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## Solid phase extraction and metabolic profiling of exudates from living copepods

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Copepods are ubiquitous in aquatic habitats. They exude bioactive compounds that mediate mate finding and induce defensive traits in prey organisms. Little is, however, known about the chemical nature of the copepod exometabolome that contributes to the chemical landscape in pelagic habitats. Here we describe the development of a closed loop solid phase extraction setup that allows for extraction of exuded metabolites from live copepods. We captured exudates from male and female *Temora longicornis* and analyzed the content with high resolution LC-MS. Chemometric methods revealed 87 compounds that constitute a specific chemical pattern either qualitatively or quantitatively indicating copepod presence. The majority of the compounds were present in both female and male exudates, but nine compounds were mainly or exclusively present in female exudates and hence potential pheromone candidates. Copepodamide G, known to induce defensive responses in phytoplankton, was among the ten compounds of highest relative abundance in both male and female extracts. The presence of copepodamide G shows that the method can be used to capture and analyze chemical signals from living source organisms. We conclude that solid phase extraction in combination with metabolic profiling of exudates is a useful tool to develop our understanding of the chemical interplay between pelagic organisms.

# 1 **Solid phase extraction and metabolic profiling of exudates**

## 2 **from living copepods**

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21 **Abstract**-Copepods are ubiquitous in aquatic habitats. They exude bioactive compounds that  
22 mediate mate finding and induce defensive traits in prey organisms. Little is, however, known  
23 about the chemical nature of the copepod exometabolome that contributes to the chemical  
24 landscape in pelagic habitats. Here we describe the development of a closed loop solid phase  
25 extraction setup that allows for extraction of exuded metabolites from live copepods. We  
26 captured exudates from male and female *Temora longicornis* and analyzed the content with high  
27 resolution LC-MS. Chemometric methods revealed 87 compounds that constitute a specific  
28 chemical pattern either qualitatively or quantitatively indicating copepod presence. The majority  
29 of the compounds were present in both female and male exudates, but nine compounds were  
30 mainly or exclusively present in female exudates and hence potential pheromone candidates.  
31 Copepodamide G, known to induce defensive responses in phytoplankton, was among the ten  
32 compounds of highest relative abundance in both male and female extracts. The presence of  
33 copepodamide G shows that the method can be used to capture and analyze chemical signals  
34 from living source organisms. We conclude that solid phase extraction in combination with  
35 metabolic profiling of exudates is a useful tool to develop our understanding of the chemical  
36 interplay between pelagic organisms.

37

38 **Key Words**-Copepod exudate, Metabolomics, Infochemicals, *Temora longicornis*,  
39 Exometabolome.

40

**INTRODUCTION**

41 Copepods are the most abundant mesozooplankton in the oceans and constitute a central link  
42 between primary production and higher trophic levels in the pelagic food web (Mauchline et al.  
43 1998; Turner 2004). In addition, copepods change the chemical composition of the surrounding  
44 water by taking up and giving off chemical compounds. Common excretion products such as  
45 carbon dioxide, ammonium, urea, and dissolved organic carbon (DOC) released during “sloppy  
46 feeding” (Møller et al. 2003) dominate the chemical signature, but copepods also produce  
47 species- and gender-specific compounds that act as inter- and intraspecific signals. Female  
48 copepods attract males using sex pheromones (Heuschele & Selander 2014). Mate finding  
49 involves near field tracking of chemical trails and general search behavior triggered by  
50 background levels of female exudates (Doall et al. 1998; Heuschele & Kiørboe 2012). Sex  
51 pheromones increase mate encounter rates several fold compared to random encounters (Kiørboe  
52 2008).

53

54 Prey organisms sense copepod exudates and respond by expressing defensive traits. Colonial  
55 phytoplankton adjust colony size to evade predation (Bergkvist et al. 2012; Long et al. 2007 ),  
56 and harmful algal bloom-forming dinoflagellates increase toxin content (Selander et al. 2006;  
57 Wohlrab et al. 2010) or change swimming behavior to avoid copepod encounters (Jiang et al.  
58 2010; Selander et al. 2011). Phytoplankton exposed to copepod exudates becomes more resistant  
59 to grazing and harmful metabolites may affect a wide variety of organisms in the pelagic food  
60 web (Bergkvist et al. 2012; Long et al. 2007; Selander et al. 2006). Copepod exudates  
61 consequently modulate pelagic food webs in many unforeseen ways, and beyond the direct  
62 consumption of prey.

63

64 Despite the many documented effects of copepod exudate our understanding of the chemical  
65 composition is mainly limited to un-characterized dissolved organic matter and nitrogen  
66 excretion products (Breckels et al. 2013; Perez-Aragon et al. 2011; Saba et al. 2011). A group of  
67 eight taurine conjugated lipids that induce toxin formation in dinoflagellates, copepodamides, are  
68 hereto the only identified info-chemicals from copepod sources (Selander et al. 2015). Metabolic  
69 profiling provides an opportunity to characterize exudates from copepods in greater detail. In  
70 mass spectrometric metabolomic profiling the mass of all detectable compounds is recorded with  
71 high accuracy providing a list of exuded compounds of unknown identity but with a known  
72 molecular mass. Samples from different organisms or sexes can subsequently be cross referenced  
73 to identify compounds that are associated with an organism or a specific biological activity.  
74 Candidate compounds can then be tested for biological activity and active compounds  
75 structurally determined. As an example, the first diatom pheromone, diproline, was recently  
76 identified by comparing the metabolic profiles of exudates from sexual and asexual cultures of  
77 the diatom *Seminavis robusta* (Gillard et al. 2013). Diproline was found in higher concentrations  
78 in the sexual culture, and subsequently proven active in bioassays. The species examined here,  
79 *Temora longicornis*, has been suggested to produce sex pheromones based on the observation  
80 that males track the chemical wake of females (Doall et al. 1998). Males, however, sometimes  
81 track their own wakes, which suggest that the signal compound(s) guiding males may not be  
82 entirely sex specific. In fact, *Temora longicornis* males even pursue females of the partially  
83 sympatric species *Temora stylifera* showing that the signals to some extent overlap between  
84 species (Goetze 2008). Furthermore, *Temora longicornis* exude compound(s) that suppress chain  
85 formation in diatoms (Bergkvist et al. 2012). Together these observations make *Temora*  
86 *longicornis* an interesting target for exudate profiling.  
87

88 We designed a sampling devise to capture and analyze exudates from live male and female  
89 *Temora longicornis*. The main objective was to obtain more detailed information about the  
90 copepod exometabolome. We specifically looked for differences in exudate composition  
91 between the two sexes to test the hypothesis that the sexual dimorphism in copepod morphology  
92 and behavior (Gilbert & Williamson 1983) also manifest in terms of a gender specific exudate  
93 profile.

94

## 95 MATERIALS AND METHODS

### 96 *Sampling and Sorting of Copepods*

97 Copepods were collected with oblique tows from the surface to ~20 meters depth in the  
98 Kosterfjord outside Sven Lovén Centre for Marine Sciences, Tjärnö on the west coast of Sweden  
99 in January 2013. A 200- $\mu\text{m}$  mesh size WP-2 net equipped with a non-filtering cod end was used  
100 to reduce physical stress. The catch was directly transferred to the laboratory. Adult males and  
101 females were pipetted to separate jars under dissecting microscopes. The copepods were fed a  
102 mixture of *Rhodomonas* sp., *Chaetoceros* sp., and *Skeletonema* sp. before and between trials.

103

### 104 *Closed Loop Extraction of Copepod Exudates*

105 To obtain a low and constant background of dissolved organic compounds in the incubation  
106 water we prepared a batch of purified seawater (pSW) that was used in all incubations. The water  
107 (33 psu) was filtered through GF/F filter (Whatman) and pumped ( $\sim 7 \text{ ml min}^{-1}$ ) through two  
108 serial isolate ENV+ (Biotage) 500 mg solid phase extraction (SPE) columns. The SPE  
109 purification reduced the background of retainable DOC and increased the signal to noise ratio in  
110 copepod incubations considerably (data not shown). The pSW was stored refrigerated ( $\sim 5^\circ\text{C}$ ) in  
111 the dark.

112  
113 Animals for each replicate extraction were defecated for 30-45 minutes in 50-mL polypropylene  
114 cylinders with a 200- $\mu$ m nylon mesh bottom standing in glass beakers with pSW. This was done  
115 to decrease the contribution of compounds associated with fecal pellets in the samples. The mesh  
116 bottom cylinders were subsequently sequentially dipped in two freshly prepared glass beakers  
117 with pSW to eliminate carry over from culture water, feed algae, or fecal pellets. After the final  
118 rinse the copepods were gently flushed into an Erlenmeyer flask in 150 ml pSW. Exudates were  
119 stripped from the incubation water using a closed loop solid phase extraction system (Fig. 1). The  
120 number of animals used per incubation ranged from 239 to 619, with an average of 376  
121 individuals. The high density of copepods was necessary to obtain sufficient signal to noise ratio.  
122 Water was drawn through an ENV+ (Biotage 100 mg) solid phase extraction (SPE) column  
123 followed by a serially connected 100 mg C8 SPE (Biotage) by a peristaltic pump ( $\sim 1.5$  ml min<sup>-1</sup>).  
124 The ENV+ is a polystyrene resin functionalized with phenolic groups. It does not collapse in  
125 water and retains more polar compounds compared to the C8 column. The combination of ENV+  
126 and traditional silica based reversed phase (RP) packing has been successfully used to retain info-  
127 chemicals from parasitic copepods (Ingvarsdottir et al. 2002) and functionalized polystyrene  
128 resins generally retain a wider range of DOC from natural seawater samples compared to silica  
129 based RP resins (Dittmar et al. 2008). Columns were activated with 3 ml methanol followed by 5  
130 ml distilled water prior to use. The water from the pump was reintroduced into the incubation  
131 chamber through a 100 mg ENV+ column to minimize contamination from the pump or tubing  
132 (Fig. 1). The inlet to the serial columns was covered by a 30  $\mu$ m nylon plankton mesh to avoid  
133 copepods and eggs from entering the columns. Control samples were obtained as above, but  
134 without copepods in the incubations. We obtained five replicate extractions from each treatment  
135 (male, female, and control).



136  
137 At the end of the overnight (average incubation time 12 h) incubation the return line was  
138 removed and sampling continued until ~50 ml was left in the flask. The duration of the  
139 incubation was noted, and the columns removed. The columns were desalted with 1 mL MQ  
140 water and eluted with 1.5 ml 100 % LC-MS grade methanol (Lichrosolv, Merck) with a 30 s soak  
141 step after 0.75 ml to increase yield. The combined eluate (from the ENV+ and C8 SPE) was  
142 evaporated under a stream of nitrogen at 30 °C. The sample was resolved in 150 µl methanol and  
143 stored in sealed HPLC vials with inserts at -20 °C until use in bioassays and analysis.

144  
145 The copepods from each incubation were photographed (Canon 7D equipped with a 65 mm  
146 macro lens, Sigma) in a Petri dish on a light table for enumeration before transfer to larger  
147 volume beakers and supplemented with food. Used animals were allowed to feed during the day  
148 and were reused the following night. Freshly picked and reused animals were used in separate  
149 incubations. We could not see any systematic difference between fresh and reused copepods in  
150 the exudate profiles. Incubations were done in a dark thermostatic room at 10 °C.

151  
152 *Mass Spectrometry*

153 Metabolic profiling samples were analyzed using an Agilent 1100 HPLC equipped with a  
154 Lichrosphere 2.1 \*150 mm, 3 µm C18 silica column (Poroshell, Agilent) with a Q-ToF 6540 MS as  
155 detector. The eluent gradient started at 5% acetonitrile and 0.1% formic acid in water (Eluent A)  
156 that was maintained for one minute followed by a linear gradient up to 95% acetonitrile and 0.1%  
157 formic acid (Eluent B) over 14 minutes. 95% Acetonitrile was maintained for 4 minutes and the  
158 column re-equilibrated for 7 minutes in 100% eluent A before the following injection. Injection  
159 volume was 4 µl and eluent flow rate 250 µl min<sup>-1</sup>. Positive mass spectra were acquired in 4 GHz

160 High Resolution mode with 2 spectra  $s^{-1}$  sampled over a scan range of 80 to 1100  $m/z$ . ESI  
161 conditions were gas temperature 300 °C, drying gas 8  $l\ min^{-1}$ , nebulizer 40 psi, sheath gas  
162 temperature 350 °C, sheath gas flow 11  $l\ min^{-1}$ , nozzle voltage 500 V, fragmentor 120 V, and  
163 skimmer 65 V. Inspection of resulted TIC chromatograms showed that two samples (one control  
164 and one female) dramatically deviated from replicate samples and were excluded from further  
165 analysis.

166  
167 The same settings were used for tandem MS experiments targeting compound #10 with  $m/z$   
168 434.28 and collision energy set to 40 V.

#### 170 *Multivariate Analysis*

171 Mass spectra were de-convoluted by the molecular feature extraction algorithm (MFE) of the  
172 Agilent MassHunter Qualitative Analysis software (MH Qual., version B.05.00). MFE locates  
173 groups of co-variant ions, and each group represents a unique feature. The features are defined as  
174 time-aligned ions (i.e. isotopes, adducts, dehydrations, and/or dimers) summarized to the  
175 calculated neutral mass, possess an abundance and a retention time (RT), and are subsequently  
176 treated as compounds (Sana et al. 2008). We allowed for  $H^+$ ,  $Na^+$ ,  $K^+$  and  $NH_4^+$  adducts and a  
177 neutral dehydration. Compounds with absolute peak heights of 10,000 or higher were selected  
178 and stored as compound exchange format (CEF) files for compound alignment in Mass Profiler  
179 Professional (Agilent Technology, MPP, version B.12.01). Alignment parameters were 0.2 min  
180 RT window and 5 ppm + 2 mD mass tolerance. To reduce false-positive and false-negative  
181 detection rates from the untargeted MFE, we created a composite list of ions generated in MPP  
182 for a recursive, targeted analysis of raw data using the ‘find by ion’ algorithm of MH Qual. The  
183 settings for adduct ions, neutral mass, and aggregates (i.e. dimers) were the same as for MFE.

184 The recursive CEF files were re-imported into MPP for alignment using the previous setting. The  
185 aligned data were filtered by considering only compounds (i.e. metabolites) occurring in at least 3  
186 replicates within any experimental group, and the resulted aligned compound list was exported as  
187 a text file for further statistical analysis.

188  
189 Multivariate data analysis was performed with the software SIMCA (version 13.0.0.0, Umetrics,  
190 Sweden). Principal component analysis (PCA) was used to generate an overview and to analyze  
191 for systematic differences in metabolite content. The metabolite data were modeled and  
192 interpreted using orthogonal partial least squares with discriminant analysis (*OPLS-DA*) (Bylesjo  
193 et al. 2006; Trygg & Wold 2002). *OPLS-DA* is a supervised multivariate data projection method  
194 used to relate a set of predictor variables (*X* or metabolites in this study) to a response matrix (*Y*)  
195 that represents predefined sample classes (i.e. control, female and male copepods). This method  
196 can then be used to predict class identity and to extract specific features among the predictor  
197 variables (metabolites) distinguishing between the predefined sample classes. *OPLS* operates by  
198 separating the systematic variation in *X* into two parts: one that is linearly related to *Y*, and thus  
199 can be used to predict *Y*, and one that is orthogonal and uncorrelated to *Y*. To determine copepod  
200 specific compounds, *OPLS-DA* was carried out between controls and males in one model, and  
201 between controls and females in a separate model. Correlation coefficients obtained from the  
202 *OPLS-DA* models were used to identify metabolites significantly more abundant in males and/or  
203 females, using correlation coefficients greater or equal to 0.75 as a cut-off. The chromatographic  
204 peaks of the metabolites were visually inspected for each sample, and noise peaks together with  
205 peaks with high intensity in controls were removed. The resulting metabolite data set, normalized  
206 to the numbers of incubated copepods, was further analyzed with both *PCA* and *OPLS-DA* for  
207 systematic differences between male and female copepods. Statistically significant metabolites

208 related to differences between male and females were selected from both loadings plots showing  
209 *OPLS-DA*-derived correlation (Fig. 4A) and covariance (Fig. 4B) coefficients, the latter with  
210 jack-knifed 95 % confidence intervals calculated from cross validation (Wiklund et al. 2008). The  
211 correlation loading profile relates to the effect and reliability of each metabolite for class  
212 separation, and covariance loading profile relates to the contribution or magnitude of each  
213 metabolite. Thus, the combination of covariance and correlation information is an effective  
214 method of finding metabolites of interest (Wiklund et al. 2008).

215 The quality of the *OPLS-DA* models was assessed by the parameters  $R^2X$  and  $Q^2$ , which represent  
216 the total explained variations for X matrix and the model predictability, respectively (Bylesjo et  
217 al. 2006; Wiklund et al. 2008). The models were certified using a 7-fold cross -validation method  
218 and a permutation test, using the default option of SIMCA for cross-validation. A model was  
219 considered significant if the  $Q^2$  value was significant ( $P < 0.05$ ) through permutation. The data  
220 sets were Pareto-scaled prior to all *PCA* and *OPLS-DA*. For calculations of elemental  
221 compositions of specific metabolites, the molecular formula generator (MFG) algorithm included  
222 in the Agilent MassHunter Workstation software was used. MFG uses a wide range of MS  
223 information including accurate mass measurements, isotope abundances, and spacing between  
224 isotope peaks to produce a list of candidate molecular formulas that are ranked according to their  
225 relative probabilities (Sana et al. 2008).

226

### 227 *Bioassay of Exudate Samples*

228 To bioassay female exudate samples for sex pheromone activity, 50  $\mu\text{l}$  of the combined female  
229 exudates were dried down and resolved in 100  $\mu\text{l}$  pSW with slightly elevated (34 psu compared  
230 to the 33 psu of incubation water) salinity. 50  $\mu\text{l}$  of this solution was added to a 100  $\mu\text{l}$  pipet tip  
231 ending with a 0.25 mm  $\varnothing$  PEEK capillary to restrict flow. The pipette tip was allowed to empty

232 due to gravitation into a NUNC cell culture bottle (inner dimensions: 65x38x22 mm) with 55 ml  
233 seawater and 50 inexperienced male copepods. The setting was kept in the dark at 10 °C and  
234 illuminated from below with infrared light. The formation of stable scented trails was verified in  
235 test trials with fluorescent dye (fluorescein). Each replicate was filmed for 10 minutes with a  
236 camera (Sony DCR-TRV738E) directed towards the side of the aquaria (perpendicular to the  
237 light path of the infrared light providing dark field visualization of copepods) and analyzed for  
238 the occurrence of trail following behavior, i.e., the occurrence of fast directional swimming  
239 patterns coinciding with the location of the chemical trail. It is likely that only a fraction of the  
240 females were emitting pheromones, and that starvation and poor recoveries of compounds  
241 through sample preparation may have lowered the concentration further. To compensate for this  
242 the amount added over the 10 minutes corresponded to the exudates from 125 females over 12  
243 hours.

244

## 245 **RESULTS**

### 246 *Chemical Analysis*

247 The analyses revealed a high complexity of compounds with signals observed over the entire  
248 polarity range of the LC-gradient. With a few exceptions, the copepod-derived compounds were  
249 present in amounts not directly visible in the TIC chromatogram (Fig. 2). Despite the efforts to  
250 purify the medium, several signals were observed in the copepod-free controls, and these were  
251 excluded from further data evaluation as described below. Raw data in the form of an aligned  
252 peak list is available in supplemental information S1.

253

254 A *PCA* representation showed a clear separation between copepod samples and controls (Fig.  
255 3A). The first two principal components explain 21.3 and 19.7 % of the total variance,

256 respectively. Male and female exudates samples cluster together showing that differences  
257 between the sexes cannot be identified in this untreated data-set. Orthogonal partial least square  
258 discriminant analysis (*OPLS-DA*) showed a clear separation between control and male samples,  
259 as well as between control and female samples (table 1). The separation could be attributed to  
260 115 metabolites significantly more abundant in male and/or female copepod exudate samples  
261 compared to controls (correlation coefficients  $\geq 0.75$ ), and removal of noise peaks and peaks that  
262 were also present in relatively high intensity in copepod-free controls decreased the number to  
263 87. *PCA* on this reduced metabolite data set revealed differences also between male and female  
264 copepod samples (Fig. 3B), with 71.0 and 10.9 % of the variance explained by the two principal  
265 components. To identify the metabolites responsible for the differentiation between female and  
266 male samples, loading plots with *OPLS-DA*-derived correlation and covariance coefficients with  
267 jack-knifed 95 % confidence intervals were generated (Fig. 4A, B, table 1). The resulting plots  
268 showed that exudation of metabolites normalized to the number of copepods were higher for  
269 females compared to males (Fig. 4B, positive values indicates more in female than male  
270 samples). The difference was mainly quantitative and only two metabolites were unique for  
271 female exudates. A single metabolite had higher abundance in male exudates, suggested by  
272 negative correlation and covariance coefficients with jack-knifed confidence interval not  
273 overlapping zero. That metabolite, however, had a relatively low correlation coefficient. Most of  
274 the metabolites were only slightly more abundant in female samples indicated by their low  
275 covariance, but seven metabolites were distinct in that they had high covariance and correlation  
276 (Fig. 4A, B).

277 A complete compound list showing all 87 metabolites considered to be of copepod origin is given  
278 in table 2. The seven compounds most strongly associated with female copepods are highlighted

279 together with the two compounds exclusively found in female samples. As male *Temoras* are  
280 known to track female wakes we tested the exudates in a bioassay monitoring mate finding  
281 behavior in males exposed to an artificial trail tainted with the female exudates. No mate  
282 tracking behaviors were, however, observed in the trail following bioassays of the combined  
283 ENV+ and C8 SPE extracts indicating that other or additional compounds are involved in mate  
284 finding in *Temora*.

285  
286 The most evident copepod compound in the TIC chromatogram, compound 10 (Fig. 1, table 2)  
287 with high relative abundance in both male and female exudates had an accurate mass and  
288 retention time (13.1 min) matching that of dehydrated copepodamide G (table 2 compound 10,  
289 m/z 433.2865, [M-H<sub>2</sub>O], Δ 0.7 ppm). Similar to copepodamide G compound 10 also showed an  
290 additional dehydration (m/z 416.2833, [M-2H<sub>2</sub>O+H]<sup>+</sup>, Δ 0.5 ppm) in positive mode. MS/MS  
291 experiments further revealed a fragment of m/z 126.0219, indicating the presence of taurine  
292 (C<sub>2</sub>H<sub>8</sub>NO<sub>3</sub>S, Δ 4.7 ppm) also present in Copepodamide G (Fig. 5, Selander et al. 2015).

293

## 294 DISCUSSION

295 Copepods release only minute amounts of metabolites into their environment. To overcome the  
296 limitations associated with the analytics of such dilute cues we developed a closed loop  
297 extraction technique with serial solid phase extraction columns to cover a wide polarity range.  
298  
299 Metabolic profiling did provide more comprehensive information about the copepod  
300 exometabolome than has previously been available. The approach is especially useful for small

301 and manually sorted organisms as mass spectrometry is a sensitive technique that requires little  
302 material. Yet, the method has some significant constraints. Only compounds that are retained and  
303 detectable will be included in the analysis. The most polar metabolites and proteins would for  
304 example not be recovered on the column resins used here. By using different types of columns  
305 resins, more targeted extractions can be envisioned that could be paired with adapted LC  
306 separation techniques. Sequestering the more polar analytes in sea water is a challenge that can  
307 be addressed, e.g., by extraction and separation using hydrophilic/lipophilic balanced interaction  
308 chromatography (Spielmeyer & Pohnert 2010). Anion and cation exchange may also be useful,  
309 especially in fresh water organisms, as the high salt concentration in seawater will compromise  
310 its function. Collection over several hours enables high extraction success and covers metabolites  
311 that are released during brief periods. At the same time, sampling over long time periods may  
312 increase the risk of degradation and confounding effects of starvation. Analyte sequestering time  
313 is consequently a tradeoff between quantity and quality that will have to be carefully considered  
314 for each individual case.

315  
316 Despite the fact that we pre-purify instrumentation and depleted seawater in organic constituents  
317 by solid phase purification we detected only minor amounts of copepod-released metabolites  
318 compared to background signals (Fig. 1). This is typical for plankton studies, where low  
319 abundance signals are encountered in a complex matrix (Barofsky et al. 2009; Vidoudez &  
320 Pohnert 2012). For organisms that tolerate crowding, it is possible to improve the signal to noise  
321 ratio by reducing the incubation volume e.g. by keeping organisms in the SPE reservoir. Weber  
322 and colleagues (2013) found that isotope labelling of autotrophic organisms enhance the  
323 possibility to filter out low concentration compounds from the labeled organism from complex



324 chemical backgrounds. This could in principle be done with copepods too, but the labeling  
325 procedure requires copepods reared on isotope enriched food.

326  
327 The most probable elemental composition was calculated for each metabolite, based on the  
328 accurate mass and isotope distribution pattern. Full structure elucidation requires more  
329 experiments and often more materials. Here we only identified the most obvious peak in the  
330 chromatogram; compound 10 (table 2, Fig. 5) as copepodamide G. The copepodamides are  
331 known to induce toxin production in harmful algal bloom forming dinoflagellates. Their presence  
332 here demonstrates that the method is indeed sensitive enough to sequester and analyse  
333 infochemicals. Copepodamide G was among the ten compounds of highest relative abundance in  
334 both sexes which further indicates that copepodamides may be among the leading features of the  
335 copepod exometabolome.

336  
337 The hypothesized difference between male and female exudates was present but subtle. Only two  
338 out of the 87 compounds were exclusively found in female exudates (table 2). Most compounds  
339 were, however, more abundant in female exudates, possibly reflecting higher metabolic turnover  
340 in the larger and egg-producing females. The high level of similarity between male and female  
341 exudates may contribute to the low specificity described for mate finding behavior in *Temora* and  
342 other copepods, where males and heterospecifics are sometimes followed by males (Goetze 2008;  
343 Goetze & Kiørboe 2008).

344  
345 Female exudates did not trigger mate search behavior in male *T. longicornis*, which suggests that  
346 other or additional compounds are needed to trigger mate attraction. A lack of response could  
347 result if pheromones were not retained, or did not survive sample preparation. It could also result

348 if the *Temora* females were recently mated and therefore not emitted pheromones (Heuschele &  
349 Kiørboe 2012), or if males were not responsive. Thus, it is not possible to exclude that the  
350 metabolites listed here may be involved in mate finding in *T. longicornis*. Copepod pheromones  
351 can be predicted to be of low molecular weight as smaller molecules form longer trails per unit  
352 mass exuded (Bagoien & Kiørboe 2005). Aquatic info-chemicals, however, encompass diverse  
353 classes of molecules, and molecular masses ranging from a molecular weight below 100 Da to  
354 protein signals larger than 31.5 kDa (Pohnert et al. 2007). Metabolomic approaches targeting  
355 unknown infochemicals should consequently aim to cover as much as possible of the  
356 exometabolome to be useful.

357  
358 Chemical interactions in the aquatic realm sometimes supersede the direct trophic interactions  
359 between organisms (Cyr & Pace 1993; Hay 2009; Preisser et al. 2005). Deciphering the chemical  
360 interplay between plankton organisms is consequently key to further our understanding of the  
361 pelagic ecosystem. Furthermore, insights to chemical signaling opens up applied possibilities  
362 such as design of artificial baits, pest management, and aqua culture tools similar to the  
363 development in terrestrial chemical ecology.

364  
365 In summary, the closed loop solid phase extraction and metabolomic profiling of exudates from  
366 living copepods did provide detailed information on the composition of the copepod  
367 exometabolome. The high relative abundance of copepodamide G shows that the method can be  
368 used to target info-chemicals from small plankton organisms. There is a subtle difference in the  
369 composition of male and female exudates, but negative trail following assays indicates that the  
370 sex pheromone of *Temora longicornis* involved other or additional compounds. Future studies

371 should strive to include more polar compounds, larger compounds, and volatiles to create a more  
372 comprehensive view of the copepod exometabolome.

373

#### 374 **Acknowledgements**

375 See publisher's webpage.

376

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**Table 1**

**Table 1** Parameter values of OPLS-DA models.

OPLS-DA model	No <sup>a</sup>	R2X(cum) <sup>b</sup>	R2X <sup>c</sup>	Q2(cum) <sup>d</sup>
control vs. male	1P + 2O	0.537	0.213	0.785
control vs. female	1P + 2O	0.631	0.292	0.823
male vs. female	1P + 2O	0.874	0.465	0.87

<sup>a</sup> No, the number of predictive (P) and orthogonal (O) components.

<sup>b</sup> The R2X(cum) value is the predictive and orthogonal variation in model samples (X) explained by the model.

<sup>c</sup> The R2X value is the amount of variation in X which is correlated to Y (response matrix)

<sup>d</sup> The Q2(cum) value describes the predictive ability of the model, based on sevenfold-cross validation.

**Table 2**

**Table 2** Metabolites from female and male *Temora longicornis*. The two compounds exclusively found in female exudates are indicated in red, and metabolites that are significantly more abundant in females but also present in male exudates in grey.

No.	RT (min)	Mass <sup>1</sup>	Fold change <sup>2</sup>	Elemental composition with scores <sup>3</sup>
1	1.59	136.0382	0.5	C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O (98.0), C <sub>4</sub> H <sub>8</sub> O <sub>5</sub> (93.6)
2	1.70	152.0331	0.4	C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O <sub>2</sub> (98.0), C <sub>4</sub> H <sub>8</sub> O <sub>6</sub> (92.4)
3	4.86	284.1373	0.9	C <sub>13</sub> H <sub>20</sub> N <sub>2</sub> O <sub>5</sub> (99.6), C <sub>14</sub> H <sub>16</sub> N <sub>6</sub> O (93.2), C <sub>8</sub> H <sub>17</sub> N <sub>10</sub> P (91.8)
4	6.17	304.1495	7.4	C <sub>10</sub> H <sub>20</sub> N <sub>6</sub> O <sub>5</sub> (98.9), C <sub>11</sub> H <sub>16</sub> N <sub>10</sub> O (91.8), C <sub>12</sub> H <sub>25</sub> N <sub>4</sub> OPS (91.0)
5	6.75	264.1473	1.6	C <sub>14</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub> (98.8)
6	9.34	163.0993	0.8	C <sub>10</sub> H <sub>13</sub> NO (98.8)
7	10.61	230.1514	1.6	C <sub>12</sub> H <sub>22</sub> O <sub>4</sub> (99.0)
8	11.88	334.2144	0.3	C <sub>20</sub> H <sub>30</sub> O <sub>4</sub> (99.2), C <sub>21</sub> H <sub>26</sub> N <sub>4</sub> (92.4), C <sub>14</sub> H <sub>31</sub> N <sub>4</sub> O <sub>3</sub> P (90.5)
9	12.76	380.2533	1.6	N/A
10	13.08	433.2863	0.9	C <sub>22</sub> H <sub>43</sub> NO <sub>5</sub> S (99.1), C <sub>23</sub> H <sub>39</sub> N <sub>5</sub> OS (95.2), C <sub>17</sub> H <sub>40</sub> N <sub>9</sub> PS (95.1)
11	13.08	505.2166	0.9	C <sub>23</sub> H <sub>39</sub> NO <sub>7</sub> S <sub>2</sub> (94.6), C <sub>24</sub> H <sub>43</sub> NO <sub>2</sub> S <sub>4</sub> (92.9), C <sub>27</sub> H <sub>41</sub> NP <sub>2</sub> S <sub>2</sub> (90.1)
12	14.15	765.4143	1.3	C <sub>36</sub> H <sub>63</sub> NO <sub>16</sub> (99.5), C <sub>31</sub> H <sub>60</sub> N <sub>9</sub> O <sub>11</sub> P (98.5), C <sub>34</sub> H <sub>51</sub> N <sub>15</sub> O <sub>6</sub> (98.4)
13	14.23	362.2431	1.7	C <sub>18</sub> H <sub>30</sub> N <sub>6</sub> O <sub>2</sub> (95.5), C <sub>19</sub> H <sub>41</sub> P <sub>3</sub> (93.9)
14	14.97	364.2599	1.2	C <sub>16</sub> H <sub>37</sub> N <sub>4</sub> O <sub>3</sub> P (97.8), C <sub>18</sub> H <sub>32</sub> N <sub>6</sub> O <sub>2</sub> (93.7), C <sub>22</sub> H <sub>36</sub> O <sub>4</sub> (90.1)
15	14.98	661.1729	1.2	C <sub>25</sub> H <sub>41</sub> ClNO <sub>13</sub> PS (96.1), C <sub>30</sub> H <sub>47</sub> ClNOP <sub>3</sub> S <sub>3</sub> (95.7), C <sub>20</sub> H <sub>36</sub> O <sub>3</sub> (98.3)
16	15.20	364.2592	1.3	C <sub>18</sub> H <sub>32</sub> N <sub>6</sub> O <sub>2</sub> (98.3)
17	15.20	364.2592	1.4	C <sub>18</sub> H <sub>32</sub> N <sub>6</sub> O <sub>2</sub> (98.3), C <sub>19</sub> H <sub>43</sub> P <sub>3</sub> (94.3), C <sub>16</sub> H <sub>37</sub> N <sub>4</sub> O <sub>3</sub> P (92.7)
18	15.73	364.2589	1.4	C <sub>18</sub> H <sub>32</sub> N <sub>6</sub> O <sub>2</sub> (97.2), C <sub>19</sub> H <sub>43</sub> P <sub>3</sub> (94.4)
19	16.82	174.1042	1.7	N/A
20	16.82	602.3516	2.1	C <sub>33</sub> H <sub>46</sub> N <sub>8</sub> OS (93.0)
21	16.82	367.1243	1.5	N/A
22	16.82	226.1720	1.7	C <sub>17</sub> H <sub>22</sub> (99.0)
23	16.82	176.1194	1.6	N/A
24	16.82	312.1701	1.9	C <sub>16</sub> H <sub>20</sub> N <sub>6</sub> O (99.1), C <sub>16</sub> H <sub>28</sub> N <sub>2</sub> S <sub>2</sub> (96.6), C <sub>13</sub> H <sub>29</sub> O <sub>6</sub> P (95.3)
25	16.82	130.0778	1.7	N/A
26	16.82	272.1775	1.8	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub> (99.6), C <sub>12</sub> H <sub>25</sub> N <sub>4</sub> OP (91.3)
27	16.82	134.0725	1.6	N/A
28	16.82	634.2966	2.2	C <sub>38</sub> H <sub>50</sub> O <sub>2</sub> S <sub>3</sub> (96.5), C <sub>32</sub> H <sub>51</sub> N <sub>4</sub> OPS <sub>3</sub> (95.8), C <sub>30</sub> H <sub>46</sub> N <sub>6</sub> O <sub>5</sub> S <sub>2</sub> (94.0)
29	16.82	132.0943	1.7	N/A
30	16.82	344.1074	2.1	C <sub>19</sub> H <sub>20</sub> O <sub>4</sub> S (93.2)
31	16.82	618.3222	1.8	C <sub>36</sub> H <sub>47</sub> N <sub>2</sub> O <sub>5</sub> P (97.9), C <sub>34</sub> H <sub>50</sub> O <sub>8</sub> S (96.6), C <sub>38</sub> H <sub>52</sub> OP <sub>2</sub> S (96)
32	16.82	118.0778	1.1	C <sub>9</sub> H <sub>10</sub> (92.7)
33	16.82	158.1093	1.6	N/A
34	16.82	908.5126	20.9	C <sub>52</sub> H <sub>78</sub> O <sub>9</sub> P <sub>2</sub> (99.4), C <sub>53</sub> H <sub>74</sub> N <sub>4</sub> O <sub>5</sub> P <sub>2</sub> (98.7), C <sub>46</sub> H <sub>64</sub> N <sub>14</sub> O <sub>6</sub> (98.6)

35	16.82	144.0935	1.7	N/A
36	16.82	146.1091	1.7	N/A
37	16.82	186.1402	1.6	N/A
38	16.82	254.1667	1.6	C <sub>18</sub> H <sub>22</sub> O (99.1)
39	16.83	328.1416	1.4	C <sub>20</sub> H <sub>25</sub> PS (95.4)
40	16.83	92.0621	1.4	N/A
41	17.21	274.1937	1.6	C <sub>18</sub> H <sub>26</sub> O <sub>2</sub> (98.7)
42	17.73	300.2092	1.0	C <sub>20</sub> H <sub>28</sub> O <sub>2</sub> (97.2)
43	18.05	610.2795	0.8	C <sub>35</sub> H <sub>38</sub> N <sub>4</sub> O <sub>6</sub> (99.5), C <sub>37</sub> H <sub>45</sub> N <sub>2</sub> P <sub>3</sub> (98.7), C <sub>36</sub> H <sub>34</sub> N <sub>8</sub> O <sub>2</sub> (97.8)
44	18.31	300.2085	1.1	N/A
45	18.79	326.2249	0.7	C <sub>22</sub> H <sub>30</sub> O <sub>2</sub> (94.9)
46	19.00	435.3027	1.1	C <sub>23</sub> H <sub>41</sub> N <sub>5</sub> OS (94.0), C <sub>24</sub> H <sub>42</sub> N <sub>3</sub> O <sub>2</sub> P (90.4), C <sub>22</sub> H <sub>45</sub> NO <sub>5</sub> S (90.4)
47	20.80	274.1943	1.7	C <sub>18</sub> H <sub>26</sub> O <sub>2</sub> (92.0)
48	21.51	552.3253	1.4	C <sub>38</sub> H <sub>40</sub> N <sub>4</sub> (98.5), C <sub>37</sub> H <sub>44</sub> O <sub>4</sub> (96.8), C <sub>30</sub> H <sub>46</sub> N <sub>6</sub> P <sub>2</sub> (96.4)
49	21.63	622.4023	0.7	C <sub>42</sub> H <sub>54</sub> O <sub>4</sub> (99.6), C <sub>43</sub> H <sub>50</sub> N <sub>4</sub> (96.7), C <sub>36</sub> H <sub>55</sub> N <sub>4</sub> O <sub>3</sub> P (95.1)
50	21.99	598.4036	0.6	C <sub>41</sub> H <sub>50</sub> N <sub>4</sub> (98.6), C <sub>40</sub> H <sub>54</sub> O <sub>4</sub> (97.0), C <sub>33</sub> H <sub>56</sub> N <sub>6</sub> P <sub>2</sub> (96.0)
51	22.70	534.2645	2.4	C <sub>31</sub> H <sub>39</sub> N <sub>2</sub> O <sub>4</sub> P (99.8), C <sub>33</sub> H <sub>34</sub> N <sub>4</sub> O <sub>3</sub> (95.3), C <sub>32</sub> H <sub>35</sub> N <sub>6</sub> P (95.1)
52	23.11	595.1772	1.7	C <sub>36</sub> H <sub>26</sub> ClN <sub>5</sub> O <sub>2</sub> (93.1), C <sub>36</sub> H <sub>34</sub> ClNOS <sub>2</sub> (92.2), C <sub>29</sub> H <sub>26</sub> ClN <sub>11</sub> S (91.5)
53	23.54	534.3936	2.0	C <sub>33</sub> H <sub>50</sub> N <sub>4</sub> O <sub>2</sub> (94.8), C <sub>32</sub> H <sub>54</sub> O <sub>6</sub> (91.4), C <sub>31</sub> H <sub>55</sub> N <sub>2</sub> O <sub>3</sub> P (90.5)
54	24.07	560.4090	1.6	C <sub>35</sub> H <sub>52</sub> N <sub>4</sub> O <sub>2</sub> (99.4), C <sub>34</sub> H <sub>56</sub> O <sub>6</sub> (96.1), C <sub>33</sub> H <sub>57</sub> N <sub>2</sub> O <sub>3</sub> P (93.3)
55	24.21	596.3877	1.6	C <sub>41</sub> H <sub>48</sub> N <sub>4</sub> (98.8), C <sub>40</sub> H <sub>52</sub> O <sub>4</sub> (97.4), C <sub>33</sub> H <sub>54</sub> N <sub>6</sub> P <sub>2</sub> (94.3)
56	24.48	581.3995	1.2	N/A
57	24.47	596.4063	N/A	C <sub>34</sub> H <sub>48</sub> N <sub>10</sub> (98.3), C <sub>31</sub> H <sub>57</sub> N <sub>4</sub> O <sub>5</sub> P (97.6), C <sub>33</sub> H <sub>52</sub> N <sub>6</sub> O <sub>4</sub> (96.5)
58	24.73	563.3923	1.5	N/A
59	24.73	546.3871	N/A	C <sub>40</sub> H <sub>50</sub> O (92.0)
60	24.82	652.2914	1.4	C <sub>38</sub> H <sub>36</sub> N <sub>8</sub> O <sub>3</sub> (98.5), C <sub>37</sub> H <sub>52</sub> O <sub>2</sub> P <sub>4</sub> (97.1), C <sub>35</sub> H <sub>45</sub> N <sub>2</sub> O <sub>8</sub> P (96.8)
61	24.83	581.4016	1.2	C <sub>43</sub> H <sub>51</sub> N (91.2)
62	25.02	565.4044	1.5	C <sub>38</sub> H <sub>51</sub> N <sub>3</sub> O (95.1), C <sub>36</sub> H <sub>56</sub> NO <sub>2</sub> P (95)
63	25.04	640.4123	0.7	C <sub>36</sub> H <sub>57</sub> N <sub>4</sub> O <sub>4</sub> P (99.1), C <sub>37</sub> H <sub>53</sub> N <sub>8</sub> P (97.4), C <sub>34</sub> H <sub>62</sub> N <sub>2</sub> O <sub>5</sub> P <sub>2</sub> (97.4)
64	25.16	565.4049	2.1	C <sub>36</sub> H <sub>56</sub> NO <sub>2</sub> P (98.7), C <sub>38</sub> H <sub>51</sub> N <sub>3</sub> O (94.9), C <sub>30</sub> H <sub>57</sub> N <sub>5</sub> OP <sub>2</sub> (90.4)
65	25.16	548.4030	2.9	C <sub>40</sub> H <sub>52</sub> O (96.8), C <sub>32</sub> H <sub>58</sub> N <sub>2</sub> OP <sub>2</sub> (90.5)
66	25.37	588.4407	1.6	C <sub>37</sub> H <sub>56</sub> N <sub>4</sub> O <sub>2</sub> (98.2), C <sub>35</sub> H <sub>61</sub> N <sub>2</sub> O <sub>3</sub> P (93.6), C <sub>36</sub> H <sub>60</sub> O <sub>6</sub> (92.6)
67	25.59	639.4035	0.4	N/A
68	25.64	563.3889	1.9	C <sub>36</sub> H <sub>54</sub> NO <sub>2</sub> P (98.5), C <sub>38</sub> H <sub>49</sub> N <sub>3</sub> O (95.9)
69	25.72	639.4068	0.5	N/A
70	25.88	790.4619	1.4	N/A
71	25.89	785.5103	1.5	C <sub>47</sub> H <sub>63</sub> N <sub>9</sub> O <sub>2</sub> (98.5), C <sub>48</sub> H <sub>74</sub> N <sub>3</sub> P <sub>3</sub> (98.0), C <sub>44</sub> H <sub>72</sub> N <sub>3</sub> O <sub>7</sub> P (97.9)
72	26.09	981.6413	2.0	C <sub>52</sub> H <sub>92</sub> N <sub>3</sub> O <sub>12</sub> P (99.4), C <sub>47</sub> H <sub>89</sub> N <sub>11</sub> O <sub>7</sub> P <sub>2</sub> (98.8), C <sub>48</sub> H <sub>100</sub> N <sub>5</sub> O <sub>5</sub> P <sub>5</sub> (98.6)
73	26.09	986.5968	2.0	C <sub>53</sub> H <sub>74</sub> N <sub>14</sub> O <sub>5</sub> (99.0), C <sub>60</sub> H <sub>84</sub> N <sub>4</sub> O <sub>4</sub> P <sub>2</sub> (98.7), C <sub>55</sub> H <sub>86</sub> O <sub>15</sub> (97.7)
74	26.20	787.5238	1.8	C <sub>45</sub> H <sub>73</sub> NO <sub>10</sub> (97.7), C <sub>40</sub> H <sub>70</sub> N <sub>9</sub> O <sub>5</sub> P (96.1), C <sub>40</sub> H <sub>80</sub> N <sub>5</sub> O <sub>2</sub> P <sub>3</sub> S (95.7)
75	26.61	366.3292	0.9	C <sub>27</sub> H <sub>42</sub> (97.7)
76	26.72	605.4007	1.5	C <sub>45</sub> H <sub>51</sub> N (93.6)
77	26.75	835.5826	1.8	C <sub>58</sub> H <sub>78</sub> NOP (98.5), C <sub>50</sub> H <sub>84</sub> N <sub>3</sub> OP <sub>3</sub> (98.3), C <sub>49</sub> H <sub>73</sub> N <sub>9</sub> O <sub>3</sub> (97.8)

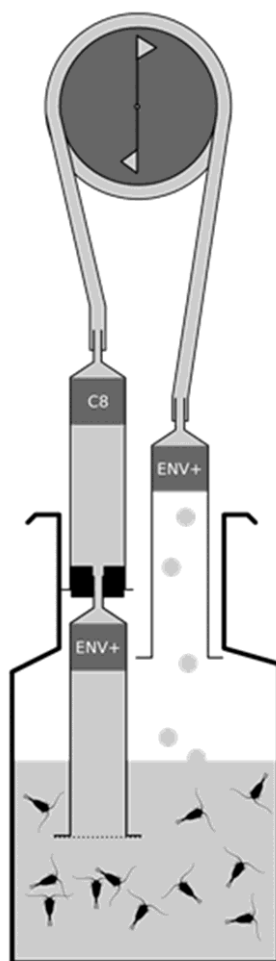


78	27.43	739.5623	1.8	C <sub>43</sub> H <sub>83</sub> NO <sub>2</sub> P <sub>2</sub> S (99), C <sub>36</sub> H <sub>73</sub> N <sub>11</sub> O <sub>3</sub> S (96.4), C <sub>39</sub> H <sub>81</sub> NO <sub>9</sub> S (96.2)
79	27.74	384.3400	1.4	C <sub>27</sub> H <sub>44</sub> O (93.0)
80	27.99	549.4112	2.5	N/A
81	28.01	652.5047	1.9	C <sub>39</sub> H <sub>75</sub> OP <sub>3</sub> (96.0), C <sub>36</sub> H <sub>69</sub> N <sub>4</sub> O <sub>4</sub> P (95.3), C <sub>35</sub> H <sub>73</sub> O <sub>8</sub> P (94.3)
82	28.35	565.4058	2.1	N/A
83	28.38	549.4112	2.3	C <sub>36</sub> H <sub>56</sub> NOP (95.7), C <sub>35</sub> H <sub>55</sub> N <sub>3</sub> S (90.2)
84	28.75	565.4719	1.9	C <sub>35</sub> H <sub>59</sub> N <sub>5</sub> O (93.4), C <sub>33</sub> H <sub>64</sub> N <sub>3</sub> O <sub>2</sub> P (92.8), C <sub>34</sub> H <sub>63</sub> NO <sub>5</sub> (91.8)
85	29.03	428.3664	1.6	C <sub>29</sub> H <sub>48</sub> O <sub>2</sub> (95.3)
86	29.86	916.5733	2.2	C <sub>54</sub> H <sub>81</sub> N <sub>2</sub> O <sub>8</sub> P (98.6), C <sub>49</sub> H <sub>88</sub> N <sub>6</sub> P <sub>4</sub> S (97.9), C <sub>52</sub> H <sub>84</sub> O <sub>11</sub> S (97.4)
87	29.86	937.5355	2.1	N/A

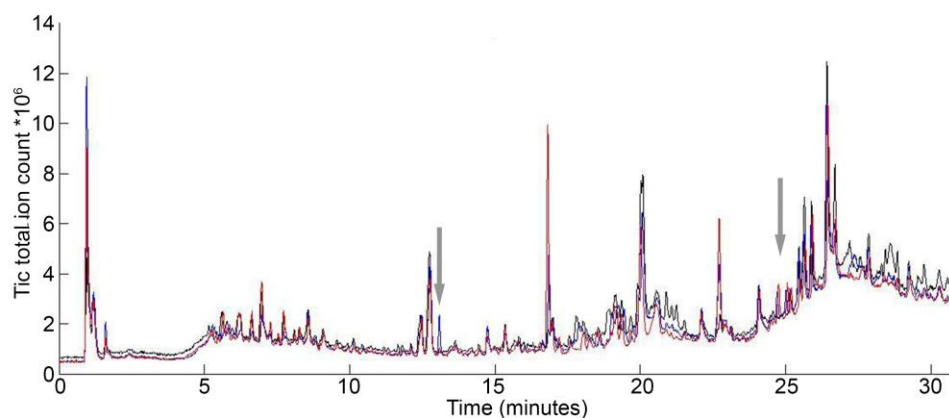
<sup>1</sup> Calculated neutral mass

<sup>2</sup> Fold change female/male. Metabolite 57 and 59 were not detectable for males

<sup>3</sup> The three highest ranked candidate molecular formulas. Only candidates with scores  $\geq 90$  were included

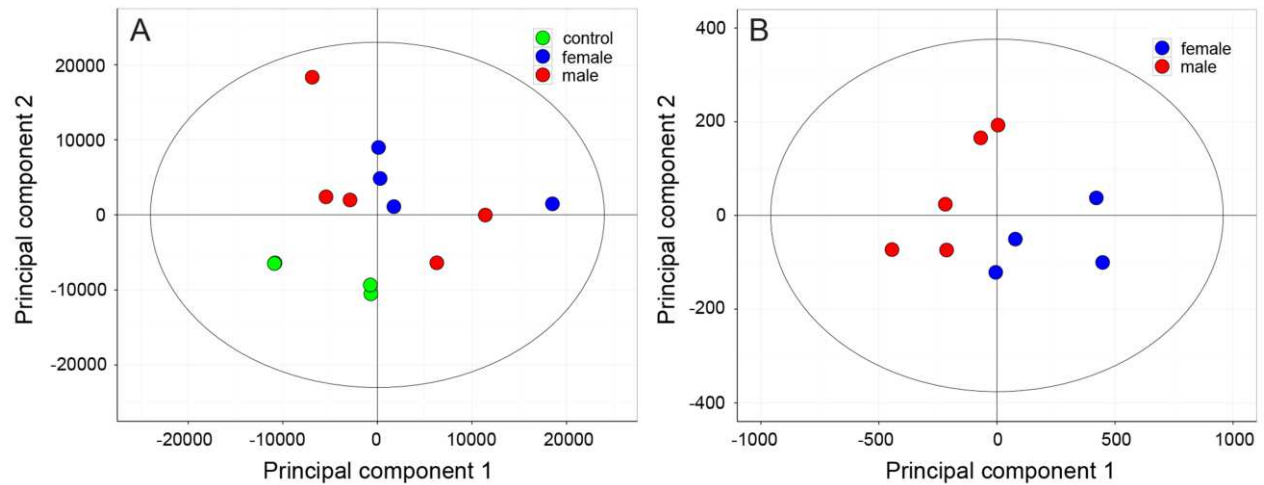
**Figure 1**

**Figure 1** Sampling device used to collect exudates from copepods. Water is drawn through the partially immersed ENV+ and the following C8 SPE before reintroduced into the incubation chamber. The ENV+ column on the outlet end serves to minimize contamination from pump or hosing. The hatched line in the opening of the first SPE column represents a 30  $\mu\text{m}$  nylon plankton mesh that prevents copepods and copepod eggs from entering the column.

**Figure 2****Figure 2**

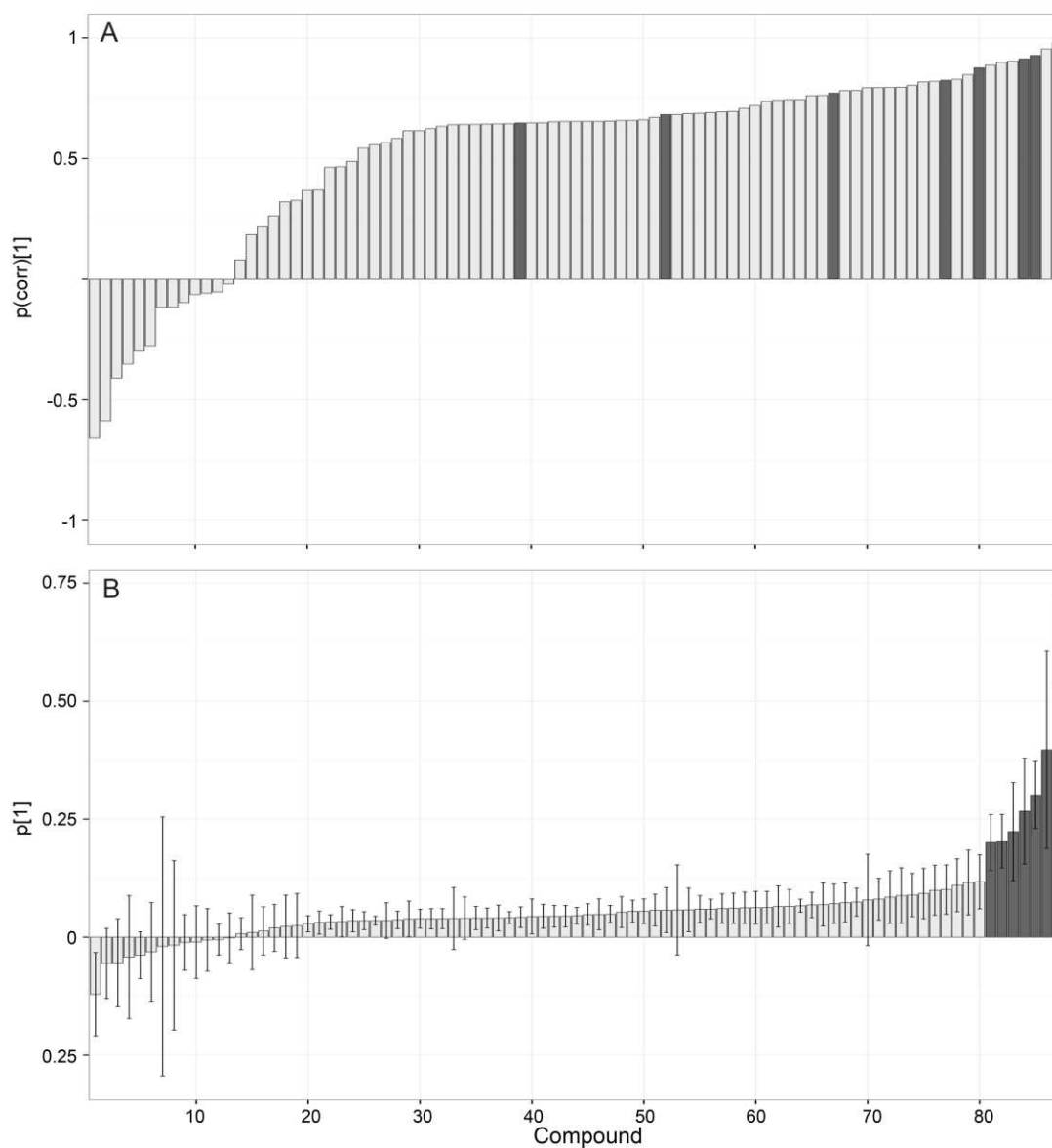
Representative chromatograms from male exudates (blue), female exudates (red), and copepod free controls (black). Prominent peaks exclusive to copepod samples were always visible after 13 minutes (compound 10 in table 2 with a monoisotopic mass of 433.29) as well as at 24-25 minutes (compounds 58 and 63 in table 1, monoisotopic mass 563.39 and 640.41). The majority of the 87 compounds detected were, however, too low in concentration to be observed without extracting specific masses.

Figure 3

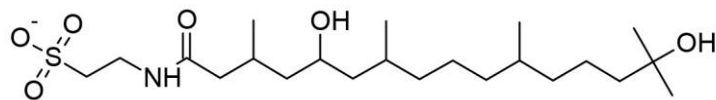


**Figure 3 A:** PCA based on Pareto-scaled raw data showing the separation between sea water controls (green), male (red) and female (blue) exudate samples. **B:** PCA on the reduced data-set containing only compounds indicated to be of copepod origin in the control-male and control-female OPLS-DA analysis show a systematic difference also between male and female exudates.

Figure 4



**Figure 4** Differences between male and female exudates compound by compound. The OPLS-DA loading plots of metabolites exuded from female and male copepods. The loading plot **A** represents the effect and reliability (correlation,  $p(\text{corr})[1]$ ), and **B** the contribution or magnitude (covariance,  $p[1]$ ) of each metabolite to class separation. Jack-knifed confidence intervals (95 %) in the covariance plot **B** were calculated from cross-validation. Metabolites with high  $p(\text{corr})[1]$  and  $p[1]$  values are shaded.

**Figure 5****Figure 5** Structure of copepodamide G (compound 10).