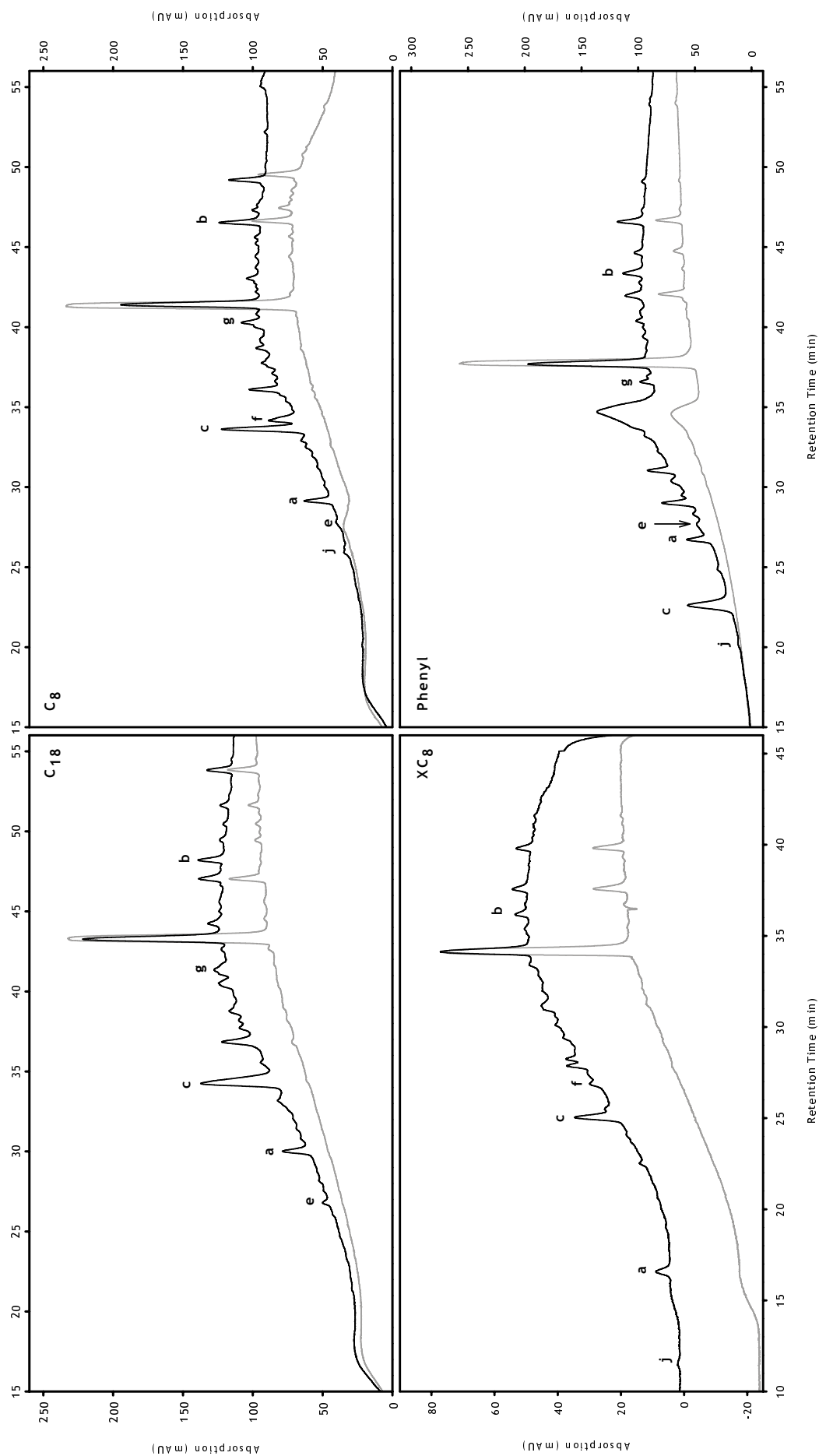


Figure 5. HPLC profiles of *Daphnia* test water extract V (—) and control water extract VI (---, trace is in another scale) analysed on four different columns at 210 nm with an MeOH-H₂O gradient. C₁₈ = Alltima C₁₈; C₈ = Alltima C₈; Phenyl = Hypersil Phenyl-2, XC₈ = Xterra® C₈

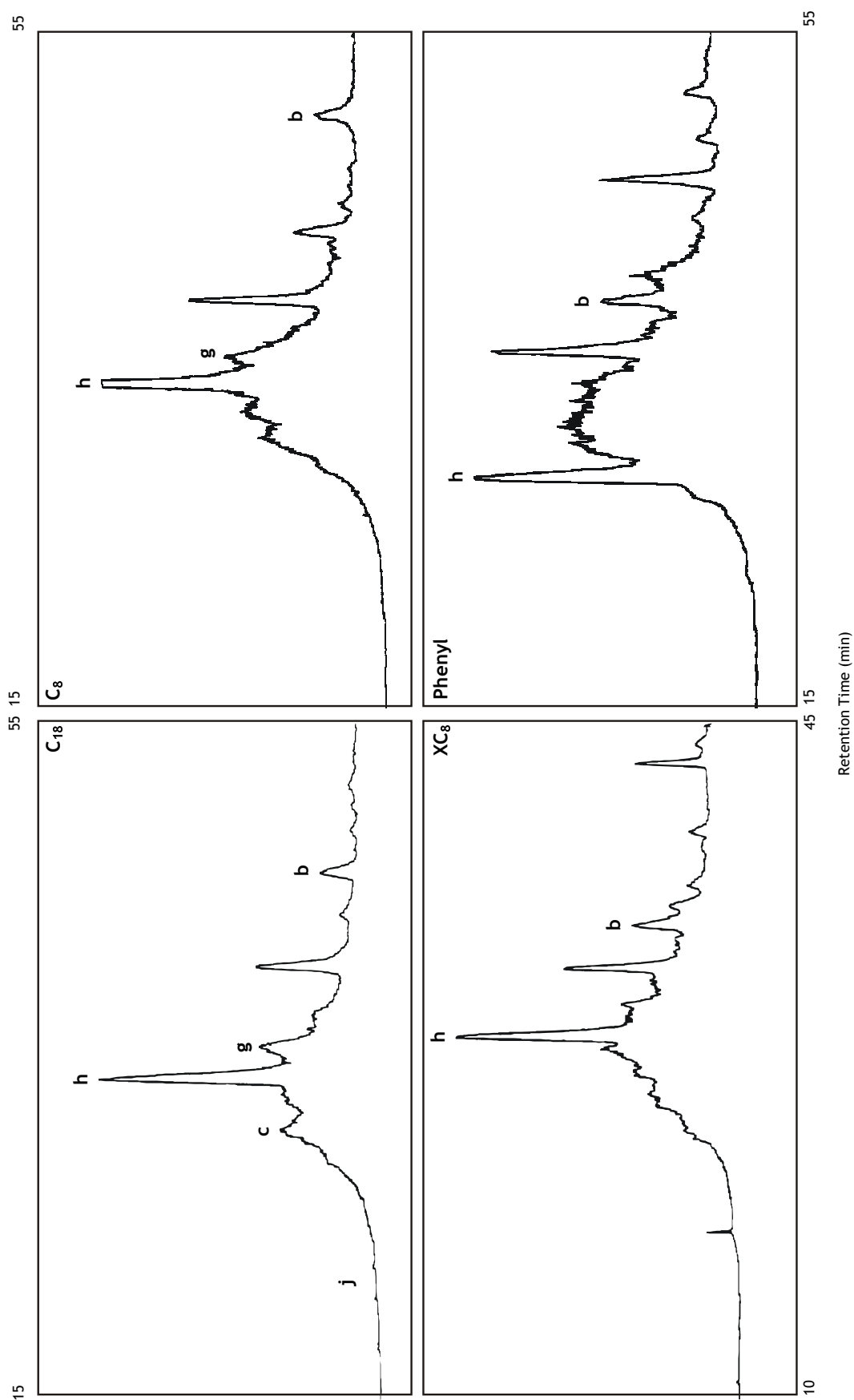


Figure 6. HPLC-ELSD profiles of *Daphnia* test water extract V analysed on four different columns with an ACN- H_2O gradient.
 C₁₈ = Alltima C₁₈, C₈ = Alltima C₈, Phenyl = Hypersil Phenyl-2, XC₈ = Xterra® C₈

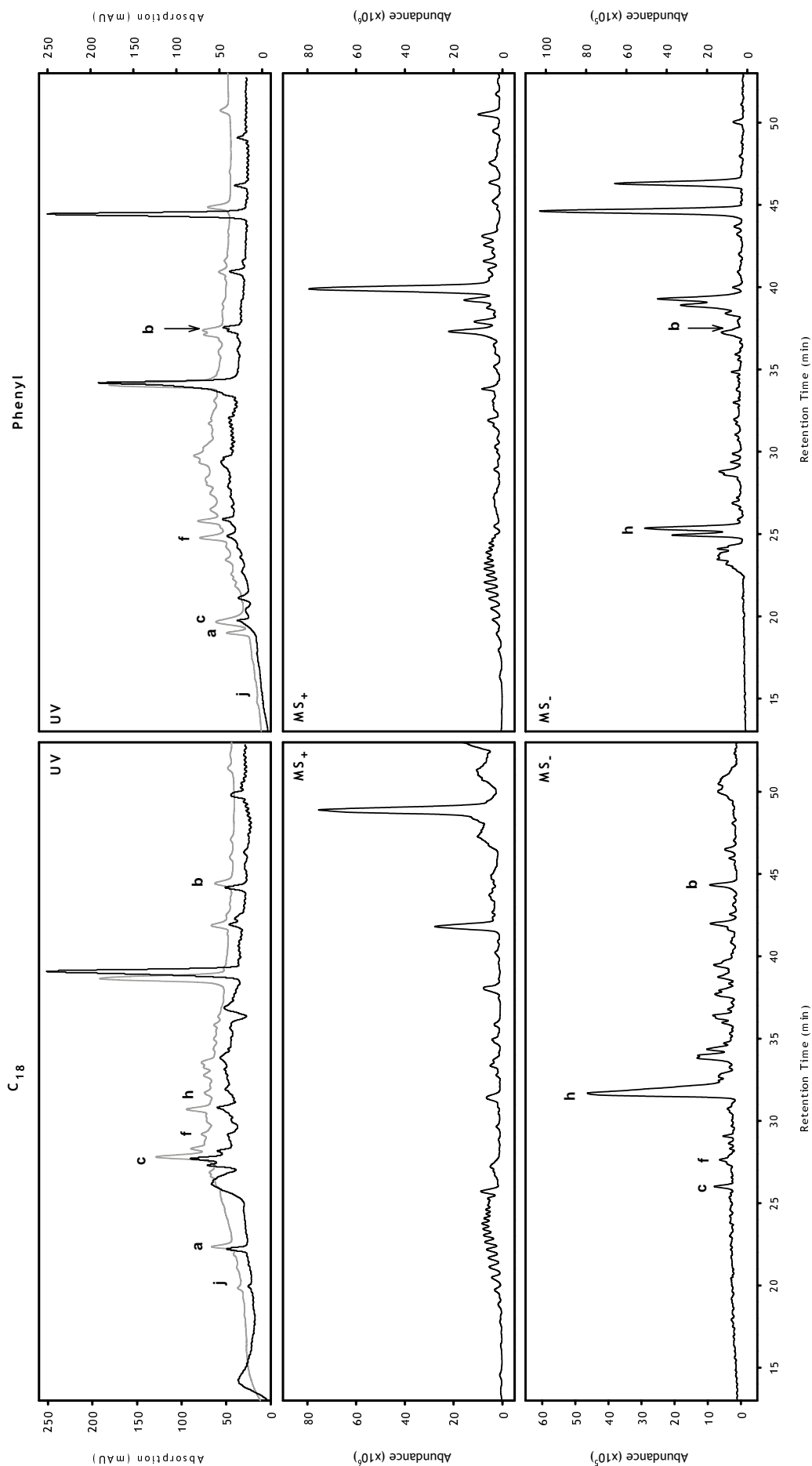


Figure 7. LC-MS profiles of *Daphnia* test water extract V analysed with UV (UV_{HPLC}, —) and UV_{LC-MS} (—), NI and PI mode on two different columns (i.e. an Alltima C₁₈ and a Hypersil Phenyl).



B, **F** and **H** are given in Figure 8. Of these three, only peak **B** was found in the active region 'Fraction C' (see Chapter 4). It is unclear whether the main ions mentioned before are in fact pseudo molecular ion peaks. The assignment of a molecular ion can be

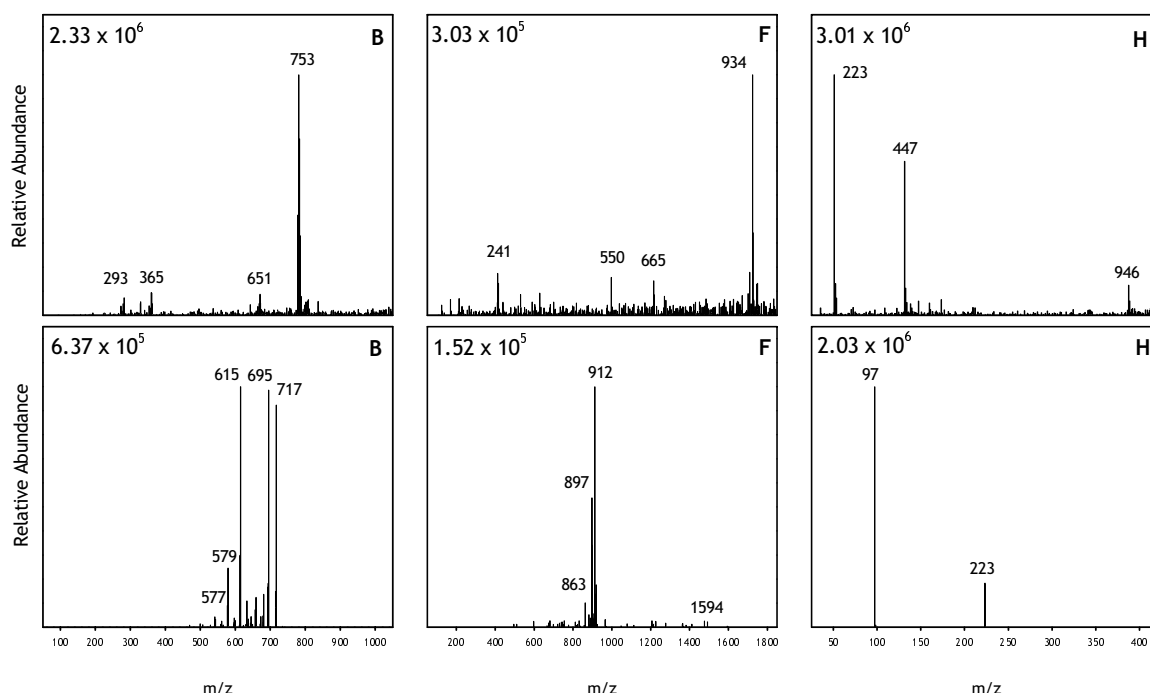


Figure 8. Mass spectra of peaks **B**, **F** and **H**.

Parent ion spectrum (upper panel) and the CID (35%) daughter ion spectrum of the base peak from the parent ion spectrum (lower panel). Spectra were obtained with ESI in NI mode.

checked with the nearest fragment ions (for example peaks at m/z of $[M-1]$, $[M-2]$, $[M-15]$ and $[M-18]$). In the case of peak **B** these appear to be absent, whereas losses of 3-14 Da and 21-25 Da that indicate impurities or an incorrect assignment of the molecular peak appear to be absent as well¹⁵. For peak **F** the nearest fragment ions are absent, but an important fragment can be observed at -22. It is unclear whether this is a contaminant or possibly an $[M-Na]^-$ -fragment. This unusual fragment for negative mode has been reported before¹⁶⁻¹⁸ and could be indicative of a -COOH or -SO₃H group. Furthermore the CID spectrum is very reproducible between different extracts of *Daphnia* test water, decreasing the probability of an impurity. Unfortunately CID spectra are not comparable to spectra obtained by EI and therefore cannot be identified using the available EI libraries¹⁹. However since LC-MS is still a relatively young technique, probably CID libraries will become available in years to come, although the mass spectra are so much determined by experimental conditions that universal libraries might not be feasible¹⁵. Although an MS-detector is often termed universal, this is not true, since some compounds will not ionise at all, while others will only be visible in PI or NI mode. Lampert et al. reported that the colony inducing compound is smaller than 500 Da¹². That would imply that peaks **B** and **F** could not be responsible for the colony inducing activity of this compound, but membrane ultrafiltration with a molecular weight cut-off of 500 Da was used to obtain this value. The nominal molecular weight cut-off of an ultrafiltration



membrane does not refer to its absolute retention, but to an arbitrarily selected point on the retentivity scale. Some larger compounds can also pass the membrane, especially if they are more linear in shape. Other factors that can play a role are configuration, lot-to-lot variability and type of membrane material²⁰. Other peaks (**A**, **C**, **G**) could be found in the UV trace obtained with the LC-MS analysis, but unfortunately injected material or ionisation was not sufficient to discover these peaks in the NI and PI profiles. Peak **C** can be found in the NI mode profile of the C₁₈ column but coelutes with some other compounds. It was impossible to determine which ion belonged to peak **C**. Peaks **E**, **J**, **K** and **L** could not even be recognised in the UV trace obtained with the LC-MS. Interestingly the profiles of both columns in PI mode undulate strongly between 20 and 25 minutes. The mass spectrum shows a Gaussian curve with mass increments of 44 (possibly PEG, a known detergent²¹). Peak **A** falls within this region.

Forensic sciences deal with similar problems as described in this chapter. Van Boxlaer et al. stated that LC-MS may be less suited in profiling analysis of unknowns²², but the development of improved LC-MS probes, techniques and methods continues. Some groups are making progress with the development of a 'general unknown screening' (GUS) method using a single-quadrupole instrument^{23,24}, although it still has some disadvantages. Another group has developed a GUS method for a quadrupole time-of-flight (Q-TOF) instrument²⁵. This method was able to simultaneously obtain qualitative and quantitative data as well as product ion spectra in a single acquisition. The automatic function switching ability of the Q-TOF could become an important tool in the identification and quantification of unknown components (either forensic screening, elucidation of biologically active compounds or metabolomics).

LC-NMR

This technique still has several drawbacks, such as its insensitivity compared to UV or MS detection, the need for solvent suppression and the inability to obtain ¹³C-NMR spectra without a reasonable amount of sample¹⁹. Recently the introduction of SPE cartridges within the LC-NMR system has solved some of these problems²⁶⁻²⁸. The use of SPE cartridges can enable the trapping of higher amounts of the analyte of interest and makes solvent replacement possible prior to introduction into the NMR²⁶.

Now that some peaks unique for extracts of *Daphnia* test water have been recognised, analysis by LC-NMR could provide more detailed structural information of the compound in question. Especially peak **B** was suitable for this purpose as it seemed to be present in high amounts and was well separated from neighbouring peaks. It was unclear whether the *Daphnia* water extracts could yield enough material (e.g. total 90% extract of V weighed ca. 26 mg) for NMR analysis. The relative peak area of peak **B** (in extract V) was only 1.2% (ca. 0.31 mg) but during LC-MS and HPLC-DAD-ELSD experiments some material was lost. To increase the amount of peak **B** that was transferred to the NMR, multiple runs (8) were trapped on the same cartridge (Figure 9). This was possible because peak **B** eluted relatively late from the column and has therefore a non-polar nature. More care has to be taken when trying to multi-trap compounds that elute earlier from the column. The obtained NMR spectrum unfortunately showed that the peak was not completely



pure. Large signals at 4.8, 3.3 and 2.0 ppm indicate HDO, CD₂HOD, and CHD₂CN respectively. Although the sample was transferred to the probe with CD₃OD, residual acetonitrile from the gradient, that was also trapped, was also eluted to the probe. Very small signals were observed between 8.2 and 5.0 ppm. In this region signals normally indicate the presence of an olefinic and aromatic group. In that case these signals are

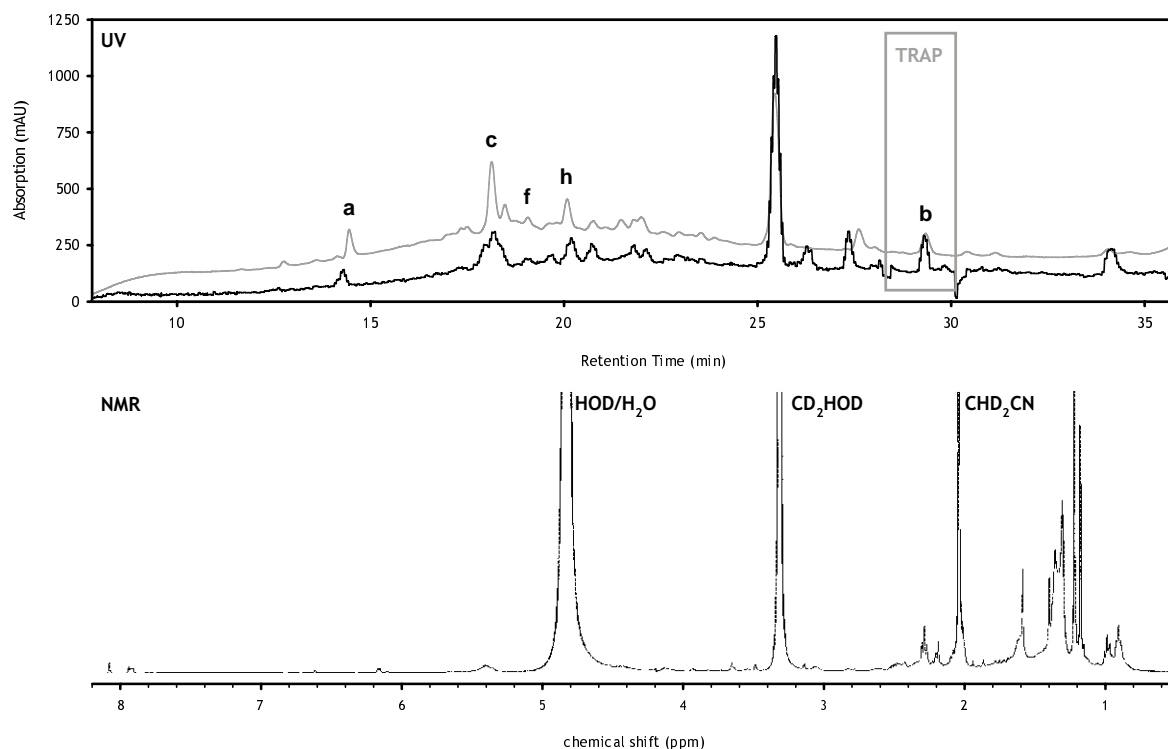


Figure 9. LC-NMR chromatogram and spectrum

Upper panel: UV (210 nm) profiles of *Daphnia* test water extract V obtained with the normal HPLC system (—) and the LC-NMR system (---). Lower panel: 400 MHz ¹H NMR spectrum of peak B after multiple trapping (8x) on the same SPE cartridge obtained after 2000 scans. The signals at 4.84, 3.31 and 2.02 indicate respectively H₂O, MeOH and ACN.

part of a contaminant as the UV spectrum of peak B has a λ_{max} of 227 nm. Aromatics would show an absorption at higher wavelengths. If these weak signals indicate something else (double bonds) then they could be part of the spectrum for peak B, but that remains unclear. Between 2.3 and 0.9 ppm some very strong signals were detected, but they did not lead to a successful interpretation of the NMR spectrum. The high mass that was detected with the LC-MS (NI, $[M-H]^-$? 752.8) indicates a relatively large molecule. Even a steroid like ecdysone (moulting hormone in *Crustaceae*) only has a molecular weight of 464.6, but the NMR spectrum does resemble the observed spectrum of peak B slightly²⁹ and the NMR spectrum of a more non-polar steroid (such as stigmasterol³⁰) resembles it somewhat better. The area between 5.0 and 2.3 ppm is empty, therefore this compound is not a glycoside.

Bioassay peak B

After analysis by LC-NMR, peak B was pumped out of the probe in CD₃OD (ca. 540 μ l) and

collected. An aliquot of this sample was dried and taken up in ultrapure water. Then the sample was tested in a bioassay for biological activity (see Fig. 10).

The detergent (see Chapter 1), FFD-6, was used as a positive control. Although peak **B** did not reach the same level of activity as FFD-6, it was significantly different from the negative control. Furthermore when the results are compared with Figure 2, it is clear that no biological activity was lost during HPLC experiments with the SPE extracts. The MPV from peak **B** has a similar value as the MPVs from the SPE extracts (Fig. 2). Therefore peak **B** could be (partly) responsible for the colony formation in *Scenedesmus*.

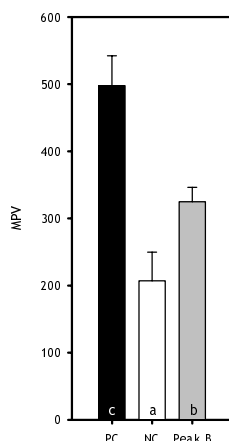


Figure 10. Peak **B**

Colony formation activity of peak **B** (■) expressed as mean particle volume (μm^3). Positive control (FFD-6, ■) and negative control (□). Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).

Furthermore, an aliquot was sent to a separate lab (Institute for Limnology, University of Konstanz) to test the sample independently with a second bioassay. Peak **B** was tested at two different concentrations but both proved to be incapable to induce colony formation in this bioassay ($P = 0.144$). Though the observed P-value is not statistically significant it is low enough to carefully argue there is a tendency for increased particle size in the sample of peak **B**. Although *Daphnia* test water and the positive control (FFD-6) were active, these responses were not very big, which could have influenced the outcome of the bioassay at Konstanz.

GC-MS

Additionally all extracts were analysed by a GC-MS. Previous research and results described in Chapter 4 have led to the conclusion that the active compound is most likely not a volatile compound. Therefore the extracts were silylated before injection into the GC-MS. Quite detailed chromatograms were obtained (Figure 11).

First all chromatographic profiles of the 90% MeOH extracts of active *Daphnia* test water were compared with profiles of controls. Peaks were rejected that occurred in both profiles. After this manual inspection of the 90% MeOH extracts, small peaks were found

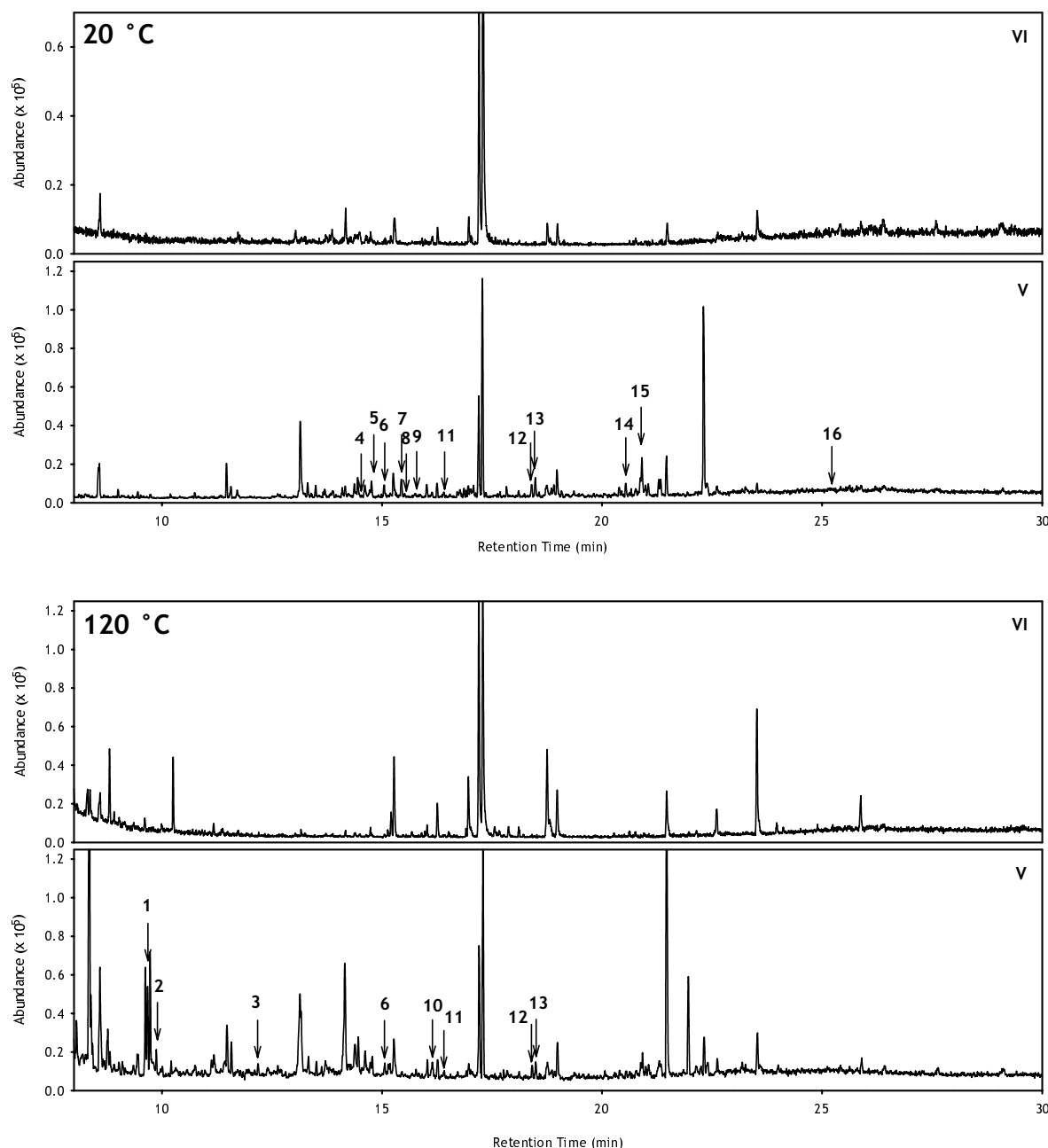


Figure 11. GC-MS profiles of silylated *Daphnia* test water extract V and control water extract VI with (120 °C) and without (20 °C) heating.

that were present only in the *Daphnia* test water extracts and not in the controls. Furthermore, peaks were discarded that were found in two or less extracts. Then only a small number of peaks were left that were present in three or more of the *Daphnia* test water extracts (Table 5).

At first a split ratio of 50:1 was used and although very large peaks come of the column during the first ten minutes, after that peaks were very small. The NIST and Wiley libraries were used to search for identifiable compounds within the *Daphnia* test water extracts. Although the libraries did come up with some hits, several spectra were obtained from very small peaks and consequently were just too weak to find a reliable



Table 5. Spectra, presence and quantity of peaks unique to *Daphnia* test water extracts silylated with BSTFA-TMCS at 20 °C

<i>Daphnia</i> test water						
	III	IV	V	VII	IX	<i>m/z</i>
4	●	●	tr	tr	tr	73 (100), 75 (57), 44 (30), 147 (21), 223 (18), 149 (16), 221 (15), 131 (14), 117 (14)
5 Azelaic acid	●	●	●	●	●	73 (100), 75 (64), 55 (27), 147 (25), 117 (20), 129 (19), 201 (11), 204 (5), 217 (5), 152 (5), 317 (3)
6 Phthalate	●	●	●	tr	tr	73 (100), 75 (96), 223 (43), 149 (24), 44 (21), 221 (18), 147 (18), 163 (11), 117 (8)
7 Veratroyl formic acid	●	tr	●	tr	●	73 (100), 165 (76), 75 (50), 147(22), 282 (14), 267 (9), 193 (5), 234 (2)
8	●	●	tr		tr	73 (100), 242 (66), 75 (37), 270 (20), 135 (16), 45 (16), 59 (12), 147 (11)
9 Sebacic Acid	●	●	tr	tr	●	73 (100), 75 (55), 147 (24), 55 (22), 129 (19), 117 (17), 331 (7), 93 (7), 217 (5), 185 (2), 204 (2)
11 Phthalate	●	●	tr	●	●	149 (100), 73 (55), 75 (32), 44 (17), 57 (8), 129 (7), 103 (6), 205 (2), 223 (1)
12			●	●	●	73 (100), 75 (28), 147 (26), 129 (20), 95 (15), 103 (14), 171 (12), 211 (6), 313 (6), 301 (4) , 273 (1)
13			●	●	●	73 (100), 75 (28), 147 (28), 129 (23), 171 (16), 103 (15), 93 (10), 211 (9), 313 (9), 301 (5) , 273 (2)
14			●	●	●	73 (100), 75 (87), 55 (49), 95 (30), 45 (48), 129 (47), 147 (30), 117 (29)
15		tr	●	●	●	73 (100), 75 (35), 147 (25), 129 (20), 81 (16), 55 (12), 103 (12), 204 (3), 221 (3), 317 (3), 333 (1)
16	tr	●		●	tr	73 (100), 502 (23), 282 (17), 251 (16), 75 (14), 207 (12), 57 (11), 45 (9), 147 (5), 487 (5), 117 (4), 129 (4), 519 (4), 430 (2)

tr = trace amount

Table 6. Spectra, presence and quantity of peaks unique to *Daphnia* test water extracts silylated with BSTFA-TMCS at 120 °C

<i>Daphnia</i> test water						
	III	IV	V	VII	IX	<i>m/z</i>
1	tr	●	●	●	●	73 (100), 121 (63), 44 (63), 174 (53), 268 (42), 178 (30), 57(31), 152(23), 322(11), 337 (7)
2 Aliphatic alcohol	●		●	●	●	73 (100), 97 (60), 75 (63), 103 (60), 55 (58), 83 (58), 111 (20), 126 (15)
3 Dodecanol	●	●	●	●	●	75 (100), 73 (84), 243 (55), 103 (30), 69 (22), 55 (21), 97 (18), 89 (15), 147 (14), 199 (3)
6 Phthalate	●	●	●	tr	tr	73 (100), 75 (96), 223 (43), 149 (24), 44 (21), 221 (18), 147 (18), 163 (11), 117 (8)
10 Monosaccharide	●		●	●	tr	73 (100), 204 (35), 75 (35), 147 (30), 191 (18), 217 (14), 45 (14), 117 (11)
11 Phthalate	●	●	●	●	●	149 (100), 73 (55), 75 (32), 44 (17), 57 (8), 129 (7), 103 (6), 205 (2), 223 (1)
12		tr	●	●	●	73 (100), 75 (28), 147 (26), 129 (20), 95 (15), 103 (14), 171 (12), 211 (6), 313 (6), 301 (4) , 273 (1)
13			●	●		73 (100), 75 (28), 147 (28), 129 (23), 171 (16), 103 (15), 93 (10), 211 (9), 313 (9), 301 (5) , 273 (2)

tr = trace amount



hit. Therefore replicate injections were made at a split ratio of 10:1 and splitless to improve peak height and hence the obtained mass spectra. Some peaks that at first seemed like promising candidates turned out to be derivatives of phthalic acid, a known plasticiser (peaks 6 and 11) and not likely to be the bioactive compound from *Daphnia*. Other compounds that were tentatively identified by the Wiley library were dodecanol **97**, veratroylformic acid **98**, azelaic acid **99** and sebacic acid **100** (peaks 3, 7, 5, and 9,

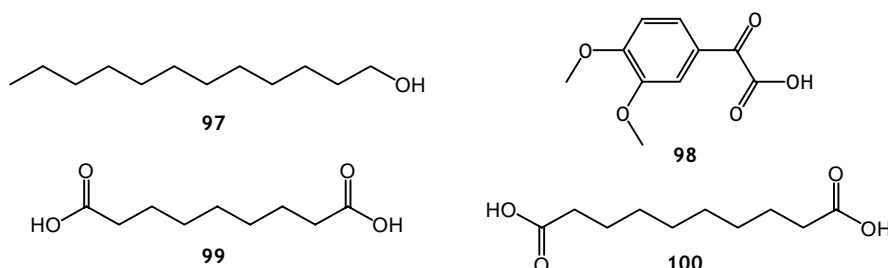


Figure 12. Structures of tentatively identified compounds extracted from *Daphnia* test water

97. Dodecanol; 98. Veratroylformic acid; 99. Azelaic Acid; 100. Sebacic acid

Fig. 12). The latter two compounds have previously been observed in cyanobacteria^{31,32}. Dodecanol **97**, azelaic acid **99** and sebacic acid **100** were tested in a bioassay and did not exhibit colony formation activity (Fig. 13). Dodecanol even had a deleterious effect on growth. However concentrations tested in the bioassay could differ from the natural concentrations and therefore misrepresent the observed MPV's. Furthermore another aliphatic alcohol, hexadecanol was reported to possess some colony formation activity and no negative impact on growth rate³³. Toxicity tests on fish indicate that hexadecanol is indeed less toxic than dodecanol³⁴.

Peak 10 showed a pattern characteristic for monosaccharides, but due to the weakness of

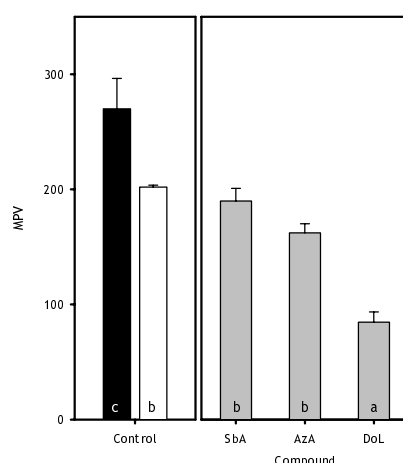


Figure 13. Sebacic acid, Azelaic acid and Dodecanol

Colony formation activity of Sebacic acid, Azelaic acid and Dodecanol (resp. SbA, AZA, DoL, ■) expressed as mean particle volume (μm^3). Positive control (FFD-6, ■) and negative control (□). Error bars represent 1 SD ($n=3$). Similar symbols (a-c) indicate homogeneous groups that are not statistically different at the 95%-level (Tukey).



the mass spectrum it was impossible to positively identify which saccharide. The pattern of fragmentation of peak 2 resembled several aliphatic alcohols, however the $[M^+]$ and $[M-15]$ fragments were missing. This could indicate a more complicated aliphatic alcohol with additional groups that interfere with the formation of $[M^+]$ and $[M-15]$ fragments.

In an attempt to speed up the identification of interesting peaks, software (metAlign) that was developed initially for peak comparisons in proteomics was tried. This software (metAlign) was developed jointly by the Institute for Food Safety (RIKILT, Wageningen) and Plant Research International (PRI, Wageningen) to assist the analysis of large amounts of chromatographic and spectral data. Originally developed for the analysis of proteomics where several datasets containing information on hundreds of compounds have to be compared to find out which biochemical processes have significantly altered between mutants and wild-type lines^{35,36}, this program is being discovered by other fields, especially chemical ecology as a tool to cut down analysis time³⁷. It filters out significant statistical differences between predefined classes of chromatograms (i.e. *Daphnia* test water versus control water).

The program runs an algorithm, which subsequently runs data smoothing, estimation of local noise, baseline correction, calculation of peak amplitudes, alignment using 'landmark peaks', iterative fine alignment and finally filtering for significant differences. It has been found to outperform other software such as Massfrontier and manual inspection with respect to precision and time³⁶, although it failed with the extracts mentioned in this chapter where differences between *Daphnia* test water extracts versus control water extracts were analysed. The program came up with only a handful of peaks that appeared to be unique to *Daphnia* test water, however upon closer examination it appeared that all were also present in the controls. This is probably due to experimental set-up where the data was split into two groups, *Daphnia* test water extracts (5 extracts) and controls (2 extracts). The active group as a whole was then compared with the inactive group as a whole and all peaks that were unique to either of the two groups were identified. As soon as a particular peak was absent from one of the extracts that made up the larger group, then the peak would not be recognised. This could account for the lack of identified peaks as some peaks can be so small they fall underneath the detection threshold. Furthermore most of these peaks were observed in the 100% MeOH extract, which is less likely to contain the colony-inducing compound. Finally, the result of the metAlign software could be due to the imbalance between active (5) and control (2) treatments (i.e. number of chromatograms).

Accurate Mass

No significant signals were observed when peak **B** was analysed with HRMS, even though the temperature program was extended to 360 °C. This indicates that the compound is non-volatile. Another analysis method more suitable to non-volatile compounds did yield accurate mass measurements (LC-ESI-QTOF), but the observed masses were different from the earlier mentioned $[M-H]^-$ of 752.8. Further analysis is needed to determine which mass can be attributed to peak **B**.



Conclusion

An HPLC method was developed for the analysis of *Daphnia* test water and control extracts. Chromatographic profiles of all active extracts were compared to control extracts (differential diagnosis). The control consisted of fresh WC medium without *Daphnia*. These extracts were on the whole very clean and often did not show a lot of peaks in the chromatographic profiles obtained by HPLC. Possibly the instability of the kairomone at room temperature (biological degradation, Chapter 2) can be used to obtain more useful control extracts. More information can potentially be gained by extracting one half of biologically active *Daphnia* water immediately (biologically active extract), while the other half is kept at room temperature for a day and is subsequently extracted the same way as the first half (biologically inactivated extract). Excretory products of *Daphnia* that have nothing to do with colony formation (but are also absent from the traditional culture media controls) can then be ruled out. Four different HPLC columns were compared aided by a mixture of ten known natural products. These columns were first run with two gradient programs, namely an ACN-H₂O and MeOH-H₂O gradient. The ACN-H₂O gradient performed better with all columns and an isocratic hold had to be included for both Alltima columns (C₁₈ and C₈) when analysis was performed with MeOH-H₂O gradient to avoid the loss of non-polar compounds such as alizarin **95** and geranic acid **96**. Within the groups of chromatographic profiles obtained by ACN-H₂O the Alltima C₁₈ performed best. None of the ten compounds coeluted. Both C₈ columns (Xterra and Alltima) showed coelution of two compounds, although these were different pairs of compounds. On the phenyl column 2 x 2 compounds coeluted. Finally selectivity (i.e. order of elution) was similar for the C₁₈, Alltima C₈ and phenyl columns, but somewhat different for the Xterra C₈. Still all *Daphnia* test extracts were analysed with the abovementioned columns and again the C₁₈ column showed the best resolution in the UV trace. Samples were analysed with a diode-array to obtain UV spectra of all peaks of interest, however these UV spectra alone did not allow the identification of the compounds. ELSD chromatographic profiles were also obtained but they were not very informative. Furthermore a HPLC-ESI-MS method was developed to identify as wide a range of compounds as possible. Measurements were made in PI and NI mode with a post-column modifier to obtain sufficient ionisation (resp. NH₄HCO₃ and NH₃). Some compounds were detected with the MS detector and yielded a possible molecular weight and a fragmentation pattern obtained by 35% CID. None of them could be detected in both NI and PI mode. Furthermore the obtained masses were not accurate enough to obtain structural information, for example the [M-H]⁻ of peak **F** varied between extracts V, VII and IX between 933.6 and 933.9. Consequently UV detection remains the preferred method of choice for the analysis of unknown compounds especially if analysis can be performed by diode-array or at low wavelengths (210 nm). In an attempt to gain more information on a peak that was identified as unique to *Daphnia* test water extracts (**B**), LC-NMR was tried. Focus was on peak **B** as this peak was well separated and larger than some of the other candidates. By means of standard HPLC analysis this peak was trapped on an SPE cartridge and later transferred to the NMR. This method was successful in that it was possible to concentrate small amounts of material on the same cartridge. Thus it



Table 7. Summary of several spectroscopic techniques

λ_{\max}	LC-MS		NMR (ppm)	HRMS
	MS	MS/MS		
B 227	753	717, 695 615, 579, 577	See Fig. 8	nd
F 215, 244, 259, 297	934	912, 897, 863		
H 230	223	97		

nd=not detected

was possible to obtain an NMR spectrum. It was unfortunately not possible to draw definite conclusions from this spectrum. Information from other techniques such as accurate mass (elemental composition) from high resolution EI-mass spectrometry (HRMS) or LC-QTOF could be helpful for further interpretation of this spectrum but have not yielded results so far. A summary of the results is given in Table 7.

Finally dried extracts were silylated for analysis by GC-MS. Comparing controls with *Daphnia* test water extracts gave approximately 16 peaks not found in control water. EI spectra could only be obtained for some of these peaks (due to low concentrations) and these were tentatively identified.

Further research can pick up where this study has stopped. Extraction procedures will need to be further optimised to obtain higher concentration factors and cleaner extracts. Fractionation in combination with a bioassay will be the best way to proceed. That way active fractions from both techniques can be compared.

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Chapter 6. Discussion



Introduction

Five years ago at the start of *Daphnia* - *Scenedesmus* project six objectives were formulated (see Table 1). At that time chemical communication between plants and animals in an aquatic environment was still an unknown scientific area and only one other group had done some preliminary chemical experiments¹. Although more biological information has been gathered over recent years, chemically orientated experiments have only been reported by the groups of Von Elert² and Yasumoto³. Both were unable to isolate and identify the kairomone(s). Therefore everything found in the course of this project would be new and would potentially lead to more insight in aquatic chemical communication.

Table 1. Initial objectives of the *Daphnia*-*Scenedesmus* project (ca. 1997)

- 1 Production of larger quantities of the kairomone(s) for further experiments
- 2 Elucidation of the structure of the colony inducing kairomone(s)
- 3 Development of a sensitive quantitative assay for the kairomone
- 4 Determination when, how and by which organisms the compound(s) are produced
- 5 Preparing a hypothesis on the biogenetic origin of the compound
- 6 Elucidation of the structure of kairomone(s) in other *Daphnia*-phytoplankton systems

The ultimate aim of this project was to reach all six objectives, but the most crucial aim was the elucidation of the structures of the compound(s) responsible for the colony formation activity.

Increasing the availability of larger quantities of the kairomone

A method for the production of extracts from large volumes of *Daphnia* test water was developed. This method allowed the extraction and concentration of large amounts of *Daphnia* water. Although the compound has not been identified yet, concentrated extracts (20.000x) were produced that could be used by groups performing more biologically orientated research. However as the compound has not been identified yet, targeted extraction or synthesis to produce larger amounts of the kairomone has not been possible. The extracts did allow extensive analysis with several chromatographic methods.

Elucidation of the Structure of the kairomone

Isolation and identification of the colony inducing kairomone has not been achieved. Elucidation of kairomone(s) from aqueous environments is not straightforward which has been discovered by many other groups active in this field. Likewise in the *Daphnia*-*Scenedesmus* system no compounds have been identified yet. However some progress was made.

The highly concentrated extracts that were obtained within this project provided more detailed chromatograms than other groups have obtained but at some point the bioassay



failed to produce reliable information on colony forming activity. Thus chromatographic analysis had to be performed initially on water that was produced in the way that had previously yielded biologically active water, but once the bioassay was working again some extracts (90% aqueous MeOH) were tested afterwards and proved indeed to be active. In an ideal situation biological activity would have been verified before chromatographic analysis was started and for all *Daphnia* water extracts (including 50% and 100% aqueous MeOH).

It was apparent that although *Daphnia* test water was concentrated 20.000x, most peaks were still small. Only peaks that were also present in control extracts were easily identified within different extracts and chromatographic profiles. The amount of data on the active extracts was maximised by analysing the obtained extracts in different ways. Each of the chromatographic techniques used during this project has its own advantages and disadvantages. The use of different detectors gave structural information on several peaks, particularly a few larger ones (e.g. **B**, **F**, **H**).

Especially LC-MS and LC-NMR seemed promising. Unfortunately the obtained MW's by LC-MS did not provide the desired information and the NMR spectrum obtained after multiple trapping revealed that peak **B** was not completely pure. Additionally due to the complete lack of knowledge on the nature of the compound, interpretation of these spectra was complicated. ESI-MS spectra libraries are not yet available and may never become available, as spectra are more dependent on external conditions than spectra obtained with EI. Furthermore the used LC-MS was a low-resolution instrument delivering an accuracy of 0.1 m/z units in the m/z range of 50-1000. An analyte with a nominal mass of say 123 ± 0.1 yields hundreds of potential empirical formulas, whereas the determination of an accurate mass of 123.1234 ± 0.001 reduces the number of formulas to less than twenty and can then be helpful with assigning signals in the NMR spectrum. Recently, high-resolution mass analysers such as QTOF have become more popular. These instruments record spectra quickly with a mass accuracy approaching that of classical double focussing instruments⁴.

Recently a new powerful technique was introduced to improve the resolution between separated peaks. It was first developed as comprehensive two-dimensional GC (GCxGC), but has now been transferred to HPLC as comprehensive two-dimensional LC (LCxLC). It entails a separation on two different columns, where the separation of the first column is completely transferred onto the second column. These two columns have to be as different as possible (orthogonal) to ensure the separation of coeluting peaks from column 1 on column 2. This technique is becoming popular in protein and proteomics research, where usually the first dimension comprises an ion exchange or size exclusion column and the second dimension a traditional reversed phase column⁵⁻⁷. Data analysis can be difficult as large sets of data are produced and some peak overlap remains. Chemometrics and specialist software to analyse and deconvolute the data may be necessary^{8,9}. Nonetheless this technique shows potential for the analysis of complex samples and trace analysis (such as *Daphnia* water extracts).

Finally, the reliability of the bioassay has to be addressed. The bioassay seemed to be rather robust, as small differences in the protocol did not seem to influence colony



formation. However, over time significant differences between positive and negative controls declined. It is unclear whether fewer colonies are being formed and/or if they are smaller. Until this has been determined, future work to isolate and identify the kairomone responsible for colony formation will be very difficult as this kind of work is compromised without a suitable bioassay. Though some work can be done without the aid of the bioassay at some point peaks or fractions will have to be tested for colony inducing activity, which can really only be done with a bioassay.

Other objectives

The objects (no. 3-6) were not achieved as they rely completely on the isolation and identification of the colony inducing compound. Until this is achieved, development of a sensitive quantitative assay, determination of the biogenetic origin as well as elucidation of kairomone(s) in other *Daphnia*-phytoplankton systems will be impossible. Especially the elucidation of kairomone(s) in other systems is expected to become much easier once one of these compounds is identified, because similar compounds are thought to be responsible for colony formation in other *Daphnia* species. Chemical screening then becomes an option and analysis with LC-MS and LC-NMR becomes more straightforward as more is known about the nature of a possible kairomone. The interspecific and intraspecific specificity of the compound(s) can then also be determined.

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Appendix Ia. History of chemical communication terminology

Year	Term	
1932	Ectohormone	Substance having an effect on other organisms beneficial to the emitter ¹
1959	Pheromone	Secreted substance that causes specific reaction in another member of the same species ^{2,3}
1968	Allomone	Chemical substance produced or acquired by an organism which when it contacts an individual of another species evokes in the receiver a behavioural or physiological reaction beneficial to the emitter ⁴
1970	Allelochemic	Significant to organisms of a species different from the source, for reasons other than food ^{5,6}
1970	Xenomone	Broad term, paralleling pheromone but applicable to interspecific chemical signals ⁷
1970	Kairomone	Secreted substance that evokes in the receiver a behavioural or physiological reaction beneficial to the receiver but not the emitter ⁸
1971	Semiochemical	Chemical involved in chemical interactions between individual organisms ⁹
1976	Synomone	Secreted substance that evokes in the receiver a behavioural or physiological reaction beneficial to the receiver and the emitter ¹⁰
1976	Apneumone	Substance emitted by non-living material that evokes a behavioural or physiological reaction beneficial to the receiver but detrimental to an organism which may be found on or live in the non-living material ¹⁰
1985	Isomone	Chemical signal (semiochemical) system which regulates the growth pattern in corals ¹¹
1988	Infochemical	Chemical that conveys informations between two individuals evoking in the receiver a behavioural or physiological response that is adaptive to the emitter, receiver or both ¹²

Appendix Ib. Terminology according to the widely used system of Dicke & Sabelis¹²

Interaction			Benefits
Intraspecific	Pheromone	-/+	Receiver
		+/-	Emitter
		+/+	Both
Interspecific	Allelochemical	Kairomone	Receiver
		Allomone	Emitter
		Synomone	Both

Appendix II. Composition of Culture Media

Nutrient	WC ¹³ mg L ⁻¹	Modified WC ¹⁴ mg L ⁻¹	Modified WC ¹⁵ mg L ⁻¹	COMBO ¹⁶ mg L ⁻¹	CHU 12 ¹⁷ mg L ⁻¹	Modified CHU 12 ¹⁸ mg L ⁻¹	APW ¹⁹ mg L ⁻¹	Z8 ²⁰ mg L ⁻¹	20% Z8 ²¹ mg L ⁻¹	RT ²² mg L ⁻¹
TES		85	115							85
NaNO ₃	85	85.01	85	85				496	93.4	50
CaCl ₂ · 2 H ₂ O	36.76	36.76	36.8	36.76			58.8	36		39
Ca(NO ₃) ₂ · 4 H ₂ O					30	43.18			11.8	
Ca(OH) ₂										30
K ₂ HPO ₄ · 3 H ₂ O	8.71	8.71	11.4	8.71	5	?		39	6.2	
Na ₂ EDTA	4.36	4.36	4.36	4.36		2		1	0.741	5
Mg	36.97	36.97	37	36.97	75	75		75	5	20
Si	28.42	28.42	21.2	28.42		5.75	11.6			10
NaSiO ₃ · 5 H ₂ O										
K ₂ SiO ₃					25					
B	24	24	1	24				0.248	0.062	0.572
H ₃ BO ₃										13
CO ₃										
CaCO ₃					20	20		20	4.2	
Na ₂ CO ₃										
NaHCO ₃	12.6	12.6	12.6	12.6			16.8	0.0095	0.0024	0.075
Br										
KBr										
NaBr				0.016						
Cl					5	5	3.7			0.10
KCl							29.2			
NaCl										
I				0.0033				0.0066	0.00066	0.006
KI				0.31						0.87
LiCl										
NH ₄ Cl										
Fe	3.15	1	3.15	1	0.5	0.39			0.56	1.935
FeCl ₃ · 6 H ₂ O								6		
Ferric citrate								6		
Citric acid										
Mn	0.18	0.18	0.18	0.18		0.072				0.072
MnCl ₂ · 4 H ₂ O										
MnSO ₄ · 4 H ₂ O								0.178	0.0446	
Zn	0.022	0.022	0.022	0.022				0.0046	0.00574	
ZnSO ₄ · 7 H ₂ O										
ZnCl ₂						0.021				
V	0.018	0.0018		0.0018						
Na ₃ VO ₄							0.054	0.0016		
VOSO ₄ · 6 H ₂ O									0.000178	
V2O5										

Appendix II. Continued

Nutrient	WC ¹³ mg L ⁻¹	Modified WC ^{T14} mg L ⁻¹	Modified WC ^{F15} mg L ⁻¹	COMBO ¹⁶ mg L ⁻¹	CHU 12 ¹⁷ mg L ⁻¹	Modified CHU 12 ¹⁸ mg L ⁻¹	APW ¹⁹ mg L ⁻¹	Z8 ²⁰ mg L ⁻¹	20% Z8 ²¹ mg L ⁻¹	RT ²² mg L ⁻¹
Co	CoCl ₂ · 6 H ₂ O	0.01	0.012	0.01	0.012	0.0082		0.012	0.00292	0.20
	Co(NO ₃) ₂ · 6 H ₂ O									
Mo	Na ₂ MoO ₄ · 2 H ₂ O	0.0063	0.022	0.006	0.022	0.025		0.012	0.00292	0.125
	(NH ₄) ₆ Mo ₇ O ₂₄ · 4 H ₂ O									
Cu	CuSO ₄ · 5 H ₂ O	0.0025	0.001	0.01	0.001			0.007	0.00176	
	CuCl ₂ · H ₂ O								0.0025	0.065
Se	H ₂ SeO ₃									
	Na ₂ SeO ₃ · 5 H ₂ O		0.0016		0.0016					0.002
Rb	RbCl									
W	Na ₂ WO ₄ · 2 H ₂ O									
Cd	Cd(NO ₃) ₂ · 4 H ₂ O							0.0026	0.00066	
Cr	Cr(NO ₃) ₃ · 9 H ₂ O							0.0123	0.0031	
Ni	NiSO ₄ (NH ₄) ₂ SO ₄ · 6 H ₂ O								0.00082	
Al	Al ₂ (SO ₄) ₃ K ₂ SO ₄ · 24 H ₂ O							0.0158	0.00396	
Sr	SrCl ₂ · 6 H ₂ O							0.0379	0.00948	
Vit. B ₁₂		0.00055								
Biotin		0.005								
Thiamine HCl		0.1								

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Summary

Infochemicals play an important role in interactions between living organisms in aquatic environments. Although the presence of these chemical cues is confirmed in more and more systems, the chemical structures of the compounds involved remain predominantly elusive. To create more accurate prey-predator interaction models and to advance the research on chemical communication it is essential to identify these compounds.

An overview of chemical cues involving *Daphnia* (either as producer or receiver) is given and the progress towards their isolation and structure elucidation is described (*Chapter 1*). Most of the research so far has concentrated on the elucidation of kairomones produced by predators of *Daphnia* (especially *Chaoborus* and several species of fish). Although some progress has been made these cues have not been isolated and identified yet. Less study has been devoted to the isolation of the infochemical exuded by *Daphnia* that causes colony formation in its prey *Scenedesmus*. Several compounds have been suggested for roles in the interactions of *Daphnia*, e.g. trimethylamine (fish) and urea (*Scenedesmus*) but both compounds have not been universally accepted as true candidates. Colony formation in *Scenedesmus* only occurs when unicellular test populations are exposed to either *Daphnia* or water that had contained *Daphnia*. It was concluded that the responsible cue had a chemical rather than a mechanical nature, since filtered *Daphnia* water also showed the colony formation activity.

This colony formation was the basis for the development of a bioassay, which is essential for the isolation and identification of the compound(s) responsible for the colony formation (bioassay-directed fractionation and identification). Unfortunately over time a gradual decline of the difference between negative and positive controls was observed (*Chapter 2*). Several conditions, such as time (48 hours), temperature (20°C), algae strain, culture medium, location, incubator, Erlenmeyer size (50 ml), bacterial growth (axenic) and microevolution were investigated. Additionally some general properties of the kairomone, such as thermal decomposition (none), biodegradation (within one day) and concentration (colony formation activity dropped between a dilution factor of 3.3 and 1.6 % v/v) were tested for their effects on the ability to form colonies. A correlation between any of the above mentioned factors and the gradual decline of the difference between negative and positive controls was not found. Given that the bioassay was performed under such variable and not strictly controlled circumstances, this particular bioassay seems to be rather robust. However this does not defer from the fact that the quality of the bioassay did decline over time. Until the variable is identified that is responsible for the observed decline in difference between positive and negative controls, more care should be taken to standardise as many variables as possible. Despite its drawbacks, a bioassay still remains the best option to guide isolations of bioactive compounds through controlled experiments as long as observed differences are statistically significant.

Several sample pre-treatment methods (both traditional and some recently developed) were tested to find the most suitable and practical one for the analysis of *Daphnia* test water (*Chapter 3*). The chosen methods (liquid-liquid extraction, solid-phase extraction, stir-bar sorptive extraction, solid-phase disk extraction) were compared with the aid of a test mixture with ten known natural compounds differing in polarity ($\log K_{o/w}$ between -4.34 and 3.70). Methods included off-line and on-line sample pre-treatment. A suitability index and averaged standard deviation were calculated for each method to enable comparisons between the different methods. Depending on the aim a different pre-treatment method would be more suitable. For the preparative isolation of bioactive constituents from 20 L batches of *Daphnia* test water syringe SPE was chosen, because this method was most suitable for scaling up. For an analytical approach stirred SPE or SPDE would be more suitable.

Consequently an SPE analysis protocol was developed that could elute the active compound in one fraction. To reach this goal several different elution profiles and their reproducibility were studied using end-capped C_{18} material (*Chapter 4*). However since knowledge on the active compound is limited, some of the experiments were repeated using other silica based sorbents (MF C_{18} , non-endcapped C_{18} , C_8 , C_2 , CN) and two polymeric sorbents (ENV⁺ and Oasis[®] HLB). Based on a comparison of all the SPE results, the end-capped C_{18} sorbent was used for further experiments. Extracted compounds from *Daphnia* water were removed from the cartridges by eluting subsequently with 50%, 85% aqueous methanol and finally with pure methanol. Colony formation activity was most often recovered from the 85% aqueous MeOH fraction, indicating that the kairomone is moderately non-polar.

Biological activity was lost when active *Daphnia* water was partitioned at pH 12.0 against ethyl acetate. The aqueous and organic layer were both inactive, either by inactivation of the kairomone by the basic conditions in the aqueous layer or possibly more than one compound is present with synergistic effects. At lower pH (2.0 and 7.0) biological activity was recovered from the organic layer. This could be an indication that the active compound contains an anionic group. Experiments performed with ion exchange materials (SAX, SCX and Amberlite IRA-400) focused initially on the anion exchanger (SAX). However colony formation activity was recovered from the unretained fraction in contrast to what had been reported previously. This unexpected result prompted extraction with a cation exchanger (SCX). To exclude problems related to pH sensitive silica based sorbents, experiments were repeated on a resin based sorbent (Amberlite IRA-400), however a similar result was obtained as with the SAX sorbent. No satisfying explanation was found for the presence of biological activity in the unretained fractions and absence from the retained fractions, but different counterions on the ion exchangers could play a role.

The enriched extracts obtained by SPE (C_{18}) were fractionated by high performance liquid chromatography. One fraction showed a significantly higher biological activity relative to the control ('Fraction C'). Further fractionation yielded three active fractions (C2, C3, C6). This could be an indication that more than one compound is responsible for the activity. The active compound is found to elute most likely in the region between 40-55%

aqueous acetonitrile by comparing all available HPLC results. Several natural products were biologically inactive when screened in the bioassay. They were, ecdysterone and juvenile hormone III (important hormones in other *Crustaceae*), urea (proposed as kairomone in *Daphnia* -*Scenedesmus* system), and geranic acid (reference compound). Although an assumption was made to ignore possible synergistic or additive effects in these experiments, the possibility of synergism or additivity should not be ignored, given that most likely more than one active fraction is present.

At this point, due to the lack of reproducible and significant results from bioassay guided separations, another way to identify possible candidates for the role of kairomone had to be used. Differential diagnosis was chosen. Chromatograms of biologically active extracts were compared with chromatograms of non-active control extracts to determine and recognise unique peaks (i.e. peaks only present in active *Daphnia* test water extracts). In an attempt to maximise the available data on the unknown colony inducing compound(s) several techniques were applied simultaneously (*Chapter 5*). Extracts were analysed with high-performance liquid chromatography and gas chromatography-mass spectrometry. Several small unique peaks were recognised in the silylated extracts of *Daphnia* test water with GC-MS analysis. Some of these were tentatively identified as dodecanol, azelaic acid, sebacic acid and veratroylformic acid, but they proved to be biologically inactive.

HPLC detection was performed not only with ultraviolet spectroscopy but also with evaporative light scattering and by electrospray ionisation-mass spectrometry to avoid overlooking compounds without a UV chromophore. LC analysis on four columns with different packings ensured that peaks were well separated on at least one column. Chromatograms with the best resolution were obtained on a C₁₈ column with an ACN-H₂O gradient and UV detection. High noise levels and low sensitivity reduced the usefulness of ELS detection. Several peaks unique to 90% aqueous MeOH extracts of *Daphnia* test water were detected, but unfortunately not identified. Some of the recognised unique peaks (**B**, **G**, **K**) eluted in previously identified active regions ('Fraction C').

Especially peak **B** ($[M-H]^- = 752.8$?, λ_{\max} 227 nm) was present in high amounts and well separated from neighbouring peaks. Therefore this peak was further analysed by liquid chromatography-nuclear magnetic resonance. Peaks from several extracts were trapped onto one SPE cartridge. In this way a sufficient amount of analyte could be transferred into the NMR probe to allow the recording of 1-dimensional ¹H-spectrum. Unfortunately the spectrum did not lead to elucidation of the structure of peak **B**. One aliquot of this collected fraction was therefore analysed by high-resolution mass spectrometry and liquid-chromatography-quadrupole time-of-flight mass-spectrometry to obtain an accurate mass, while another aliquot was checked for biological activity in a bioassay. Unfortunately analysis with HRMS was unsuccessful and analysis with LC-QTOF has not yet been successful. The peak with a possible pseudo molecular mass of 752.8 ($[M-H]^-$) could not be detected.

The other aliquot that was tested for biological activity in the bioassay showed significant differences between the negative control, positive control and peak **B**. This

peak could therefore play a role in the induction of colonies in *Scenedesmus*, although it is still unclear whether it acts alone. Should peak **B** prove to be (partly) responsible for colony formation in *Scenedesmus* then the most important objective of this study has been partly reached, namely the isolation of kairomone(s) in the *Daphnia-Scenedesmus* system. This information will enable and facilitate research into the other objectives.

Samenvatting

Chemische stoffen spelen een belangrijke rol bij interacties tussen verschillende levende organismen. En al is het bewijs voor het bestaan van steeds meer van dit soort stoffen geleverd, de identificatie van de bijbehorende chemische structuren blijft achter. Voor de voortgang van onderzoek naar chemische communicatie is het belangrijk deze stoffen te identificeren.

In *Hoofdstuk 1* vindt u een overzicht van interacties waarbij de aanwezigheid van signaalstoffen in water is aangetoond. Het vinden van de chemische structuren van deze stoffen heeft zich vooral op de vijanden van watervlooien (vooral vissen en insecten) geconcentreerd. Minder onderzoek is gedaan naar de stoffen die kolonievorming induceren bij het voedsel van de watervlo, namelijk de groen alge. Eén van de doelstellingen van dit project was dan ook de isolatie en identificatie van deze signaalstof.

Kolonievorming treedt op wanneer de eencellige groen alge in aanraking komt met watervlooien of water waaruit de watervlooien verwijderd zijn. Hieruit werd geconcludeerd dat het om een chemische prikkel gaat en niet om een mechanische. Dit verschijnsel vormde de basis voor de biotoets (*Hoofdstuk 2*). Een biotoets is een test waarmee biologische activiteit (in dit proefschrift is dat kolonievorming) vastgesteld kan worden van chemische mengsels. Kolonies waren te herkennen aan hoge meetwaarden en de alge in zijn eencellige vorm aan lage meetwaarden. Hoge waardes duiden dus op de mogelijke aanwezigheid van een signaalstof. Echter de verschillen tussen beide groepen werden gaandeweg dit onderzoeksproject steeds kleiner en er werd geprobeerd de reden hiervan te achterhalen, daartoe werden diverse biotoets condities (onder andere: duur, stam, incubator, temperatuur, medium, bacteriegroei en locatie) onderzocht. Daarnaast werd een aantal algemene eigenschappen van de signaalstof bepaald (stabiliteit, biologische afbraak en concentratie). Een verband tussen één van de hierboven genoemde condities en de geleidelijke afname werd niet gevonden.

Grote variaties van de diverse factoren hadden weinig effect op de resultaten, dus kan deze biotoets toch als redelijk robuust beschouwd worden. De oorzaak voor de afname van de verschillen in de meetwaarden tussen de eencellige alge en kolonies werd echter nog steeds niet gevonden en het is beter om de variatie binnen de verschillende condities van de biotoets zo klein mogelijk te houden, tenminste totdat de oorzaak van de afname gevonden wordt. De biotoets blijft de eerste keus om tot een oplossing te komen van wetenschappelijke vraagstukken zoals de identificatie van een signaalstof, zolang de verschillen tussen positieve en negatieve controles maar groot genoeg zijn.

Om tot de beste analysemethode voor watervlo water te komen, werd in *Hoofdstuk 3* een aantal verschillende extractiemethoden vergeleken, namelijk uitschudden (LLE), solid-phase extraction (SPE), stir-bar sorptive extraction (SBSE), solid-phase disk extraction (SPDE). Met behulp van een testmengsel, bestaande uit 10 verschillende

natuurstoffen werden de methodes met elkaar vergeleken. Het bleek dat 'stirred' SPE en 'cartridge' SPE het beste waren voor de extracties van kleine hoeveelheden stof, maar dat voor de extractie van grote hoeveelheden (soms wel 20 L) 'syringe' SPE beter geschikt was. SPE is een techniek waarbij een monster (in dit geval watervlo water) over absorptiemiddel wordt geleidt. Aan dit absorptiemiddel worden sommige stoffen geadsorbeerd, maar andere niet. Hierdoor is het mogelijk specifiek bepaalde stoffen te scheiden van anderen.

Vervolgens werd er een SPE-methode ontwikkeld om de signaalstof in één fractie op te vangen (*Hoofdstuk 4*). De experimenten werden uitgevoerd met verschillende concentraties organische oplosmiddelen en verschillende soorten adsorptiemiddel (endcapped C₁₈, MF C₁₈, non-endcapped C₁₈, C₈, C₂, CN, ENV⁺ and Oasis[®] HLB). Endcapped C₁₈ werd uiteindelijk gebruikt voor verdere experimenten (andere absorptiemiddelen waren niet beter) en geëxtraheerd met verschillende concentraties methanol in water (50%, 85%) en pure methanol (100%). De signaalstof werd meestal teruggevonden in de 85% waterige methanol fractie, wat erop wijst dat deze matig niet-polair is.

De kolonievormende activiteit gaat echter verloren als het watervlo water bij hoge pH (12,0) met ethylacetaat wordt uitgeschud. De waterlaag en organische laag waren dan beiden niet actief. Dit komt mogelijk doordat de signaalstof in de sterk basische waterlaag omgezet wordt of dat meer dan één signaalstof verantwoordelijk is voor kolonievorming (mogelijk met additieve of synergistische effecten). Na uitschudden bij zure tot neutrale pH (2.0 en 7.0) blijkt de kolonievormende activiteit verschoven te zijn van de waterlaag naar de ethylacetaat laag. Dit kan betekenen dat de signaalstof een anionische groep bezit. Om dit verder te onderzoeken werd watervlo water verder bestudeerd met ionenwisselaars. In eerste instantie werd daarvoor een anionenwisselaar (negatieve ionen) gebruikt, maar de signaalstof werd niet geadsorbeerd op de ionenwisselaar in tegenstelling tot eerdere publicaties. Het gebruik van een ander type anionenwisselaar kon dit niet oplossen; de signaalstof werd ook hier niet geadsorbeerd. Ten slotte werd een kationenwisselaar (positieve ionen) gebruikt, maar weer werd de signaalstof niet geadsorbeerd. Een goede verklaring hiervoor is niet gevonden, maar mogelijk dat de ionen die aanwezig waren op de ionenwisselaar voor aanvang van het experiment een rol spelen.

De extracten die verkregen werden met het C₁₈ absorptiemiddel werden geanalyseerd en gescheiden met behulp van HPLC (vloeistof chromatografie). Zo werden diverse verschillende fracties opgevangen die vervolgens getest werden in de biotoets. Eén van deze fracties ('fractie C') gaf na de biotoetsmetingen hoge meetwaarden (= kolonies) en dus de mogelijke aanwezigheid van de signaalstof. Een nieuwe analyse met HPLC van deze fractie in kleinere fracties gaf zes fracties, waarvan drie (C2, C3, C6) met kolonies. De verspreiding over drie fracties zou nog een aanwijzing kunnen zijn dat meer dan één stof verantwoordelijk is voor de kolonievorming.

Verscheidene natuurlijke producten gaven geen kolonievorming na onderzoek met de biotoets. Dit waren, ecdysterone en juveniel hormoon III (belangrijke hormonen bij andere leden van de krab familie), ureum (voorgesteld als signaalstof bij interacties

tussen watervlooien en groen alge), en geranylzuur (referentiestofje). Let wel, bij deze experimenten is aangenomen dat er geen synergistische of additieve effecten waren, toch duidt een aantal resultaten erop dat er mogelijk meer dan één actieve fractie aanwezig is.

Wegens het gebrek aan reproduceerbare resultaten uit de biotoets, werd gezocht naar een andere manier om mogelijke signaalstoffen te identificeren. Hiervoor werden water van watervlooien en controle water eerst voorbereid met SPE en vervolgens geanalyseerd met chromatografie. De chromatogrammen konden dan vergeleken worden om de pieken te vinden die alleen in de extracten van watervlo water voorkomen. In een poging om de beschikbare gegevens over de signaalstof, die kolonievorming induceert, zo goed mogelijk te gebruiken, werd een aantal technieken gelijktijdig toegepast, zoals HPLC en GC (gas chromatografie). Dit leest u in *Hoofdstuk 5*. Hierbij werden een aantal kleine unieke pieken gevonden na analyse met GC-MS. De identificatie van deze piekjes was voorlopig als volgt: lauryl alcohol, azelaïne, sebacinezuur en 3,4-dimethoxy fenylglyoxylaat. Deze stoffen gaven echter geen kolonies in de biotoets.

HPLC werd uitgevoerd met drie verschillende detectiemethoden (UV, ELSD en MS) om uit te sluiten dat stoffen per abuis onopgemerkt zouden blijven. De extracten werden op vier kolommen met verschillende stationaire fasen gescheiden, zodat de meeste pieken op tenminste één kolom goed gescheiden waren. De chromatogrammen met de beste resolutie werden verkregen op een C₁₈ kolom met een acetonitrile-ammonium formaat buffer gradiënt en UV-detectie. De lage gevoeligheid verminderde het nut van ELS-detectie. Verscheidene pieken werden alleen gevonden in de extracten van watervlo water, maar werden nog niet geïdentificeerd. Enkele (**B**, **G**, **K**) werden aangetroffen in eerder geïdentificeerde actieve gebieden (zoals 'Fractie C').

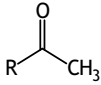
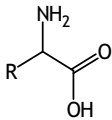
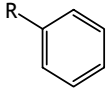



Voorals Piek **B** ($[M-H]^- = 752.8$?, λ_{\max} 227 nm) kwam in hoge concentraties voor en was bovendien goed gescheiden van naburige pieken. Deze piek werd daarom verder geanalyseerd met LC-NMR. Piek **B** van verschillende extracten (watervlo water) werd opgevangen op een SPE-patroon. Zo werd voldoende onbekende stof verkregen om in een NMR-apparaat, een 1-dimensionaal ¹H-spectrum op te nemen. Helaas leidde het spectrum niet tot opheldering van de structuur van piek **B**. Een gedeelte van deze NMR-fractie werd tevens geanalyseerd met HR-MS en LC-ESI-QTOF om de accurate massa te bepalen, terwijl een ander gedeelte op biologische activiteit in een biotoets werd gecontroleerd. De analyse met HR-MS was niet succesvol en met LC-ESI-QTOF nog niet succesvol. De piek met de pseudo-moleculaire massa van 752.8 ($[M-H]^-$) kon niet teruggevonden worden.

Het andere gedeelte van de NMR-fractie dat getest werd in de biotoets, vertoonde een relatief hoge meetwaarde (= kolonies). Deze piek speelt daarom mogelijk een rol bij de inductie van kolonies in de groen alge. Als piek **B** (gedeeltelijk) verantwoordelijk blijkt te zijn voor de kolonievorming in de groen alge dan is één van de belangrijkste doelstellingen van deze studie bereikt, namelijk de isolatie van een signaalstof die interacties tussen watervlooien en een groen alge verzorgt. Deze informatie zal onderzoek naar de andere doelstellingen toelaten en vergemakkelijken.

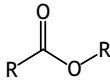
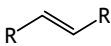
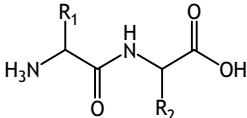
List of Abbreviations

ACN	Acetonitrile	MCV	Mean Cell Volume
AEW	Aquatic Ecology and Water Quality, Wageningen University, Wageningen	MPI	Max Planck Institute
APCI	Atmospheric Pressure Chemical Ionization	MPV	Mean Particle Volume
API	Atmospheric Pressure Ionization	MS	Mass Spectrometry
BSTFA	N,O-bis (trimethylsilyl)trifluoroacetamide	NaOH	Sodium hydroxide
BuOH	Butanol	NC	Negative Control
CD ₃ OD	Deuterated methanol	NH ₃	Ammonia
CE	Capillary Electrophoresis	NH ₄ HCO ₃	Ammonia hydrogencarbonate
CID	Collision-induced Dissociation	NI	Negative Ionisation
CPC	Cells per Colony	NIOO	Dutch Institute for Ecological Research, Centre for Limnology, Nieuwersluis
DAD	Diode Array Detector	NMR	Nuclear Magnetic Resonance
DHM	Diel Horizontal Migration	NVP	N-vinylpyrrolidone
DMS	Dimethylsulphide	OPA	o-Phthalaldehyde
DMSP	Dimethylsulfoniopropionate	PA	Polyacrylate
DVB	Divinylbenzene	PAH	Polycyclic aromatic hydrocarbon
DVM	Diel Vertical Migration	PC	Positive Control
EI	Electron Impact	PCB	Polychlorinated biphenyl
ELSD	Evaporative Light Scattering Detector	PDMS	Polydimethylsiloxane
ESI	Electro Spray Ionization	PI	Positive Ionisation
EtOAc	Ethyl acetate	PS	Polystyrene
EtOH	Ethanol	Q-TOF	Quadrupole Time of Flight
FA	Formic acid	RSD	Relative standard deviation
FMOC	Fluorenylmethyl-chloroformate	SAX	Strong anion exchanger
GC	Gas Chromatography	SBSE	Stir Bar Sorptive Extraction
GPC	Gel Permeation Chromatography	SCX	Strong cation exchanger
GR	Growth rate	SDS	Sodium dodecylsulfate
GUS	General unknown screening	SFC	Supercritical Fluid Chromatography
HCl	Hydrochloric acid	SPDE	Solid-phase Disk Extraction
HPLC	High-Performance Chromatography	SPE	Solid-Phase Extraction
HRMS	High Resolution Mass spectrometry	SPME	Solid-Phase Micro Extraction
LC	Liquid Chromatography	t-BuOMe	tert-butylmethylether
LC-MS	Liquid Chromatography-Mass Spectrometry	TFA	Trifluoroacetic acid
LLE	Liquid-Liquid Extraction	THF	Tetrahydrofuran
LPLC	Low-Pressure Liquid Chromatography	TMA	Trimethylamine
LPME	Liquid-Phase Microextraction	TMCS	Trimethylchlorosilane
MeOH	Methanol	UV	Ultra Violet

List of Terms

Acetyl		Ciliata	One of the orders of Infusoria, characterized by having cilia. In some species the cilia cover the body generally, in others they form a band around the mouth.
Acetylation	To bring an acetyl group into (an organic molecule).		
Actinastrum	see <i>Chlorophyceae</i>		
Allomone	Interspecific infochemical, chemical cue (Appendix I)	Cladocera	Order of Entomostraca. They have a bivalve shell, covering the body but not the head, and from four to six pairs of legs and two pairs of antennae, for use in swimming. They mostly inhabit fresh water (e.g. <i>Daphnia</i>)
Allelochemical	Interspecific infochemical, chemical cue (Appendix I)		
Amino acid		Coenobia	Colonies of cells formed by certain green algae, in which little or no specialisation of the cells occurs.
Amoeba	see <i>Protozoa</i>	Congener	Organism belonging to the same taxonomic genus as another organism
Anabaena	see <i>Cyanobacteria</i>	Conspecific	Organism belonging to the same species as another organism
Anatoxin	Toxin produced by <i>Anabaena</i>		
Anion	Negatively charged ion, the ion that migrates to an anode in electrolysis	Copepoda	Order of Entomostraca, including many minute Crustacea, both fresh-water and marine. They have a distinct carapace. The eggs are carried in a pair of external pouches. Some are parasites of fishes. (e.g. <i>Calanus</i>)
Anisops	Backswimmer, insect		
Aromatic		Crustacea	One of the classes of the arthropods, including lobsters and crabs, so called from the crustlike shell with which they are covered (consisting of two subclasses, Entomostraca and Malacostraca, each of which includes several orders)
Axenic	Raised under sterile conditions, not contaminated by or completely free of the presence of other organisms		
Brachionus	see <i>Rotifera</i>	Cryptomonas	see <i>Cryptophyceae</i>
Carassius	Crucian carp, Fish	Cryptophyceae	Motile usually brownish-green protozoa-like algae
Carbonyl		Cyano	
Carboxyl		Cyanobacteria	A photosynthetic bacterium of the class Coccogoneae or Hormogoneae, generally blue-green in color and in some species capable of nitrogen fixation. Cyanobacteria were once thought to be algae.
Cation	Positively charged ion, the ion that migrates to a cathode in electrolysis		
Ceratophyllum	Coontail, see <i>Macrophyte</i>	Cyclomorphosis	Originally described as a seasonal response to temperature, meanwhile shown to be an inducible defence
Chaoborus	Midge, insect		
Chlamydomonas	see <i>Chlorophyceae</i>	Daphnia	Waterflea, see <i>Cladocera</i>
Chlorophyceae	Algae distinguished chiefly by having flagella and a clear green color, their chlorophyll being masked little if at all by other pigments, commonly named: "green algae"	Derivatisation	Process of chemically modifying the functionality of an analyte in order to enable chromatographic analysis
CHU	see <i>Medium</i>	Diapause	Period during which growth or development is suspended and physiological activity is diminished, as in certain insects in response to adverse environmental conditions.

List of Terms

Diaptomid	see <i>Diaptomidae</i>	<i>Leucaspius</i>	Sunbleak, fish
<i>Diaptomidae</i>	see Copepoda	<i>Leuciscus</i>	Blageon, fish
Dinoflagellata	see <i>Dinophyceae</i>	Lipophilic	Having an affinity for, tending to combine with, or capable of dissolving in lipids
Dinophyceae	Numerous minute, chiefly marine protozoans, characteristically having two flagella and a cellulose covering and forming one of the chief constituents of plankton. They include bioluminescent forms and forms that produce red tide.	Macrophyte	Macroscopic plant, visible by naked eye
<i>Elodea</i>	Oxygen weed, see macrophyte	Medium	Nutrient substance (solid or liquid) that is used to cultivate micro-organisms
<i>Emiliana</i>	see Protozoa	Methyl	$R-CH_3$
<i>Ehippial</i>	Saddle-shaped, occupying an ehippium, usually used for resting eggs (diapause)	Methylation	To bring an methyl group into (an organic molecule).
Epiphyte	Plant that grows on another plant upon which it depends for mechanical support but not for nutrients	Microcystin	Toxin produced by <i>Microcystis</i>
<i>Esox</i>	Northern pike, fish	<i>Microcystis</i>	see Cyanobacteria
Ester		<i>Myriophyllum</i>	Millfoil, see macrophyte
<i>Euplotes</i>	see Ciliata	<i>Nannochloris</i>	see <i>Chlorophyceae</i>
Food chain	Representation of the passage of energy from a primary producer through a series of consumers at progressively higher trophic levels	<i>Nereis</i>	see <i>Polychaeta</i>
Food web	Representation of the various paths of energy flow through populations in a community, taking into account that each population shares resources and consumers with other populations	<i>Nitella</i>	Stonewort, see macrophyte
<i>Gasterosteus</i>	Threespine stickleback, fish	<i>Notonecta</i>	Backswimmer, insect
Growth rate	Rate of increase in size per unit time	<i>Notonectid</i>	see <i>Notonecta</i>
Haptophyte	see <i>Prymnesiophyceae</i>	Nucleic acid	Group of complex compounds found in all living cells and viruses, composed of purines, pyrimidines
Herbivore	Organism that consumes living plants or their parts	Oligonucleotide	Short polymer of two to twenty nucleotides composed of nucleic acids, carbohydrates, and phosphoric acid.
Hydrophilic	Having an affinity for water; readily absorbing or dissolving in water	Olefinic	
Infochemical	Interspecific infochemical, chemical cue (Appendix I)	<i>Oscillatoria</i>	see <i>Cyanobacteria</i>
Intraspecific	Arising or occurring within a species	<i>Oxyrrhis</i>	see <i>Dinophyceae</i>
Interspecific	Arising or occurring between species	Parthenogenesis	Form of reproduction in which an unfertilized egg develops into a new individual, occurring commonly among insects and certain other arthropods.
Irradiance	Emission of rays of light	Peptide	
Kairomone	Interspecific infochemical, chemical cue (Appendix I)	<i>Perca</i>	Perch, fish
<i>Lembadion</i>	Predatory ciliate	<i>Peridinium</i>	see <i>Dinophyceae</i>
<i>Lepomis</i>	Pumkinseed sunfish	phenyl	see Aromatic
		<i>Phaeocystis</i>	see <i>Prymnesiophyceae</i>
		Pheromone	Intraspecific infochemical, chemical cue (Appendix I)
		Phytoplankton	Photosynthetic or plant constituent of plankton; mainly unicellular algae

List of Terms

<i>Pimephales</i>	Bullhead, fish		
Piscivorous	Organism that consumes fish	Synergism	Working together of two compounds to produce an effect greater than the sum of their individual effects
Planktivorous	Organism that consumes plankton	Synomone	Interspecific infochemical, chemical cue (Appendix I)
Plankton	Collection of small or microscopic organisms, including algae and protozoans, that float or drift in great numbers in fresh or salt water, especially at or near the surface, and serve as food for fish and other larger organisms.	Trade-off	Relinquishment of one benefit or advantage for another regarded as more desirable
		<i>Trichormus</i>	see <i>Cyanobacteria</i>
<i>Platynereis</i>	see <i>Polychaeta</i>	Trophic level	Position in the food chain, determined by the number of energy-transfer steps to that level
<i>Polychaeta</i>	Marine annelid worms	WC	see medium
<i>Pomoxis</i>	Crappie, fish		
<i>Potagemon</i>	Pondweed, see macrophyte	Xenic	Raised under non-sterile conditions, contaminated by or not completely free of the presence of other organisms
<i>Protozoa</i>	Large group of single-celled, usually microscopic, eukaryotic organisms, such as amoebas, ciliates, flagellates, and sporozoans	Z	see medium
<i>Prymnesiophyceae</i>	Mainly unicellular marine yellow-brown algae, possessing a haptonema in addition to flagella, and the surface of their spherical cells is covered by numerous shield-like scales	Zooplankton	Plankton that consists of animals, including the crustacea, fish larvae, corals, rotifers, sea anemones, and jellyfish
<i>Pungitius</i>	Ninespine stickleback, Fish		
Recruitment	Larval or juvenile organisms that settle or move into adult habitat, or organisms entering the exploitable stage of their life cycle		
<i>Rhodeus</i>	Bitterling, fish		
<i>Rhodomonas</i>	see <i>Cryptophyceae</i>		
<i>Rotifera</i>	Order of minute worms which usually have one or two groups of vibrating cilia on the head, which, when in motion, often give an appearance of rapidly revolving wheels. The species are very numerous in fresh waters, and are very diversified in form and habits		
<i>Rutilus</i>	Roach, fish		
<i>Scardinius</i>	Rudd, fish		
<i>Scenedesmus</i>	see <i>Chlorophyceae</i>		
Silyl	$ \begin{array}{c} \text{CH}_3 \\ \\ \text{R}-\text{Si}-\text{CH}_3 \\ \\ \text{CH}_3 \end{array} $		
Silylation	see derivatisation, introduction of silyl groups		
<i>Stenostomum</i>	Flatworm		
<i>Stigeoclonium</i>	see <i>Chlorophyceae</i>		
<i>Synechococcus</i>	see <i>Cyanobacteria</i>		
<i>Synechocystis</i>	see <i>Cyanobacteria</i>		

Dankwoord

Het is bijna onmogelijk je iedereen voor de geest te halen die je tijdens je promotieonderzoek heeft bijgestaan met raad en daad. Toch wil ik een aantal mensen met naam noemen.

Als eerste natuurlijk mijn begeleiders zonder wiens vertrouwen en steun ik het al jaren geleden zou hebben opgegeven. Aede, ik heb je de eerste paar jaar niet vaak gezien omdat jij alle vertrouwen in de leiding van Teris had, maar op het *moment suprême* kon ik toch op je rekenen. Jij hebt me laten inzien dat ik me op een randgebied bevind en dat biologen en chemici elkaar niet altijd begrijpen. Helaas ben ik niet meer aan je geliefde syntheses toegekomen. Teris, als co-promotor was je mijn dagelijkse begeleider, ik kon altijd bij je terecht. Je had het zelf altijd heel druk, maar toch wist je altijd tijd te maken om mij even aan te horen. Ik heb met veel plezier voor je gewerkt en heb veel van je geleerd. Ik zal de gezellige 'sportieve' uitjes en de mensen van de Fyto-groep missen. Harm, de bedoeling was dat jij één van mijn co-promotoren zou zijn, helaas vertrok je al vrij snel naar Groningen en hoewel we probeerden contact te houden, ging dat toch niet zo makkelijk. Ik ben blij dat je nu toch betrokken bent bij mijn promotie, zij het als opponent. Ellen, als ontdekker van de chemische communicatie tussen watervlooien en algen heb je samen met Teris het projectvoorstel geschreven waar ik uiteindelijk op werd aangesteld. Op het NIOO hield je mij op afstand in de gaten en controleerde dat de biologische aspecten van dit onderzoek niet verloren gingen in alle chemische vraagstukken. Miquel, hoewel jij officieel niet mijn begeleider was en je net eigen proefschrift af had voordat ik begon, ben je toch enorm belangrijk geweest voor mij. Zo zijn we samen nog een paar dagen naar Konstanz geweest om Eric von Elert te ontmoeten. By the way, I would like to extend a special thanks to Eric for his efforts to aid me by performing bioassays at his lab in Konstanz. Als het allemaal weer even niet liep met de biotoets, kon ik bij je komen uithuilen. Bovendien had je altijd tonnen goede ideeën voor verder onderzoek (helaas kwam ik nooit verder). Een bespreking met jou kon al gauw twee uur in beslag nemen. Als laatste wil ik je bedanken voor de tijd die je gestoken hebt in het nakijken van twee hoofdstukken voor mijn proefschrift terwijl je eigenlijk niet mijn begeleider was en de stoomcursus Sigmaplot.

Een speciale dank gaat uit naar de analisten die mij geassisteerd hebben. Zonder hen hadden al die biotoetsen (> 130, 99 monsters per keer) niet gemeten kunnen worden. Klaas is een belangrijke partner geweest op het NIOO. Samen wisten we het zo te plooien dat we zo veel mogelijk biotoetsen konden draaien zonder dat ik drie keer per week naar het NIOO moest. Daarnaast zorgde je ervoor dat ik altijd voldoende *Daphnia* test water tot mijn beschikking had, wat je persoonlijk met de auto naar Wageningen kwam brengen (soms wel 20 L). In geval van nood kon ik altijd terecht bij Wendy van de leerstoelgroep WKAO om *Daphnia* test water te halen of om een biotoetsje te laten doen. Twee weken voor de leesversie moest worden ingeleverd heeft zij nog een aantal cruciale biotoetsjes gedraaid, waarvoor dank. Op OC werd ik gigantisch gesteund door Elbert. Jij maakte het mogelijk dat ik met mijn eigen experimenten met *Daphnia* test water kon doormodderen, terwijl jij een belangrijke bijdrage leverde aan de tot standkoming van Hoofdstuk 3. Daarnaast was jij de lopende encyclopedie van alles wat met chromatografie te maken had en kon ik altijd bij je terecht als er weer eens wat mis ging (en voor mijn gevoel gebeurde dat op de een of andere manier veel vaker bij mij dan bij andere AiO's). Samen met Frank hebben we regelmatig gezellig staan kletsen, terwijl de HPLC's werkten. Frank, jij had officieel niets te maken met mijn project, maar was een soort reserve encyclopedie als Elbert niet beschikbaar was. Ik heb altijd heel erg prettig met je samengewerkt en dat stuk appeltaart heb je nog te goed.

Anderen die een belangrijke bijdrage hebben geleverd aan dit proefschrift zijn natuurlijk niet in de laatste plaats de dames van de administratie en de heren van het chemicaliën magazijn. Jullie zorgden er samen voor dat aan alle randvoorwaarden voor wetenschappelijk onderzoek werd voldaan. Daarnaast wil ik Maarten bedanken die op het allerlaatste moment, zuchtend en steunend, mij geholpen heeft een aantal identificaties te peuren uit eigenlijk veel te zwakke spectra en Pieter voor zijn hulp bij de LC-NMR metingen. Ik wil Gerrit Gort graag bedanken voor de tijd die hij genomen heeft om de statische analyses te controleren en Arjen Lommen voor de tijd die hij gestoken heeft om zijn nieuwe software los te laten op mijn GC resultaten.

Ik heb een aantal kamergenoten gehad die ik wil bedanken voor de gezellige sfeer (Cindy en Audrius). In het bijzonder Matthew, jij was een altijd optimistische steunpilaar de eerste paar jaar (ik denk wel eens dat jij op een gegeven moment de enige was die nog moed had in mijn onderzoek) en Βασιλική σε ευχαριστώ πολύ για την υποστήριξη που μου παρείχες τον τελευταίο χρόνο της διατριβής μου. Βέβαια δεν ήρθες μόνης σου αλλά μου κουβάλησες τον Γιάννη και τη Σοφία και ξαφνικά το γραφείο μου γέμισε κόσμο. Πέρασαμε όμως πολύ ωραία μαζί και τουλάχιστον θυμόσουν να φτιάχνεις τσάι..... τελικά μου φαίνεται καλή ιδέα να ψάξω για Postdoc στην Ελλάδα. Sofia, muito obrigado por todos os teus esforços para fazeres as medidas de LC-QTOF no pico B. Infelizmente a máquina está mais tempo avariada do que a funcionar. Felizmente poderás realizar as medidas mais tarde e assim escrevermos um artigo juntas.

Irene, Gabi en Anthony (collega AiO's op het NIOO) hebben veel hand- en spandiensten verricht, zoals hulp bij het meten van CPC's, opzoeken van literatuur en het verrichten van kleine biotoetsjes, waarvoor dank.

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Tijdens mijn promotieonderzoek is hockey altijd een grote uitlaatklep geweest. Ik kan bijna de hele hockeyclub bedanken maar wil mij toch beperken tot Dames 5 (Sanne 1, Sanne 2, Marieke, Franka, Janine, Wies, Saskia, Sara en Roos) en Dames 2. Mariska, ooit samen begonnen in Dames 7 en sindsdien altijd samen gehockeyd, jij was altijd meer dan alleen teamgenoot. We zijn destijds ongeveer gelijk begonnen met ons promotieonderzoek, maar jij had alles toch iets beter gepland. Jij wist altijd als geen ander mij tegen mezelf te beschermen. Monika, jij verdient ook een vermelding, aangezien zonder jouw aansporing om te solliciteren dit boekje niet zou bestaan.

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Frédérique

Curriculum Vitae

De schrijfster van dit proefschrift werd op 4 juli 1973 geboren in Leidschendam. Na het behalen van het Gymnasium diploma aan de scholengemeenschap het St. Adelbert College te Wassenaar in 1991, vertrok zij voor 10 maanden naar Amerika om daar deel te nemen aan een uitwisselingsproject. Na terugkomst behaalde zij in de zomer van 1992 het certificaat Natuurkunde aan het James Boswell Instituut om vervolgens te beginnen met de studie Plantenveredeling en Plantenziektkunde aan de Landbouw Universiteit van Wageningen. Tijdens haar studie kwam de interesse in inhoudsstoffen bovendien en werd een eerste afstudeervak naar eiwitten in *Chenopodium* en vetzuren in *Calendula* op het toenmalige CPRO-DLO instituut uitgevoerd onder begeleiding van Dr. D. van Mastebroek en Ir. L. Hof. Dit afstudeervak werd vervolgd met een stage op de afdeling Farmacognosie van de Rijksuniversiteit van Leiden. Daar werd onderzoek gedaan naar de genetische variatie van dimere indool alkaloiden in *Catharanthus* sp onder begeleiding van Dr. R. van der Heijden en Prof. Dr. R. Verpoorte. Na terugkomst in Wageningen werd gestart met een tweede en laatste afstudeervak op de voormalige vakgroep Plantentaxonomie met als onderwerp de taxonomie, chemotaxonomie en fytochemie van *Atropa* sp. Dit vak werd uitgevoerd in samenwerking met de Botanische Tuinen van de Katholieke Universiteit van Nijmegen en de afdeling Farmacognosie van de RUL onder begeleiding van Prof. Dr. H. Scheffer en Dr. R van den Berg. Zij behaalde haar diploma Plantenveredeling en Plantenziektkunde in september 1997. Na een hoofdstuk voor deel 12 van het PROSEA project geschreven te hebben, werd zij in 1998 aangesteld als OiO bij het Laboratorium voor Organische Chemie (ATV) van de Wageningen Universiteit onder leiding van Dr. T.A. van Beek en Prof dr. Ae. De Groot. Het resultaat van deze aanstelling hebt u zo juist gelezen.

List of Publications

van Holthoon, F.L., T.A. van Beek, M. Lüring, E. Van Donk, and Ae. de Groot (2003). *Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone*. Hydrobiologia. 491: 241-254.

van Holthoon, F.L. (1999). *Phyllanthus sp.*, in *PROSEA 12: Medicinal and poisonous plants*, L.S. de Padua, N. Bunyap

van Holthoon, F.L., E. van der Klift, T.A. van Beek and Ae. de Groot (in prep). *Comparison of sample pre-treatment methods for natural products in aqueous samples*.

Fulfilled PhD Educational Activities:

1. Oral and written presentation was given based on literature review
an extensive systematic overview of research questions at the department of Organic Chemistry. 2001
2. Participation in the Post-Graduate courses and workshops:
 - I. Future Trends in Phytochemistry. 3rd young scientist symposium. PSE. 2000
 - II. Basic Statistics. 2001
 - III. Advanced Statistics. 2002
3. Additional Courses:
 - I. Natuurproducten. ORC, WUR. 1998
 - II. EHBO. ADM, WUR. 2001
 - III. AIO-onderwijs ORC. ORC, WUR. 1998-2002
4. Active participation in the workshop 'SNIFFS' and in the PhD-discussion group 'NWO-CW study group, Lunteren', and the 'Entomology Discussion Group'. 1998-2002.
5. Participation in annual meetings and seminars:
 - I. EPS AIO-meeting. 1998
 - II. EPS AIO-meeting. 1999
 - III. PE&RC AIO-meeting. 2001
 - IV. PE&RC AIO-meeting. 2002
6. Participation in, and contribution to, the international conferences:
 - I. Future Trends in Phytochemistry. 4th young scientist symposium. PSE, Gargnano, Italy. 2002
 - II. ISCE. ISCE, Hamburg. 2002
7. Laboratory training and working visits:
 - I. Troubleshooting HPLC. Merck 1998
 - II. Aquatic Chemical Ecology. University of Konstanz. 1999
 - III. AIO-reis Chicago. Several Universities (e.g. Purdue). 2000
 - IV. AIO-reis Switzerland. Several Universities and Companies (e.g. ETH, Novartis). 2003



Nederlandse Organisatie voor Wetenschappelijk Onderzoek

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The cover was created from watercolours painted especially for this thesis by Dutch artist, Bram Stoof.

<http://www.bramstoof.nl>

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