Distance interaction between marine cave-dwelling sponges and crustaceans

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ABSTRACT

Sponges are benthic organisms that are dominant in several ecosystems and known to produce a huge chemical diversity. The putative release of some specialized metabolites in the surrounding seawater is still a matter of debate, but the presence of such compounds in the environment of sponges is thought to influence the behaviour of various mobile organisms and may, thus, contribute to benthic ecosystem structuring and functioning. Underwater Mediterranean caves are characterized by stable environmental conditions and sessile species assemblages dominated by sponges. A two-choice test system was developed to assess the response of two cave-dwelling crustaceans (*Hemimysis margalefi* and *Palaemon serratus*) and two other species living in shallow water environments (*Leptomysis* sp. and *Palaemon elegans*) to various seawater treatments: control- seawater from an exposed coastline, control+ seawater, coming from an underwater cave, and seawater conditioned with four Mediterranean sponges commonly found at the entrance of underwater caves (*Aplysina cavernicola*, *Haliclona fulva*, *Oscarella tuberculata* and *Spongia officinalis*) or their chemical extracts. We tested the swimming behaviour of these crustacean species in three complementary experiments: (i) control seawater *vs.* cave seawater; (ii) control seawater *vs.* seawater conditioned with the sponge community, (iii) control seawater *vs.* seawater containing chemical extracts of the same sponge community.

Both cave-dwelling crustaceans were attracted by the seawater conditioned with the sponge community, while *Leptomysis* sp. spent more time in the control seawater and *P. elegans* exhibited indifferent responses. All four crustacean species avoided the seawater containing the sponge extracts. Interestingly, the responses shown by the crustaceans was affected by the time of day. A comparative and untargeted metabolomic approach was applied to the surrounding seawater to identify putative chemomarkers that could explain the crustaceans' behaviours. Among other compounds found in the seawater, a family of

metabolites with molecular formulae in accordance with those of oxylipin derivatives is released by sponges and may, therefore, serve as chemical cues acting as kairomones in the homing behaviour of cave-dwelling crustaceans.

KEYWORDS

Marine Ecology; Chemical mediation; Porifera; Oxylipins; Mediterranean Sea

INTRODUCTION

Distance interactions regulate the structure and functioning of marine ecosystems and species distribution within a complex mosaic of habitats. There is a need for better understanding of these interactions, in particular when attempting to assess the putative effects of human disturbances on system functioning (van de Koppel et al. 2015). Chemical mediation plays a key role in such distance interactions by influencing the behavioural ecology of mobile organisms (Hay 2009). Seawater can also be seen as a chemical "seascape" composed of dissolved and/or adsorbed molecules of diverse origins, a good number of which are released by marine organisms and carry crucial signals for the whole ecosystem (Cassier et al. 2000). Surrounded by this complex chemical environment, mobile animals must decipher a complex cocktail of chemical cues, some of which act as kairomones, and adjust their behavioural response by navigating, selecting habitat, locating food or prey, detecting the presence of conspecifics, or defending against competitors and/or predators (Heuschele and Selander 2014). This complexity also challenges animals' abilities to extract the information required to identify the sources of chemical cues which are crucial for their survival (Derby and Sorensen 2008). For example, fishes can detect natural chemical cues at very low concentrations (parts per billion) and, therefore, need to be able to identify the relevant cues from the higher levels of background noise (Belanger et al. 2006).

66 During the past decades, special attention has been paid to the functioning of tropical
67 coral reefs. One of the greatest challenges has been to better understand the life cycles of littoral
68 populations of marine species living in fragmented habitats in a vast and complex ocean matrix,
69 species which may have dispersal and larval development phases offshore, followed by the
70 recruitment of juveniles on randomly distributed reefs (Myrberg and Fuiman 2002). Several
71 pioneer studies demonstrated that marine fish and crustacean larvae could distinguish between
72 oceanic and reef waters (Atema et al. 2002; Lecchini et al. 2005; Gerlach et al. 2007; Dixson et
73 al. 2008). These larvae were shown to be capable of selecting a given habitat among the great
74 variety of substrates found in tropical waters (Huijbers et al. 2008; Munday et al. 2009; Lecchini
75 et al. 2010; Devine et al. 2012; Igulu et al. 2013). For example, Huijbers et al. (2008) reported
76 that fish recruits discriminate chemical cues from mangroves, seagrass beds and coral reefs and
77 that a given reef fish actively prefers the odour of its home coral reef. Lecchini et al. (2014)
78 also demonstrated that fish, crustacean and cephalopod larvae were significantly more attracted
79 by chemical cues from living corals than by dead colonies. Further studies have highlighted
80 how environmental disturbances leading to coral reef destructions, such as ocean acidification
81 (Munday et al. 2009; Devine et al. 2012; Leduc et al. 2013), seawater warming (McCormick et
82 al. 2010) or pollution (O'Connor et al. 2016), may affect marine chemical mediation.
83 Additionally, these changes in abiotic conditions may also alter the organism's metabolism and,
84 thus, its ability to produce specialized metabolites and may consequently impact chemical
85 mediation between organisms (Fisher et al. 2006; Munday et al. 2009; Leduc et al. 2013).
86 Surprisingly, even though chemicals are involved in these biotic interactions and in overall
87 ecosystem functioning, few studies have attempted to identify the chemical nature of distance
88 marine chemical cues (Lecchini et al. 2005; Lecchini et al. 2010; Lecchini et al. 2014; Rasher
89 et al. 2015).

In comparison to this knowledge gained in tropical reefs, chemical mediation in temperate benthic ecosystems has been poorly studied. Sponges can dominate in terms of biomass and diversity in numerous benthic ecosystems, both in tropical and temperate regions (Diaz and Rutzler 2001; Gerovasileiou and Voultsiadou 2012; Wulff 2012). They are also well known for their ability to produce a great chemical diversity (*e.g.* Paul et al. 2011). However, there is a lack of knowledge concerning the ecological functions of these chemical compounds which can be released into the surrounding water (Thompson 1985; Walker et al. 1985; Ternon et al. 2016). Today, sponge chemical ecology is mainly based on the assessment of repulsive properties, the so-called "chemical defences", against predators or competitors for space (Paul et al. 2011; Pawlik 2011). Even though sponges are often considered to be dominant components of benthic ecosystems, the putative role of the portion of their specialized metabolites released into the water column remains unclear and few studies have demonstrated a positive influence of these cues on the recruitment of invertebrate larvae (*e.g.* Bingham and Young 1991). We hypothesize that the great chemical diversity produced by sponges may construct a true chemical seascape which may be the basis of a still underestimated network of interactions shaping biodiversity.

We surmise that underwater caves constitute good ecosystem models in which to test this hypothesis. Sponges are predominant from the semi-dark zone where they coexist with cnidarians and bryozoans to the darkest parts of the cave where they can represent 100 % of the sessile biodiversity (Gerovasileiou and Voultsiadou 2012). In low or no light conditions, other putative producers of chemicals such as macroalgae (Amsler 2008) are absent or are restricted to the entrance of this habitat. Experimenting in underwater caves also avoids, or at least attenuates, variations of abiotic factors present in the open sea such as hydrodynamics, nutrient and resource bioavailability and, in some cases, seawater temperature (Rastorgueff et al. 2011). Underwater caves can thus be considered as *in situ* mesocosms where many ecological

processes and interactions are simplified. Finally, considering their very low resilience facing major environmental threats (Gunn et al. 2000), a better understanding of this ecosystem functioning is needed to propose suitable management and conservation plans (Gunn et al. 2000; Bussotti et al. 2006; Gerovasileiou and Voultsiadou 2012; Rastorgueff et al. 2015).

This study focuses on two dominant components of cave ecosystem functioning: sponges, which are active suspension feeders and which compose the vast majority of the sessile fauna, and swimming crustaceans which act on bentho-pelagic couplings through their migration in and out of caves (Rastorgueff et al. 2015). Our aim was to assess the influence of the chemical seascape of a cave which is most likely primarily determined by sponges on the swimming behaviour of crustaceans (*i.e.* attraction or repulsion). We combined the use of (i) an experimental device to assess the behavioural response of the crustaceans, and (ii) a metabolomics fingerprinting approach to decipher the chemical composition of the various seawater treatments. To conduct our experiments, we selected representatives of the sponge community found near the entrance in semi-dark conditions of Mediterranean underwater caves, and four crustacean species with contrasting ecological habits: two cave-dwelling crustaceans and two crustaceans inhabiting shallow water environments.

MATERIAL AND METHODS

Study location and species

This study was conducted using the aquaria facilities of the Marine Station of Endoume, Marseille, France. Four characteristic sponge species of the semi-dark community (*Aplysina cavernicola* (Vacelet, 1959), *Haliclona fulva* (Topsent, 1893), *Oscarella tuberculata* (Schmidt, 1868) and *Spongia officinalis* (Linnaeus, 1759); Supplementary Fig. S1) were collected in a cave in the Riou Archipelago at an average of 20 m depth (43°12'N; 5°22'E) and were maintained alive in aquaria. These species were selected because part of their specialized

metabolome had already been described (Garrido et al. 1997; Ivanisevic et al. 2011; Manzo et al. 2011; Nuzzo et al. 2012; Reverter et al. 2016; personal unpublished data). In the same cave, we also collected 70 L of seawater to act as a positive control in our first experiment.

The four crustacean species selected to perform the chemosensory assays were two mysid species (*Hemimysis margalefi* Alcaraz, Riera and Gili, 1986 and *Leptomysis* sp.) and two palaemonid species (*Palaemon serratus* (Pennant, 1777) and *Palaemon elegans* Rathke, 1837) (Supplementary Fig. S2). *Hemimysis margalefi* (mean length ± SD, 2.7 ± 0.1 mm) was collected in the same cave as the sponges, where it usually forms dense swarms. This cave-dwelling mysid undertakes circadian migrations in order to find food outside the cave at night or early morning (Rastorgueff et al. 2011), and we hypothesized that its return towards the cave may be influenced by a chemical seascape composed primarily of sponge chemical cues. *Leptomysis* sp. (8.3 ± 0.3 mm) was collected in shallow waters (8 m depth) adjacent to the Marine Station aquarium seawater pumping site (43°16.80'N, 5°20.95'E). This benthic community is dominated by macrophytes such as *Posidonia oceanica* and is, thus, a totally different chemical seascape which is devoid of any sponge influence. *Palaemon serratus* (13.8 ± 0.3 mm) and *P. elegans* (8.2 ± 0.1 mm) individuals were offspring of gravid females that were captured in the Mediterranean Sea and had been raised in aquaria, so were totally naïve regarding the experimental conditions. *Palaemon serratus* is known to live in semi-dark to dark environments, and we hypothesized that its occurrence in caves may be related to the chemical seascape, whereas *P. elegans* lives in very shallow waters among macroalgae assemblages, so they should not be influenced by a seascape conditioned by sponges. For the duration of their time spent in aquaria, all four crustacean species were fed every 2 days with *Artemia salina* nauplii.

Chemosensory trials

A Y-shaped glass flume providing a choice of two channels was set up according to a protocol developed by Gerlach et al. (2007) to assess the crustacean behavioural response when faced with the choice of different seawater treatments: open flow seawater as the control seawater (CS); cave seawater (+CS), considered here as a positive control; and two types of conditioned seawater, sponge conditioned seawater (SCS) and sponge chemical extracts dissolved in seawater (SCE). Two 10 L tanks were connected to the flume by pipes, creating a constant gravity-driven flow of 50 mL min^{-1} per upstream channel. Prior to the experiments, both flows were measured by adding fluorescein to one tank to ensure that the flows were similar and mixed only in the downstream compartment. The crustaceans were introduced at the downstream end of the apparatus, where they were free to remain or to select and swim towards one of the two channels connected to the tanks (Supplementary Fig. S3). Each assay was performed with a single specimen which was maintained for a 1-min acclimation period followed by a 5-min testing period. During the selection phase of the test, the position of the crustacean in the downstream compartment or in the right or left channels was regularly recorded at 5-sec intervals. After each test, the flume chamber was emptied and washed with the CS. The seawater treatments were then switched from one channel to the other, providing a control for potential channel preferences that were not associated with the water source. After seawater switching, the entire assay was repeated with a new specimen, with each individual being used in a single assay. In this way, the time (in seconds) spent by the crustacean individual in each of the three compartments of the choice flume (*i.e.* the downstream and the two channels) was recorded.

Using the protocol described above, three experiments were conducted with a minimum of 40 individuals per experiment and species, with the exception of *P. elegans*, for which only 25 individuals were tested per experiment. Moreover, these experiments were conducted both in the morning (half of the total individuals; 7 h – 11 h UTC) and in the afternoon (half of the

total individuals; 14 h – 18 h UTC) in order to assess the daily variability of the crustaceans' response to the seawater treatments. For each experiment, +CS and the two types of conditioned seawater (SCS and SCE) were tested against CS. Because palaemonids were not available for the first experiment (CS *vs.* +CS), only the two mysids were used. All four crustaceans were used for the second (CS *vs.* SCS) and the third (CS *vs.* SCE) experiments.

The SCS was expected to contain the sponge community exometabolome (i.e. the metabolites naturally released into the seawater by the four selected sponge species). The SCS was obtained by maintaining living individuals of the four sponge species, measuring about 25 cm^3 each, in a 10 L aerated-tank for 16 hours. The SCE was expected to contain the global metabolome of the same sponge community, that is to say, all metabolites contained in the tissues of the four selected sponges. This SCE was obtained from the sponges used to obtain the SCS, which were weighed, frozen, ground in a blender, filtered through a 500 µm sieve and then mixed with seawater in order to obtain a homogenous solution. The sponge community used to prepare the SCS and SCE represented a total weight of 108 ± 10 g.

To ensure that the modifications of the seawater used to prepare the SCS and SCE was due to the introduction of sponge exometabolomes or extracts only and not by a microbial community present in the pumped open flow seawater, a further CS was prepared for the metabolomic analyses by maintaining this open flow seawater for 16 hours in the same condition as the SCS.

Metabolomic analyses

An untargeted metabolomic approach was used to compare the metabolite composition of the different seawater treatments: the control seawater (CS), the cave seawater (+CS) and the two conditioned seawater treatments (SCS and SCE). .

Briefly, a volume of 500 mL of seawater samples was collected in the tanks during the two-flume choice experiments and passed successively through 0.45 µm pore-sized nylon filters and octadecyl-bonded silica extraction discs (Empore^{TM} C_{18} SPE discs) to obtain particulate and dissolved phases of each seawater. The filters were extracted for particulate phase (or eluted for dissolved phase) with methanol. The extracts were then concentrated to dryness, treated to remove salts, dissolved in methanol and filtered at 0.2 μm before metabolomic analysis. On-line Ultra High-Performance Liquid Chromatography coupled to a High-Resolution Mass Spectrometer (UHPLCHRMS) analyses were performed using a Dionex Ultimate 3000 system equipped with an autosampler and connected to a quadrupole Time of Flight (qToF) mass spectrometer equipped with an electrospray ionization interface (Bruker Impact II) to record mass spectra in the positive and negative modes (see Supplementary Information for detailed methods).

Following the UHPLC-HRMS data acquisition, base peak chromatograms (BPC) were exported as line spectra and converted into the netCDF file format to process unit mass resolution data in centroid mode with XCMS (Smith et al. 2006) on R software (3.4.2). The XCMS approach involved peak picking and integration, peak grouping for identification between samples, chromatogram alignment to avoid retention time deviation and, finally, peak filling to avoid missing values in chromatograms. XCMS generated a matrix (ions/retention time × sample) that was exported on Microsoft Excel. Home-made scripts were used to clean the matrix, removing 1) ions detected in solvent blanks (ratio pools/blanks < 10), 2) ions presenting an intensity variation above 30% for pool samples, and 3) ions with correlated intensities for pool samples (threshold = 0.8). Extracted Ion Chromatograms of variable importance in projection (VIP) chemomarkers were drawn to measure their concentrations in the different seawater treatments, taking into account the most important value of ion intensity at each VIP retention time. The heatmap based on data acquired in positive mode was finally

selected to tentatively annotate chemomarkers of seawater samples using the molecular formulae provided by Bruker Data-analysis 4.2.

Statistical analyses

Paired samples *t* tests (or Wilcoxon tests when normality and homoscedasticity conditions were not met) were performed to compare the time spent in each of the two upstream channels containing the different seawater treatments (CS *vs.* +CS; CS *vs.* SCS; CS *vs.* SCE), in order to determine the channel chosen by each crustacean species. Two-sample *t* tests (or Mann Whitney tests when normality and homoscedasticity conditions were not met) were performed to compare the time spent in the channels filled with CS, +CS, SCS or SCE between morning and afternoon, in order to determine if crustacean choice is affected by the time of day. Principal Component Analyses (PCA) were performed to explore and identify similarities between the chemical fingerprints of the different seawater samples. A permutation test (999 permutations, PPLS-DA models, based on a double cross-validation, outer loop = 4 fold cross-validation, and inner loop = 3 fold cross-validation) was performed for the differentiation of the chemical fingerprints of the different seawater samples followed by post hoc permutational pairwise test (Hervé, 2018). The classification error rate (CER, 0 – 1) was calculated to estimate the strength of the generated models. Kruskal Wallis tests, followed by a post-hoc multiple range test (Fisher's Least Significant Difference), were performed to test differences in chemomarker concentrations between seawater treatments.

Multivariate analyses (PCA) and heatmaps were performed with MetaboAnalyst 3.5 (Xia and Wishart 2016). Univariate analyses were performed with R software (version 3.4.2.) and Statgraphics centurion (version 15.2.11.0).

RESULTS

Control experiment

When both upstream channels were filled with CS, the four crustaceans showed no significant preference between upstream channels (Supplementary Fig. S4) and remained mainly in the downstream compartment (Supplementary Fig. S5). These results confirmed the equivalence of the two channels for the assay. Moreover, both mysid species exhibited higher locomotor activity than the two palaemonid species, staying twice as long in the upstream channels (Supplementary Fig. S4).

Comparative crustacean responses to the seawater treatments

During the 5-min tests of +CS *vs.* CS, most crustaceans exhibited stationary swimming behaviour at the intersection of the seawater currents from the two upstream channels before exhibiting their preference. However, occasionally after making their first choice, individuals would switch and explore the opposite channel, showing constant activity while investigating the seawater from both upstream channels. *Hemimysis margalefi* showed a clear preference for the +CS, staying 16 times longer in this channel (Fig. 1) than in the CS. In contrast, *Leptomysis* sp. demonstrated a significant preference for the CS channel, staying 4 times longer in this channel (Fig. 1) than in the +CS. The behaviour of the two mysids varied with the time of day, with *H. margalefi* having a higher preference for the +CS in the morning (Fig. 2). Interestingly, *Leptomysis* sp. had a higher preference for the CS in the afternoon (Fig. 2).

In the SCS *vs.* CS experiment, *Hemimysis margalefi* exhibited a significant preference for the SCS, staying 4 times longer in this channel (Fig. 1) than in the CS. This behaviour varied with the time of day, with a higher preference for the SCS in the morning, when it spent 7 times longer than in the CS channel, whereas this difference was only 2.5 times longer in the afternoon (Fig. 3). *Palaemon serratus* also exhibited a preference for the SCS compared to the CS channel (Fig. 1), but this attraction was less pronounced than for *H. margalefi* and was not affected by

289 the time of day (Fig. 3). Comparatively, *Leptomysis* sp. confirmed its clear preference for the
290 CS channel (Fig. 1) and, in this experiment too, their behaviour varied with the time of day,
291 with no clear preference in the morning, but with a 5-fold longer time spent in the CS channel
292 than in the SCS in the afternoon (Fig. 3). Finally, as was the case for *Leptomysis sp.*, *P. elegans*
293 showed no preference in the morning but stayed 4 times longer in the CS than in the SCS
294 channel in the afternoon (Fig. 3).
295
296 In the SCE *vs.* CS experiment, both *H. margalefi* and *P. serratus* clearly avoided the
297 SCE channel and spent about 15 times longer in the CS channel compared to the SCE channel
298 (Fig. 1). This trend was less marked for *Leptomysis* sp. and *P. elegans*, as these crustaceans
299 stayed in the CS channel only 2 to 3 times longer than they did in the SCE channel (Fig. 1). The
300 two cave-dwelling crustaceans (i.e. *H. margalefi* and *P. serratus*) presented the same
301 behavioural response regardless of the time of day, whereas *Leptomysis* sp. and *P. elegans*
302 exhibited a significant avoidance of SCE in the afternoon only (Fig. 4). Moreover, the
303 swimming behaviour appeared quite different than in the previous experiments. *Hemimysis*
304 *margalefi* and *P. serratus* did not exhibit a stationary phase at the intersection of the seawater
305 currents, but they swam more quickly and presented a faster choice, mostly in favour of the CS
306 channel. In a distinct manner, *Leptomysis* sp. showed a reduced swimming activity and spent
307 most of its time on the bottom of the downstream compartment (Supplementary Fig. S5).
308
309 ***Metabolomic analyses***
310 The comparative untargeted analyses of the CS, +CS, SCS and SCE chemical
311 fingerprints were performed by UHPLC-HRMS in both negative and positive modes and for
312 both dissolved and particulate phases. After XCMS processing and filtering, 156 ions in the
313 negative mode and 144 ions were detected in the positive mode, and these were used to compare

the chemical fingerprints of the various seawater treatments (Fig. 5, Fig. S9). As a preliminary result, no change could be detected when comparing the chemical fingerprints of the two CS, i.e. the CS freshly pumped to perform the behavioural experiment or the CS kept for 16 hours as was the conditioned seawater SCS.

The chemical fingerprints of the dissolved phases obtained in both negative and positive modes resulted in the separation of +CS on one side and CS, SCS and SCE on the other side along the first axis of the PCA (Fig. 5, Table S1). Along the second axis, the chemical fingerprints recorded in the negative mode resulted in the grouping of SCS and SCE that overlapped opposite to CS (Fig. 5), whereas in the positive mode, SCS grouped with CS opposite to SCE (Fig. 5), but SCS and CS appeared different according to the post hoc permutational pairwise test (Table S1). Chemical fingerprints of particulate phases obtained in both negative and positive modes showed similar patterns with the grouping of CS with SCS separated from both SCE and +CS (Fig. 5).

To highlight some chemical markers potentially responsible for the crustaceans' behaviour, heatmaps of the most important features that discriminate groups in both modes and phases were constructed (Fig. 6, and Supplementary Figs. S6, S7 and S8). Many features present in SCE waters in high quantities may help to explain the crustaceans' repulsion. Among them, the brominated derivatives known from *A. cavernicola* were easily identifiable on the basis of their isotopic pattern, exact mass and in comparison with standards (Fig. S10). For example, aeroplysinin-1 (m/z 335.8871, rt 400 s, M338T401 on Fig. S7) was identified mostly in the dissolved phase and aerothionin (m/z 812.8406, rt 470 s, M819T471 and M820T472 on Fig. S8, and possible stereoisomer with the same isotopic pattern at rt 500: M817T501 and M819T502, Figs. S8 and S10) was identified mostly in the particulate phase. On the contrary, only a few chemical features were found to possibly explain the attraction of *H. margalefi* by

both +CS and SCS. The chemical fingerprints obtained in the negative mode from the dissolved phases were the only ones separating CS from +CS and SCS/SCE (Fig. 5).

In the positive mode of the dissolved phase, two sets of features were tentatively annotated. The 1st set of features grouped ions present in both +CS and SCE (Supplementary Table S2, Figs. 6 and 7). Four metabolites, A ($C_{24}H_{44}N_4O_4$), B ($C_{30}H_{55}N_5O_5$), C ($C_{36}H_{66}N_6O_6$) and D ($C_{42}H_{77}N_7O_7$), were detected in this group and are likely small peptides made of leucine or iso-leucine units. Their concentrations were significantly higher in +CS, whereas the few visible differences between SCS and CS were never statistically significant (Fig. 7). The 2nd set of features grouped ions which were more concentrated in SCS and SCE, present in +CS and overall poorly represented in CS (Supplementary Table S2, Figs. 6 and 7). They were consistently significantly more concentrated in SCS than in other waters. However, although some of them were twice as concentrated in +CS than in CS, these differences were generally not supported statistically except for the metabolite L ($C_{17}H_{34}N_2O_3$) detected in higher concentration in SCS and +CS compared to CS (Fig. 7). Molecular formulae of the marker metabolites E ($C_{18}H_{36}O_6$), F ($C_{18}H_{30}O_3$), G ($C_{18}H_{34}O_5$) and H ($C_{14}H_{26}O_3$) were confidently assigned thanks to the high accuracy of the mass and the consistency of the molecular formulae provided by data analysis. These compounds likely belong to the oxylipin family, some derivatives of a family previously found in the marine environment (Qiao et al. 2011; Lamari et al. 2013; Lauritano et al. 2016). Halogenated metabolites harbouring the same number of carbons were also detected, I ($C_{18}H_{31}BrN_2O_5$), J ($C_{18}H_{34}Cl_2O_4$) and K ($C_{18}H_{33}ClO_4$).

DISCUSSION

Distance interactions due to the putative role of chemical mediation in habitat detection by fishes and crustaceans have been previously studied among benthic ecosystems dominated by macroalgae (Lecchini et al. 2007, 2010, 2013; Lecchini et al. 2011) or seagrasses

(Nagelkerken et al. 2002; Arvedlund and Takemura 2006; Huijbers et al. 2008; Igulu et al. 2013), in coral reefs (Lecchini et al. 2005; Lecchini et al. 2007; Huijbers et al. 2008; Ben-Tzvi et al. 2010; Lecchini et al. 2010; McCormick et al. 2010; Lecchini et al. 2011; Devine et al. 2012; Coppock et al. 2013; Igulu et al. 2013; Lecchini et al. 2013; Lecchini et al. 2014) and in mangroves (Huijbers et al. 2008; Igulu et al. 2013). For some model interactions, such as clown fishes and sea anemones, their description demonstrated how these processes are crucial for ecosystem functioning, and how this mediation can be affected by global change (Arvedlund and Nielsen 1996; Arvedlund et al. 1999; Dixson et al. 2008; Munday et al. 2009; Dixson 2012; Dixson et al. 2014; Lecchini et al. 2014).

Here, we report for the first time clear behaviours of cave-dwelling crustaceans attracted by cave seawater (+CS) and sponge conditioned seawater (SCS), and the concomitant presence of chemomarkers in these waters. Sponges being dominant in benthic communities such as underwater caves, we hypothesized that sponges and/or their associated microbiota may be responsible for the production of chemical cues that significantly contribute to the network of chemical interactions shaping the ecosystem. Conversely to previous studies in this field, we anticipated that only part of the chemical diversity produced by these sessile invertebrates would contribute to an overall chemical sea-scape conditioning the network of interactions. Indeed, most previous studies on sponge chemical ecology used tissue homogenates or crude extracts (*i.e.* the global metabolome) to highlight their deterrent properties against predators, competitors for space or microorganisms (Bakus and Green 1974; Thompson et al. 1985; Thacker et al. 1998; Kubanek et al. 2002; Pawlik et al. 2002; Pawlik 2011). Our experiment exposing crustaceans to SCE confirmed the strong deterrent effect of seawater containing the entire sponge metabolome. Most likely, deterrent or toxic properties of specialized metabolites present in the sponges are responsible for these behavioural responses. In line with this hypothesis, aerothionin, which is one of the discriminating compounds of the SCE, and other

388 compounds of the same family had already been identified as feeding deterrents in other
389 bioassays and may be responsible for the observed repulsive effects (Thoms et al. 2004;
390 Gochfeld et al. 2012). Interestingly, none of the main specialized metabolites previous known
391 in the four sponges tested were detected in the SCS or the +CS, so they do not appear to belong
392 to what can be called the sponge exometabolome and, thus, do not contribute to the chemical
393 seascape. So far, the exudation of major compounds has been demonstrated in the single case
394 of the sponge *Crambe crambe* (Ternon et al. 2016), but there is crucial need for new
395 experiments to better distinguish between the global metabolome, which is usually extracted
396 from organisms using solvent, and the exometabolome which is the part naturally released by
397 an organism into the surrounding water.

398 Following previous hypotheses by Bingham and Young (1991), our study demonstrates
399 for the first time that seawater conditioned by a sponge assemblage (SCS), potentially
400 containing components of the exometabolome only, exerts an attractant effect on some mobile
401 invertebrate species, leading to a specific behavioural response related to their ecology.
402 Demonstrating such a "positive" effect of sponge compounds would have been impossible with
403 only the rather traditional use of crude extracts or pure major compounds in our experimental
404 plan. This past strategy explains the important knowledge gap that exists concerning processes
405 of chemical mediation within marine benthic ecosystems, in particular where ecosystems are
406 dominated by sponges. Few studies have attempted to demonstrate the putative release of
407 metabolites into the surrounding medium (Walker et al. 1985; Porter and Targett 1988; Duque
408 et al. 2001; Ternon et al. 2016). After characterizing the exudation of chemical cues by the
409 sponge *Crambe crambe*, and assessing the toxic activity of these compounds on invertebrate
410 larvae, Ternon et al. (2016) hypothesized that this process might explain the ecological success
411 of this encrusting sponge. Here, we provide the first demonstration that some sponge
412 metabolites may also be involved in distance interactions, acting as kairomones to shape the

behaviour of mobile organisms and, thus, structuring biodiversity. We thus confirm the hypothesis of Bingham and Young (1991) that chemicals released by sponges have the potential to attract organisms. Moreover, we have demonstrated a species-specific response to conditioned seawater, since *H. margalefi* and *P. serratus* only showed a significant attraction for the seawater containing the exometabolome of the sponge community. The opposite response of *Leptomysis* sp. can be explained by the fact that the CS was pumped from heart to the *Leptomysis* sp. Habitat, which is dominated by macrophytes and macroalgae and where sponges are poorly distributed.

The responses of our cave-dwelling species support our hypothesis that chemical cues released by the sponges located at the entrance of underwater caves might be a key factor in the homing behaviour of *H. margalefi* and a refuge signal for *P. serratus*. The difference recorded between their behavioural responses is likely related to their life habits. *Hemimysis margalefi* is a cave-dweller living in swarms and is thought to migrate out of the cave for night feeding (Rastorgueff et al. 2011; Rastorgueff et al. 2015). This circadian movement is presumed to be linked to the decrease in light intensity (Passelaigue and Bourdillon 1986), at least to explain how these mysids get out of the cave at the end of the day. However, our goal was to provide clues to support the idea that the return to the cave in the night or early morning may be related to the chemical seascape of the cave, through chemical cues potentially produced by sponges guiding these mysids to the entrance of their habitat. We acknowledge that we only conducted our experiments in the morning, and future experiments could be performed in darkness to mimic if the same trend is observed during the night. Interestingly, *H. margalefi* exhibited the same positive attraction when faced with the choice of the natural cave seawater and the sponge conditioned seawater containing exometabolomes. A better understanding of these migrations of cave-dwelling organisms is crucial as they represent important vectors of organic matter transfer from the outside euphotic littoral zone to various locations inside caves, where this

438 matter becomes available to other cave dwellers such as molluscs, crustaceans and teleost fishes
439 (Rastorgueff et al. 2011). There is likely a strong link between this type of migration pattern,
440 and how it mitigates cave oligotrophy, and the diversity of sessile organisms at the origin of the
441 chemical seascape.

442 *Palaemon serratus* is a crustacean which may occasionally inhabit underwater caves. In
443 this case too, we hypothesize that the sponge seascape might influence the swimming behaviour
444 of this species and contribute to locate its habitat refuge. Interestingly, *P. serratus* spent most
445 of its time in the downstream compartment and exhibited a much less pronounced attraction for
446 the sponge conditioned seawater, compared to *H. margalefi*. In the case of palaemonids, the
447 process of chemical mediation in habitat detection might not be related to gradients of
448 waterborne molecules only. Whereas mysids are swimming crustaceans, palaemonids swim
449 close to and walk on the bottom using their well-developed legs. They continuously explore the
450 bottom, sensing more hydrophobic molecules with leg chemoreceptors. They also use pincers
451 to bring fragments of substrate into contact with chemosensory mouthparts in order to follow
452 hydrophobic cues adherent to the substrate (Mollo et al. 2014).

453 Variations of the behavioural responses with time of day differed among the species
454 tested. The cave-dwelling mysid seemed to be much more attracted by sponge cues in the
455 mornings, whereas the palaemonid showed no variation, which tends to confirm that their
456 response does not rely on the same ecological process. Daily rhythm of chemosensitivity has
457 already been reported for several arthropods, such as the Tsetse fly *Glossina morsitans*
458 *morsitans* which presents a higher chemosensitivity in early morning and late afternoon when
459 its feeding activity is the greatest (Van der Goes van Naters et al. 1998). The recent application
460 of underwater electroantennography (Machon et al. 2016) offers new perspectives of
461 chemosensitivity surveys of our model crustaceans that face various chemical cues at different
462 period of the day. Moreover, the behavioural responses might also be correlated to the

463 expression level of some chemo-receptor such as IR25a (Zbinden et al. 2017) in order for the
464 crustacean antennules to perceive chemical cues. For technical reason, most of our morning
465 behavioural experiments could only be conducted during daylight hours and we believe that the
466 combination of additional experiments performed in the darkness combined to chemosensitivity
467 surveys might further support our hypothesis on the role of the chemical seascape on diurnal
468 migrations of cave-dwelling crustaceans.

469 Very few studies in chemistry have attempted to identify the structure of the
470 hypothesized marine chemical cues involved in habitat selection (Lecchini et al. 2005; Derby
471 and Sorensen 2008; Lecchini et al. 2010; Lecchini et al. 2014). Even though we expected to
472 detect some of the previously known compounds of the four sponge models in the SCS, our
473 metabolomics approach shows that none of the specialized metabolites reported so far from the
474 global metabolome of our sponge models could be found in the SCS. Based on their molecular
475 formulae as obtained unambiguously by HRMS, the chemomarkers present in the sponge
476 conditioned seawater, and at low concentration in the cave seawater, correspond to C18-based
477 oxylipins. Further confirmation of these structures is needed, however, using available
478 standards for example. This hypothesis seems realistic as diatom oxylipins are known for their
479 multiple functions, such as sexual pheromones or food-finding cues detectable by grazers (Fink
480 et al. 2006; Kâ et al. 2014), but also as chemical-alarm cues when facing grazing pressure
481 (Ianora et al. 2004; Pohnert 2005). Thus, the ecological function of oxylipins may be very
482 broad. The putative oxylipins may be produced by any of the sponge holobionts (McClintock
483 et al. 2005) and they may be responsible for the homing behaviour of crustaceans. The four
484 peptides naturally found in high concentrations in the cave seawater are also likely produced
485 by sponge holobionts, although in this case their very low concentrations in the sponge
486 conditioned seawater do not provide information on their putative function in the cave chemical
487 seascape.

In addition to the organic compounds in the seawater which are detectable by our metabolomics approach, we assume that inorganic compounds may also influence the biological responses of our model organisms. A significant difference in nutrient concentrations between seawater treatments would only result from the fluxes generated by the sponge holobiont catabolism (for a review see Maldonado et al. 2012), releasing ammonia, nitrate or phosphate for instance during the water conditioning. However, as the sponge volume used in our experiments represented only 1% of the aquarium volume, we can reasonably consider that there was a very low probability of a significant change in nutrient concentration during the preparation of our seawater treatments.

Finally, elucidating the mechanisms and strategies used in habitat detection is crucial to predict how individual species or assemblages may respond to the degradation of their environment (Swihart et al. 2003; Jones et al. 2004). Changes affecting chemical mediation processes are likely to impact subsequent distribution of marine organisms. Sponges are known to be sensitive to global changes, and most of our study species have already suffered mass mortalities related to extreme climate events (Lejeusne et al. 2010). In a context of increasing frequency of such events (Giorgi and Lionello 2008), we can expect further alteration of sponge communities to impact distance interactions mediated by sponge chemical cues. Underwater caves are fragmented habitats and their biodiversity patterns are greatly shaped by their connectivity. Therefore, any alteration of the chemical seascape within and surrounding marine caves, directly or indirectly caused by human activities, could have severe consequences on these ecosystems. As has been evidenced in previous studies by Dixson et al. (2008, 2011), connectivity between distinct marine habitats may partly rely on chemical mediations, and not only on currents.

AUTHOR CONTRIBUTION STATEMENT

MS and TP designed the experiments. MS and MLC performed the experiments. MS, SG, MLC and OPT analysed the data. MS, SG, MLC, OPT and TP wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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761 **Figure legends**

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764 **Fig. 1** Response of the four crustaceans to: (a) underwater cave seawater (+CS) *vs.* control
765 seawater (CS), (b) sponge conditioned seawater (SCS) *vs.* CS, and (c) sponge chemical extracts
766 dissolved in seawater (SCE) *vs.* CS. Results are mean time (± SE) in seconds spent by each
767 species in the channel filled with water containing chemical cues (grey bar) and the CS channel
768 (white bar). Paired samples *t* tests (or Wilcoxon tests) were performed to compare the time
769 spent in each of the two upstream channels. Significant differences are indicated with * for $P <$
770 0.05, ** for $P < 0.01$, and *** for $P < 0.001$. HM = *Hemimysis margalefi*, LS = *Leptomysis* sp.,
771 PE = *Palaemon elegans*, PS = *Palaemon serratus*.

772

773 **Fig. 2** Response of *Hemimysis margalefi* and *Leptomysis* sp. to the underwater cave seawater
774 (+CS) *vs.* control seawater (CS) during the morning and the afternoon. Results are mean time
775 (± SE) in seconds spent by each species in the +CS (grey bar) and the CS (white bar) channels.
776 Paired samples *t* tests (or Wilcoxon tests) were performed to compare the time spent in each of
777 the two upstream channels. Two-sample *t* tests (or Mann-Whitney tests) were performed to
778 compare the time spent in the channels between morning and afternoon. Significant differences
779 are indicated with ** for $P < 0.01$, and *** for $P < 0.001$.

780

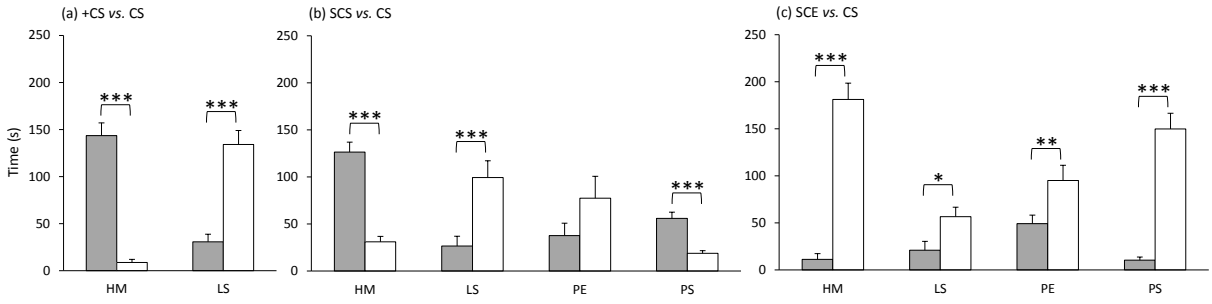
781 **Fig. 3** Response of the four crustaceans to sponge conditioned seawater (SCS) *vs.* control
782 seawater (CS) during the morning and the afternoon. Results are mean time (± SE) in seconds
783 spent by each species in the SCS (grey bar) and the CS (white bar) channels. Paired samples *t*-
784 tests (or Wilcoxon tests) were performed to compare the time spent in each of the two upstream
785 channels. Two-sample *t* tests (or Mann Whitney tests) were performed to compare the time

786 spent in the channels between morning and afternoon. Significant differences are indicated with
787 the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.
788
789 **Fig. 4** Response of the four crustaceans to sponge chemical extracts dissolved in seawater
790 (SCE) *vs.* control seawater (CS) during the morning and the afternoon. Results are mean time
791 (± SE) in seconds spent by each species in the SCE (grey bar) and the CS (white bar) channels.
792 Paired samples *t* tests (or Wilcoxon tests) were performed to compare the time spent in each of
793 the two upstream channels. Two-sample *t* tests (or Mann Whitney tests) were performed to
794 compare the time spent in the channels between morning and afternoon. Significant differences
795 are indicated with * for $P < 0.05$, and *** for $P < 0.001$.
796
797 **Fig. 5** Principal Component Analysis (PCA) based on the chemical features of the different
798 seawater samples analysed by UHPLC-MS in negative (a, c) and positive (b, d) modes, for the
799 dissolved (a, b) and particulate (c, d) phases. A permanova test (PPLS-DA models, 999
800 permutations with double-cross validation) was performed to test differentiation between
801 chemical fingerprints (post hoc permutation pairwise test in Supplementary file S). The
802 classification error rate (CER, 0 – 1) was calculated to estimate the strength of the generated
803 models. CS: control seawater; +CS: cave seawater; SCS: sponge conditioned seawater; SCE:
804 sponge chemical extract; p: particulate phase; d: dissolved phase.
805
806 **Fig. 6** Heatmap based on the variable importance in projection of the features (VIP > 1) detected
807 in the metabolic fingerprints of the dissolved phase of the seawater samples analysed by liquid
808 chromatography coupled to mass spectrometry in positive mode. X-axis displays the different
809 seawater treatments, Y-axis displays metabolite features coupling nominal mass (M as atomic
810 mass unit) with chromatographic retention time (T in seconds). Connections of samples and

811 metabolic features are based on hierarchical clustering (Euclidian distances). The first group of
812 features gathers ions present in +CS and SCE, the 2nd group of features gathers ions present in
813 SCS and SCE. CS: control seawater; +CS: cave seawater; SCS: sponge conditioned seawater;
814 SCE: sponge chemical extract.
815
816 **Fig. 7** Comparative ion intensities of the features corresponding to the chemical cues A–D (1st
817 group of features that gathers ions present in +CS and SCE) and E–N (2nd group that gathers
818 ions present in SCS and SCE) in the dissolved phase of the seawater samples analysed by liquid
819 chromatography coupled to mass spectrometry in positive mode. The metabolites E, F, G and
820 H likely belong to oxylipin family. Kruskal Wallis tests followed by a post hoc multiple range
821 test (Fisher's Least Significant Difference), $n = 4$: significant differences are indicated with
822 $*P < 0.05$, and $**P < 0.01$, and differences between treatments are shown by different letters.
823 CS control seawater; +CS cave seawater; SCS sponge conditioned seawater; SCE sponge
824 chemical extract

825 **Fig. 1**

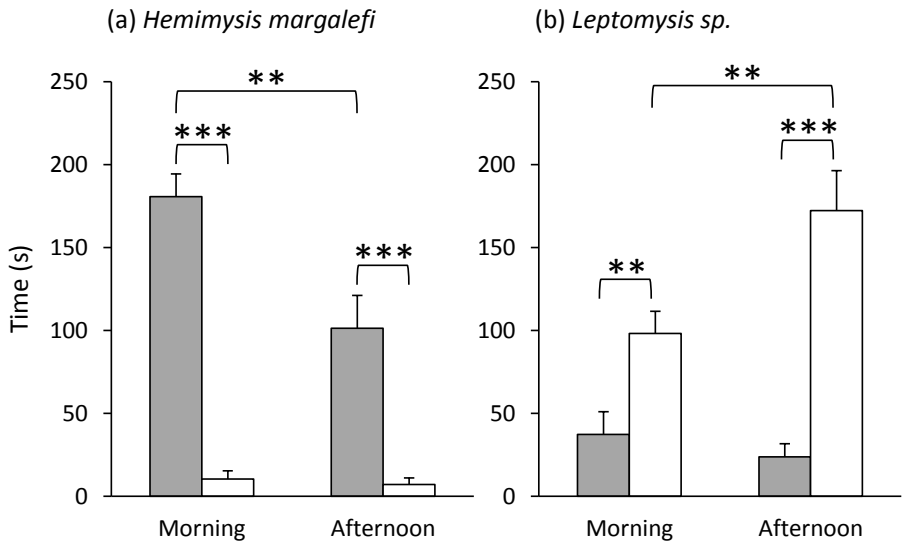
826



827

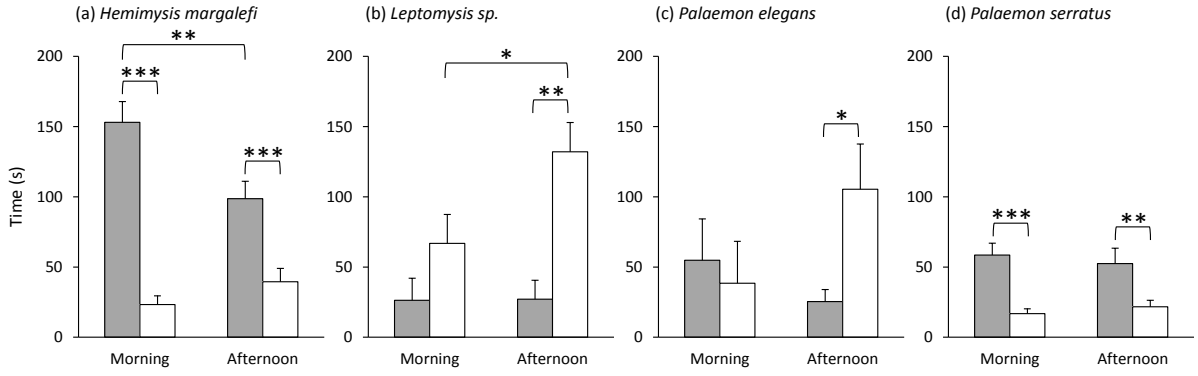
828

Fig. 2


(a) Hemimysis margalefi
(b) Leptomysis sp.
Time (s)
250
200
150
100
50
0
**
***
***
**
**
***
Morning
Afternoon
Morning
Afternoon

833 **Fig. 3**

834



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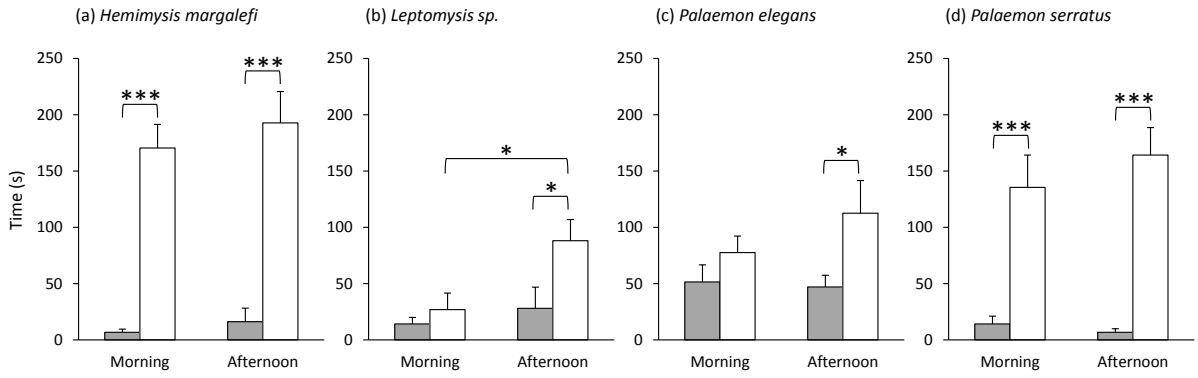
836

837 **Fig. 4**

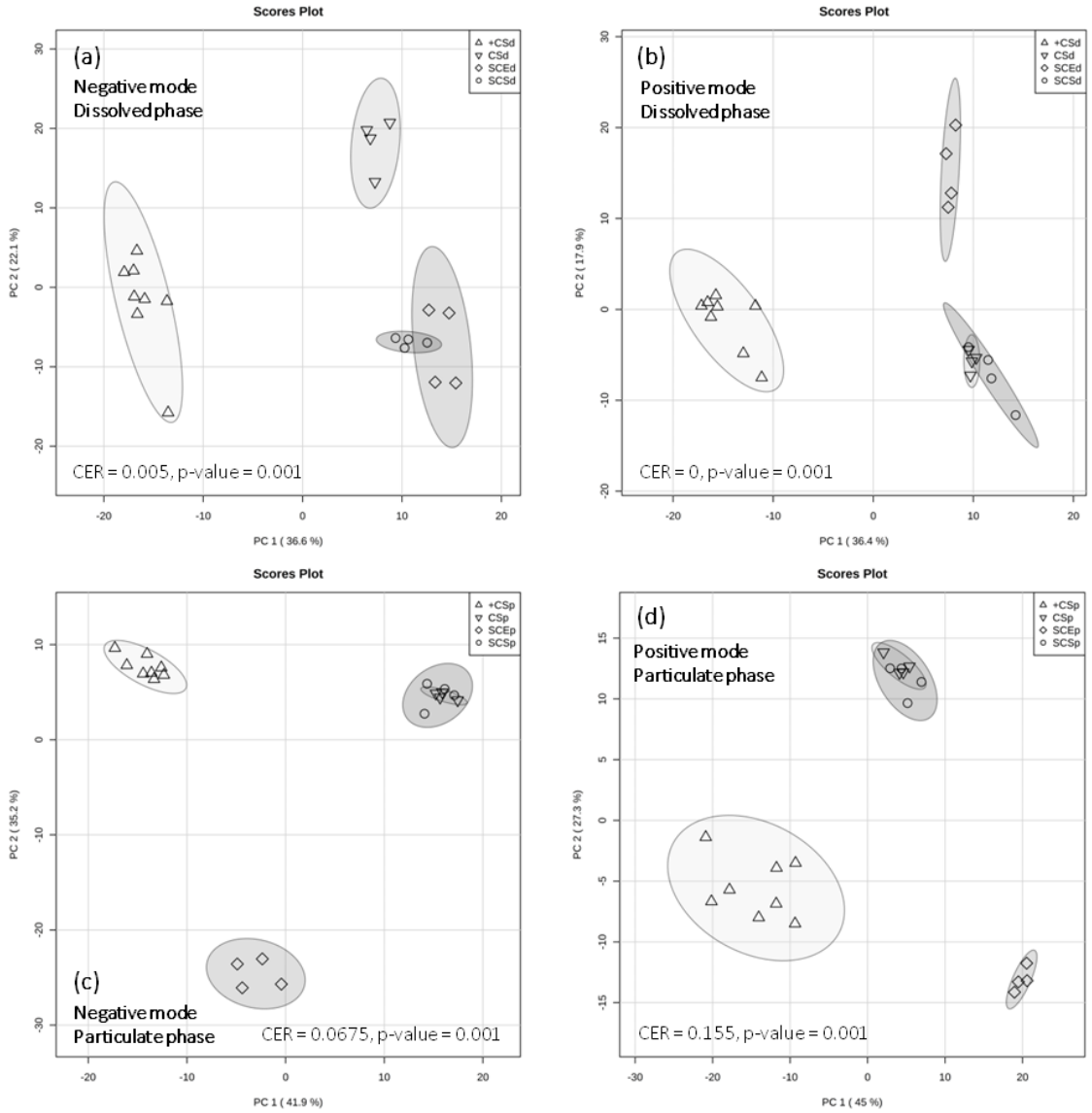
838



839

840

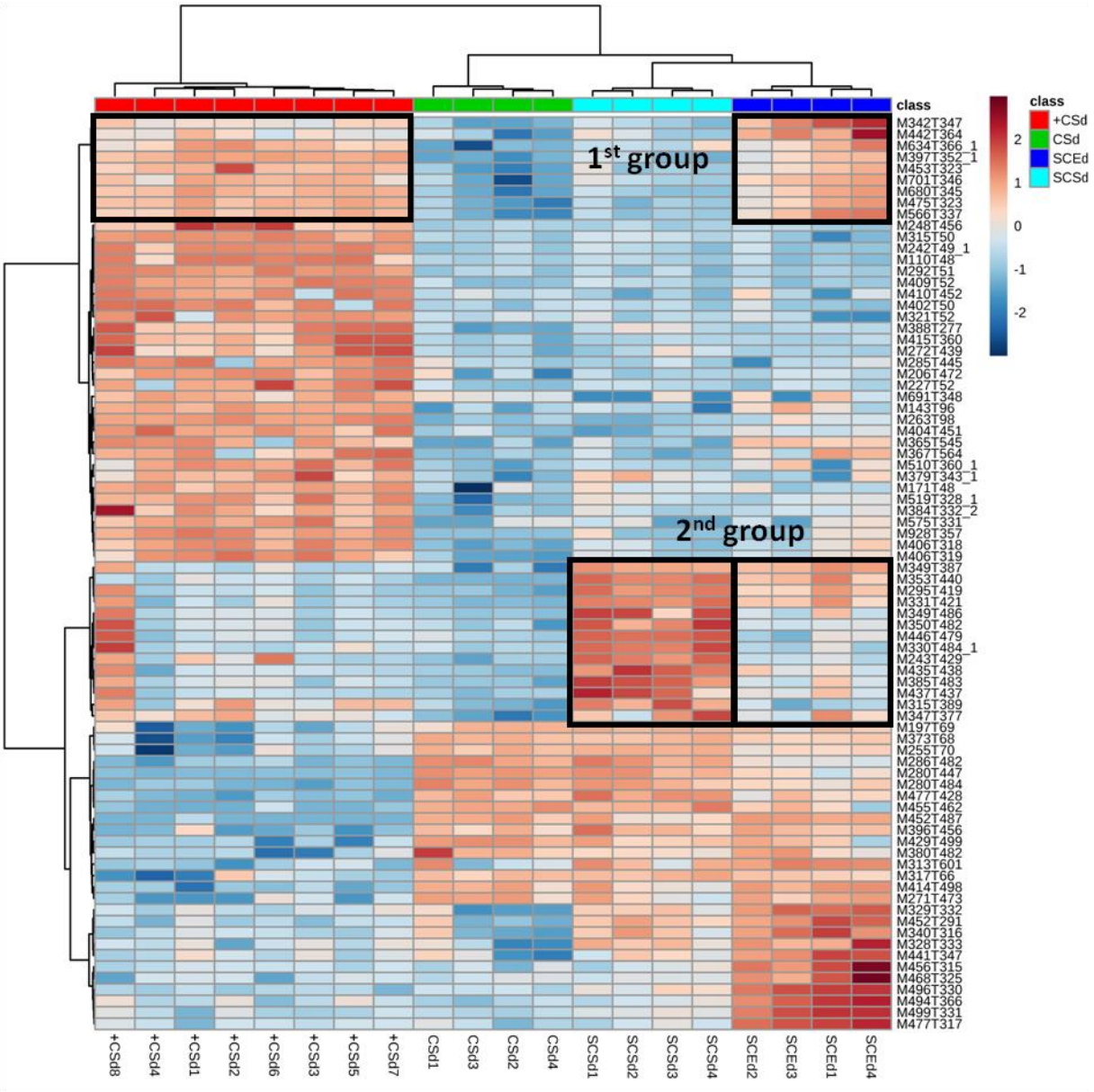
841 **Fig. 5**



842

843

844 **Fig. 6**

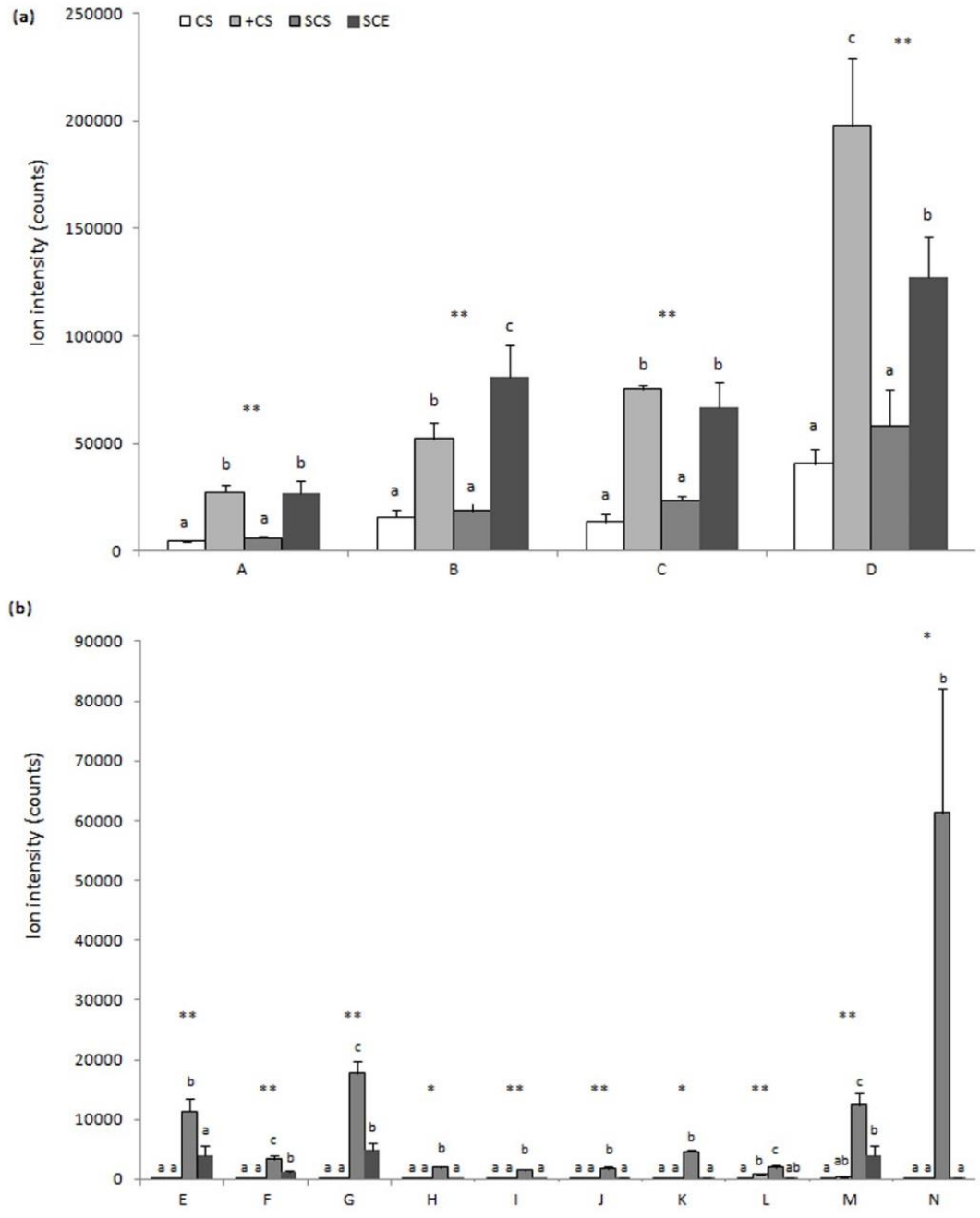


845

846

847

Fig. 7


(a)
CS
+CS
SCS
SCE
Ion intensity (counts)
250000
200000
150000
100000
50000
0
A
B
C
D
(b)
Ion intensity (counts)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
E
F
G
H
I
J
K
L
M
N