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Solid phase extraction and metabolic profiling of exudates from living copepods

Erik Selander, Jan Heuschele, Göran M Nylund, Georg Pohnert, Henrik Pavia, Oda Bjærke, Larisa Pender-Healy, Peter Tiselius, Thomas Kiørboe

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.

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Solid phase extraction and metabolic profiling of exudates from living copepods

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Abstract-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.

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

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INTRODUCTION

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

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

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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

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

MATERIALS AND METHODS

Sampling and Sorting of Copepods

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

Closed Loop Extraction of Copepod Exudates

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

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112

113 Animals for each replicate extraction were defecated for 30-45 minutes in 50-mL polypropylene
114 cylinders with a 200-µm 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 (~ 1.5 ml min^{-}
124 ^{1}). 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 µm 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).

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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 Licrosphere 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^{-1}. Positive mass spectra were acquired in 4 GHz

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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 ºC, drying gas 8 l min^{-1}, nebulizer 40 psi, sheath gas
162 temperature 350 ºC, 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.
169
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.

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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

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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 µl of the combined female
229 exudates were dried down and resolved in 100 µl pSW with slightly elevated (34 psu compared
230 to the 33 psu of incubation water) salinity. 50 µl of this solution was added to a 100 µl pipet tip
231 ending with a 0.25 mm ø PEEK capillary to restrict flow. The pipette tip was allowed to empty

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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 stabile scented trails was verified in
235 test trials with fluorescent dye (fluorescin). 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,

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

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

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

The most evident copepod compound in the TIC chromatogram, compound 10 (Fig. 1, table 2) with high relative abundance in both male and female exudates had an accurate mass and retention time (13.1 min) matching that of dehydrated copepodamide G (table 2 compound 10, m/z 433.2865, [M-H2O], Δ 0.7 ppm). Similar to copepodamide G compound 10 also showed an additional dehydration (m/z 416.2833, $[M-2H_2O+H]^+$, Δ 0.5 ppm) in positive mode. MS/MS experiments further revealed a fragment of m/z 126.0219, indicating the presence of taurine ($C_2H_8NO_3S$, Δ 4.7 ppm) also present in Copepodamide G (Fig. 5, Selander et al. 2015).

DISCUSSION

Copepods release only minute amounts of metabolites into their environment. To overcome the limitations associated with the analytics of such dilute cues we developed a closed loop extraction technique with serial solid phase extraction columns to cover a wide polarity range.

Metabolic profiling did provide more comprehensive information about the copepod exometabolome than has previously been available. The approach is especially useful for small

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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

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

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

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

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

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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

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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.

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Table 1

Table 1 Parameter values of OPLS-DA models.

OPLS-DA model	No[a]	R2X(cum)[b]	R2X[c]	Q2(cum)[d]
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

[a] No, the number of predictive (P) and orthogonal (O) components.

[b] The R2X(cum) value is the predictive and orthogonal variation in model samples (X) explained by the model.

[c] The R2X value is the amount of variation in X which is correlated to Y (response matrix)

[d] The Q2(cum) value describes the predictive ability of the model, based on sevenfold-cross validation.

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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[1]	Fold change[2]	Elemental composition with scores[3]
1	1.59	136.0382	0.5	$C_5H_4N_4O$ (98.0), $C_4H_8O_5$ (93.6)
2	1.70	152.0331	0.4	$C_5H_4N_4O_2$ (98.0), $C_4H_8O_6$ (92.4)
3	4.86	284.1373	0.9	$C_{13}H_{20}N_2O_5$ (99.6), $C_{14}H_{16}N_6O$ (93.2), $C_8H_{17}N_{10}P$ (91.8)
4	6.17	304.1495	7.4	$C_{10}H_{20}N_6O_5$ (98.9), $C_{11}H_{16}N_{10}O$ (91.8), $C_{12}H_{25}N_4OPS$ (91.0)
5	6.75	264.1473	1.6	$C_{14}H_{20}N_2O_3$ (98.8)
6	9.34	163.0993	0.8	$C_{10}H_{13}NO$ (98.8)
7	10.61	230.1514	1.6	$C_{12}H_{22}O_4$ (99.0)
8	11.88	334.2144	0.3	$C_{20}H_{30}O_4$ (99.2), $C_{21}H_{26}N_4$ (92.4), $C_{14}H_{31}N_4O_3P$ (90.5)
9	12.76	380.2533	1.6	N/A
10	13.08	433.2863	0.9	$C_{22}H_{43}NO_5S$ (99.1), $C_{23}H_{39}N_5OS$ (95.2), $C_{17}H_{40}N_9PS$ (95.1)
11	13.08	505.2166	0.9	$C_{23}H_{39}NO_7S_2$ (94.6), $C_{24}H_{43}NO2S_4$ (92.9), $C_{27}H_{41}NP_2S_2$ (90.1)
12	14.15	765.4143	1.3	$C_{36}H_{63}NO_{16}$ (99.5), $C_{31}H_{60}N_9O_{11}P$ (98.5), $C_{34}H_{51}N_{15}O_6$ (98.4)
13	14.23	362.2431	1.7	$C_{18}H_{30}N_6O_2$ (95.5), $C_{19}H_{41}P_3$ (93.9)
14	14.97	364.2599	1.2	$C_{16}H_{37}N_4O_3P$ (97.8), $C_{18}H_{32}N_6O_2$ (93.7), $C_{22}H_{36}O_4$ (90.1)
15	14.98	661.1729	1.2	$C_{25}H_{41}ClNO_{13}PS$ (96.1), $C_{30}H_{47}ClNOP_3S_3$ (95.7), $C_{20}H_{36}O_3$ (98.3)
16	15.20	364.2592	1.3	$C_{18}H_{32}N_6O_2$ (98.3)
17	15.20	364.2592	1.4	$C_{18}H_{32}N_6O_2$ (98.3), $C_{19}H_{43}P_3$ (94.3), $C_{16}H_{37}N_4O_3P$ (92.7)
18	15.73	364.2589	1.4	$C_{18}H_{32}N_6O_2$ (97.2), $C_{19}H_{43}P_3$ (94.4)
19	16.82	174.1042	1.7	N/A
20	16.82	602.3516	2.1	$C_{33}H_{46}N_8OS$ (93.0)
21	16.82	367.1243	1.5	N/A
22	16.82	226.1720	1.7	$C_{17}H_{22}$ (99.0)
23	16.82	176.1194	1.6	N/A
24	16.82	312.1701	1.9	$C_{16}H_{20}N_6O$ (99.1), $C_{16}H_{28}N_2S_2$ (96.6), $C_{13}H_{29}O_6P$ (95.3)
25	16.82	130.0778	1.7	N/A
26	16.82	272.1775	1.8	$C_{18}H_{24}O_2$ (99.6), $C_{12}H_{25}N_4OP$ (91.3)
27	16.82	134.0725	1.6	N/A
28	16.82	634.2966	2.2	$C_{38}H_{50}O_2S_3$ (96.5), $C_{32}H_{51}N_4OPS_3$ (95.8), $C_{30}H_{46}N_6O_5S_2$ (94.0)
29	16.82	132.0943	1.7	N/A
30	16.82	344.1074	2.1	$C_{19}H_{20}O_4S$ (93.2)
31	16.82	618.3222	1.8	$C_{36}H_{47}N_2O_5P$ (97.9), $C_{34}H_{50}O_8S$ (96.6), $C_{38}H_{52}OP_2S$ (96)
32	16.82	118.0778	1.1	C_9H_{10} (92.7)
33	16.82	158.1093	1.6	N/A
34	16.82	908.5126	20.9	$C_{52}H_{78}O_9P_2$ (99.4), $C_{53}H_{74}N_4O_5P_2$ (98.7), $C_{46}H_{64}N_{14}O_6$ (98.6)

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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	$C_{18}H_{22}O$ (99.1)
39	16.83	328.1416	1.4	$C_{20}H_{25}PS$ (95.4)
40	16.83	92.0621	1.4	N/A
41	17.21	274.1937	1.6	$C_{18}H_{26}O_2$ (98.7)
42	17.73	300.2092	1.0	$C_{20}H_{28}O_2$ (97.2)
43	18.05	610.2795	0.8	$C_{35}H_{38}N_4O_6$ (99.5), $C_{37}H_{45}N_2P_3$ (98.7), $C_{36}H_{34}N_8O_2$ (97.8)
44	18.31	300.2085	1.1	N/A
45	18.79	326.2249	0.7	$C_{22}H_{30}O_2$ (94.9)
46	19.00	435.3027	1.1	$C_{23}H_{41}N_5OS$ (94.0), $C_{24}H_{42}N_3O_2P$ (90.4), $C_{22}H_{45}NO_5S$ (90.4)
47	20.80	274.1943	1.7	$C_{18}H_{26}O_2$ (92.0)
48	21.51	552.3253	1.4	$C_{38}H_{40}N_4$ (98.5), $C_{37}H_{44}O_4$ (96.8), $C_{30}H_{46}N_6P_2$ (96.4)
49	21.63	622.4023	0.7	$C_{42}H_{54}O_4$ (99.6), $C_{43}H_{50}N_4$ (96.7), $C_{36}H_{55}N_4O_3P$ (95.1)
50	21.99	598.4036	0.6	$C_{41}H_{50}N_4$ (98.6), $C_{40}H_{54}O_4$ (97.0), $C_{33}H_{56}N_6P_2$ (96.0)
51	22.70	534.2645	2.4	$C_{31}H_{39}N_2O_4P$ (99.8), $C_{33}H_{34}N_4O_3$ (95.3), $C_{32}H_{35}N_6P$ (95.1)
52	23.11	595.1772	1.7	$C_{36}H_{26}ClN_5O_2$ (93.1), $C_{36}H_{34}ClNOS_2$ (92.2), $C_{29}H_{26}ClN_{11}S$ (91.5)
53	23.54	534.3936	2.0	$C_{33}H_{50}N_4O_2$ (94.8), $C_{32}H_{54}O_6$ (91.4), $C_{31}H_{55}N_2O_3P$ (90.5)
54	24.07	560.4090	1.6	$C_{35}H_{52}N_4O_2$ (99.4), $C_{34}H_{56}O_6$ (96.1), $C_{33}H_{57}N_2O_3P$ (93.3)
55	24.21	596.3877	1.6	$C_{41}H_{48}N_4$ (98.8), $C_{40}H_{52}O_4$ (97.4), $C_{33}H_{54}N_6P_2$ (94.3)
56	24.48	581.3995	1.2	N/A
57	24.47	596.4063	N/A	$C_{34}H_{48}N_{10}$ (98.3), $C_{31}H_{57}N_4O_5P$ (97.6), $C_{33}H_{52}N_6O_4$ (96.5)
58	24.73	563.3923	1.5	N/A
59	24.73	546.3871	N/A	$C_{40}H_{50}O$ (92.0)
60	24.82	652.2914	1.4	$C_{38}H_{36}N_8O_3$ (98.5), $C_{37}H_{52}O_2P_4$ (97.1), $C_{35}H_{45}N_2O_8P$ (96.8)
61	24.83	581.4016	1.2	$C_{43}H_{51}N$ (91.2)
62	25.02	565.4044	1.5	$C_{38}H_{51}N_3O$ (95.1), $C_{36}H_{56}NO_2P$ (95)
63	25.04	640.4123	0.7	$C_{36}H_{57}N_4O_4P$ (99.1), $C_{37}H_{53}N_8P$ (97.4), $C_{34}H_{62}N_2O_5P_2$ (97.4)
64	25.16	565.4049	2.1	$C_{36}H_{56}NO_2P$ (98.7), $C_{38}H_{51}N_3O$ (94.9), $C_{30}H_{57}N_5OP_2$ (90.4)
65	25.16	548.4030	2.9	$C_{40}H_{52}O$ (96.8), $C_{32}H_{58}N_2OP_2$ (90.5)
66	25.37	588.4407	1.6	$C_{37}H_{56}N_4O_2$ (98.2), $C_{35}H_{61}N_2O_3P$ (93.6), $C_{36}H_{60}O_6$ (92.6)
67	25.59	639.4035	0.4	N/A
68	25.64	563.3889	1.9	$C_{36}H_{54}NO_2P$ (98.5), $C_{38}H_{49}N_3O$ (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	$C_{47}H_{63}N_9O_2$ (98.5), $C_{48}H_{74}N_3P_3$ (98.0), $C_{44}H_{72}N_3O_7P$ (97.9)
72	26.09	981.6413	2.0	$C_{52}H_{92}N_3O_{12}P$ (99.4), $C_{47}H_{89}N_{11}O_7P_2$ (98.8), $C_{48}H_{100}N_5O_5P_5$ (98.6)
73	26.09	986.5968	2.0	$C_{53}H_{74}N_{14}O_5$ (99.0), $C_{60}H_{84}N_4O_4P_2$ (98.7), $C_{55}H_{86}O_{15}$ (97.7)
74	26.20	787.5238	1.8	$C_{45}H_{73}NO_{10}$ (97.7), $C_{40}H_{70}N_9O_5P$ (96.1), $C_{40}H_{80}N_5O_2P_3S$ (95.7)
75	26.61	366.3292	0.9	$C_{27}H_{42}$ (97.7)
76	26.72	605.4007	1.5	$C_{45}H_{51}N$ (93.6)
77	26.75	835.5826	1.8	$C_{58}H_{78}NOP$ (98.5), $C_{50}H_{84}N_3OP_3$ (98.3), $C_{49}H_{73}N_9O_3$ (97.8)

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78	27.43	739.5623	1.8	$C_{43}H_{83}NO_2P_2S$ (99), $C_{36}H_{73}N_{11}O_3S$ (96.4), $C_{39}H_{81}NO_9S$ (96.2)
79	27.74	384.3400	1.4	$C_{27}H_{44}O$ (93.0)
80	27.99	549.4112	2.5	N/A
81	28.01	652.5047	1.9	$C_{39}H_{75}OP_3$ (96.0), $C_{36}H_{69}N_4O_4P$ (95.3), $C_{35}H_{73}O_8P$ (94.3)
82	28.35	565.4058	2.1	N/A
83	28.38	549.4112	2.3	$C_{36}H_{56}NOP$ (95.7), $C_{35}H_{55}N_3S$ (90.2)
84	28.75	565.4719	1.9	$C_{35}H_{59}N_5O$ (93.4), $C_{33}H_{64}N_3O_2P$ (92.8), $C_{34}H_{63}NO_5$ (91.8)
85	29.03	428.3664	1.6	$C_{29}H_{48}O_2$ (95.3)
86	29.86	916.5733	2.2	$C_{54}H_{81}N_2O_8P$ (98.6), $C_{49}H_{88}N_6P_4S$ (97.9), $C_{52}H_{84}O_{11}S$ (97.4)
87	29.86	937.5355	2.1	N/A

[1] Calculated neutral mass
[2] Fold change female/male. Metabolite 57 and 59 were not detectable for males
[3] The three highest ranked candidate molecular formulas. Only candidates with scores ≥ 90 were included

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Figure 1

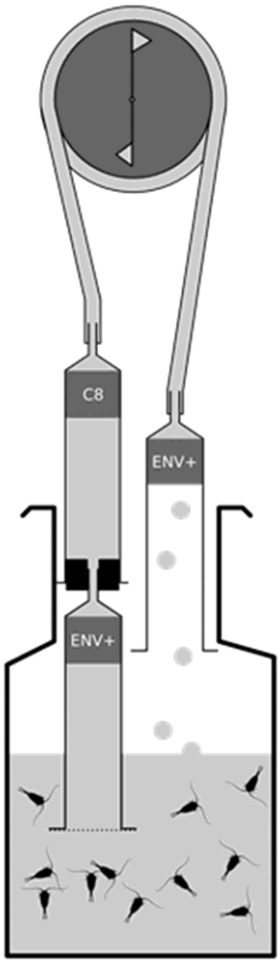

Figure 1 Sampling device used to collect exudates from copepods. Water is drawn through the partially immersed ENV+ and the following C8 SPE before reintroduced into the incubation chamber. The ENV+ column on the outlet end serves to minimize contamination from pump or hosing. The hatched line in the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

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Figure 2

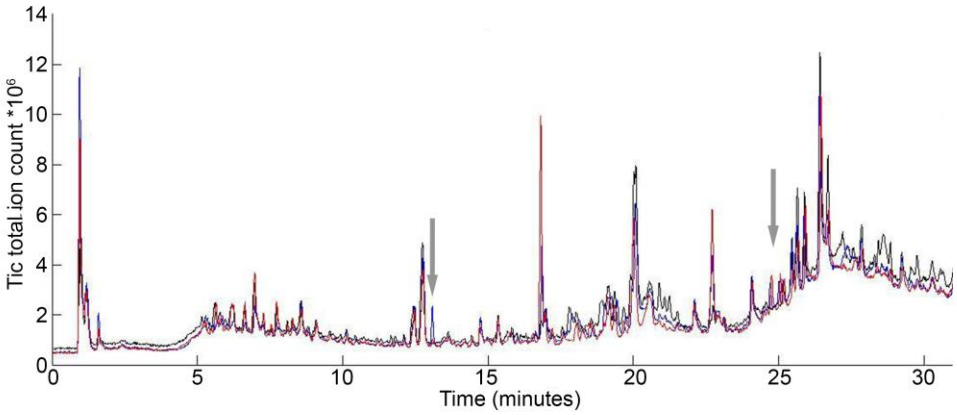

Figure 2

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.

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Figure 3

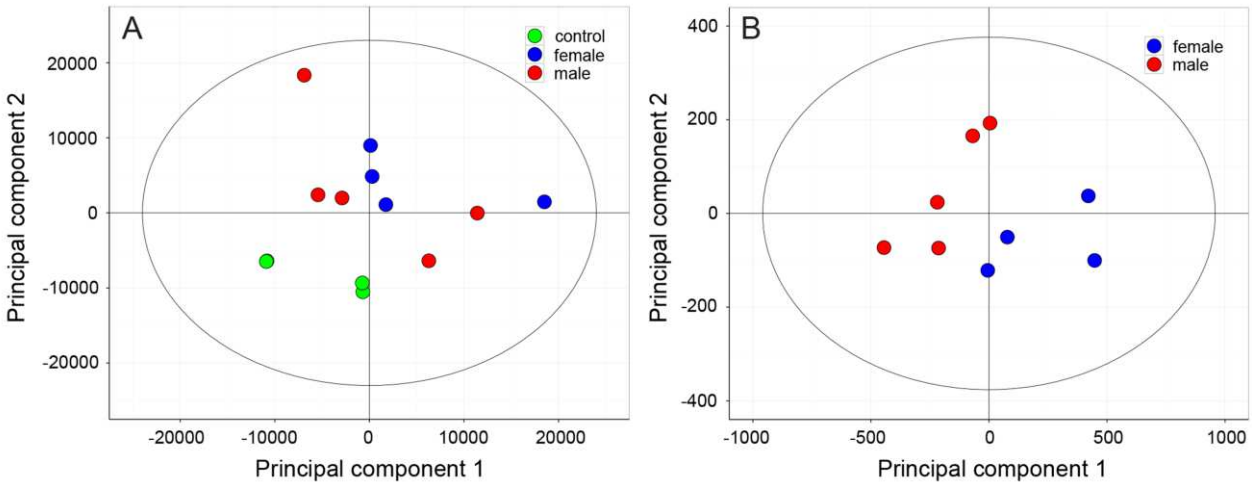

Figure 3 A: PCA based on Pareto-scaled raw data showing the separation between sea water controls (green), male (red) and female (blue) exudate samples. **B**: PCA on the reduced data-set containing only compounds indicated to be of copepod origin in the control-male and control-female OPLS-DA analysis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

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Figure 4

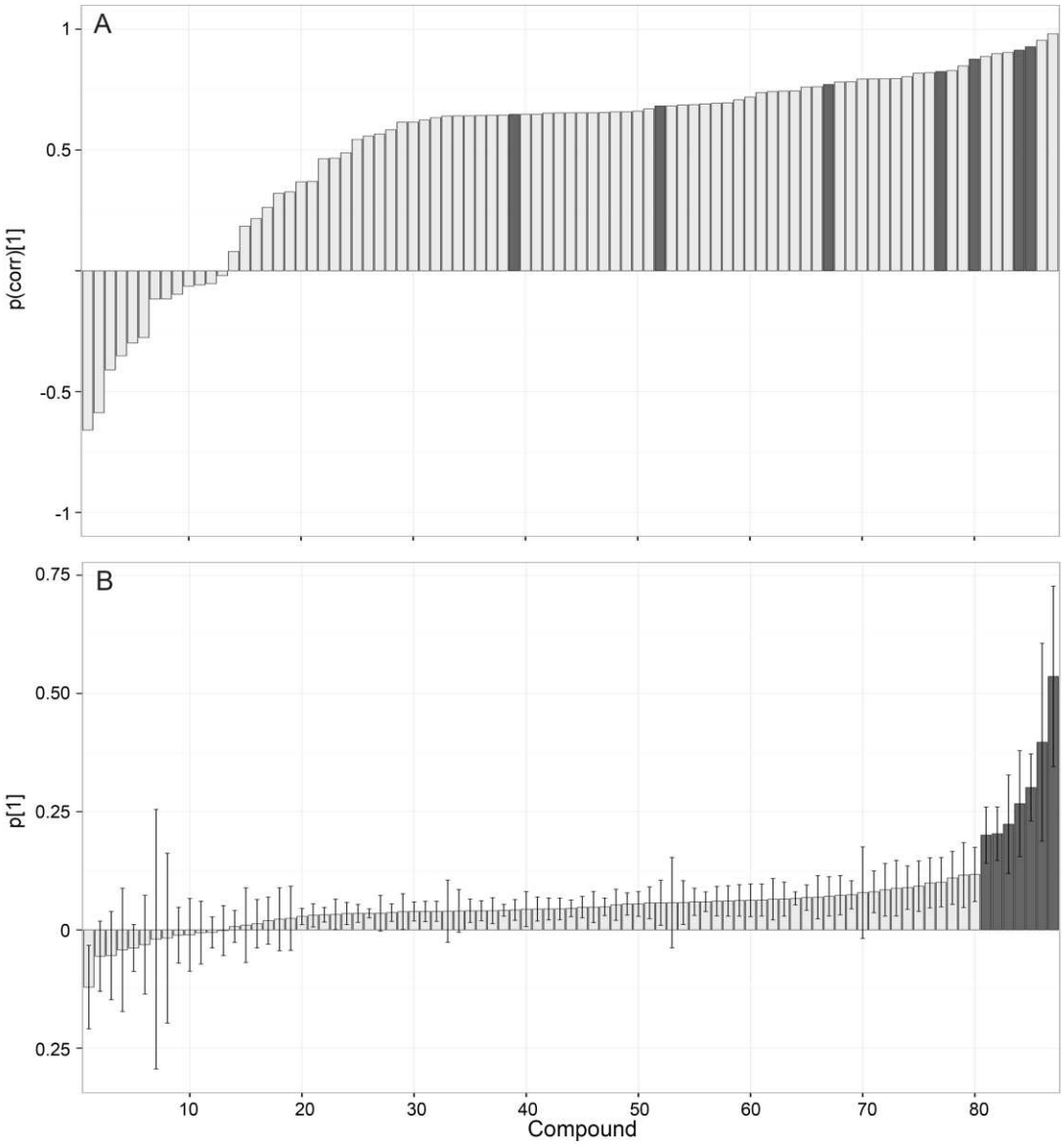

Figure 4 Differences between male and female exudates compound by compound. The OPLS-DA loading plots of metabolites exuded from female and male copepods. The loading plot **A** represents the effect and reliability (correlation, p(corr)[1]), and **B** the contribution or magnitude (covariance, p[1]) 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(corr)[1] and p[1] values are shaded.

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Figure 5

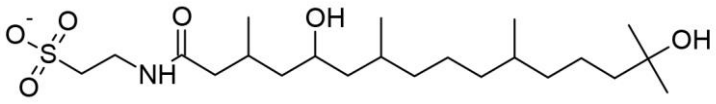

Figure 5 Structure of copepodamide G (compound 10).

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