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Metabolite profiling reveals insights into the species-dependent cold stress response of the green seaweed holobiont *Ulva* (Chlorophyta)

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ABSTRACT

The green seaweed *Ulva* (Chlorophyta) and its associated epibacterial microbiome form a functional holobiont that adapts to stress as a whole. As the macroalga provides carbon sources for bacteria and relies on algal growth and morphogenesis-promoting factors (AGMPF) released by bacteria, those cross-kingdom interactions are especially fascinating. We hypothesized that the *Ulva* holobionts from the warm-temperate Mediterranean-Atlantic and cold Antarctic habitats respond to cold stress differently depending on the production of highly polar low molecular weight compounds (LMWCs) with stress-regulating activity. We compared the microbiome of and metabolic changes in *U. compressa* (cultivar *U. mutabilis*), initially collected in Ria Formosa (Portugal), with that of *U. bulbosa* (strain AWI #1002) collected in Antarctica by performing a warm-cold temperature shift experiment. Microbiome analysis indicated significant differences between the two species and that the number of operational taxonomic units (OTUs) was lower in cultivated *U. bulbosa* than in freshly isolated *Ulva*; despite this, AGMPF-producing bacteria were detected in both holobionts. Significant differences in metabolite profiles were observed between both species using hydrophilic interaction liquid chromatography coupled with electrospray ionization mass spectrometry (HILIC-ESI-MS). Biomarkers such as dimethylsulfoniopropionate (DMSP) and taurine were identified following a temperature shift from 18 °C to 5 °C in the warm-temperate *U. mutabilis* and the cold-adapted *U. bulbosa*, respectively. Our findings show that metabolic changes in the holobiont in response to cold are species-dependent.

To evaluate the contribution of the metabolic changes of bacteria and algae to the stress response, the reductionistic model system of the tripartite community formed by *U. mutabilis* and its two essential bacteria, *Roseovarius* sp. strain MS2 and *Maribacter* sp. strain MS6, which release all essential AGMPFs, was investigated. We examined the production of polar LMWCs in the presence and absence of bacteria following a shift to cold temperatures. Among the metabolites studied, ectoine ((4*S*)-2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid) was only detected in the presence of bacteria, highlighting the role of bacteria in releasing compounds that mitigate environmental stresses through cold stress adaptation factors (CSAF). Our findings suggest that microbiome engineering will allow us to improve macroalgae adaptability to stressful situations, which can be further applied to the sustainable management of (land-based) aquaculture systems.

1. Introduction

The settlement of the earliest plants in terrestrial habitats was accompanied by exposure to a completely different habitat. To deal with changing climatic circumstances and abiotic stresses such as ultraviolet radiation, temperature change, and dehydration, extensive adaptation at the morphological, physiological, and molecular levels was required (Becker and Marin, 2009; Rensing, 2018). The evolution of heteromorphic life and the establishment of symbiotic interactions (Delaux et al., 2013; Field et al., 2015; Ghaderiardakani et al., 2020) were essential innovations in the plant terrestrialization process (Becker and Marin, 2009). Heat stress has recently attracted the attention of plant biologists not only because it may hold answers to fundamental questions about plant evolution and the transition from algae to embryophytes, but also

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because it has implications for crop yields and the associated threats to food security under global warming and the projected impacts of climate change (Fürst-Jansen et al., 2020; Ohama et al., 2017). In contrast, a significant proportion of the Earth's biosphere is permanently exposed to temperatures ≤ 5 °C (Rodrigues and Tiedje, 2008), such as polar environments (Rodrigues and Tiedje, 2008). Understanding how plants remain metabolically active at low temperatures could provide insights into stress adaptation.

Cold and temperate marine environments are inhabited by organisms of the same genus, suggesting specific adaptation processes. Several barriers inherent to permanently cold environments that could impart severe physiochemical restrictions on essential aspects of cell function include intracellular ice formation, changes in membrane fluidity, macromolecular interactions, and enzyme kinetics (Piette et al., 2011; Rodrigues and Tiedje, 2008). Cold-adapted, or cold-acclimated organisms, have thus employed different approaches to survive at lower temperatures (Rodrigues and Tiedje, 2008). For instance, in bacteria, loss of membrane fluidity is counteracted by variations in the lipid composition of their membranes. Bacteria avoid loss of membrane fluidity at cold temperatures by synthesizing unsaturated and branched fatty acids (Russell, 1983). Several studies have reported the importance of cold shock proteins, exopolysaccharides, and membrane modifications for cold adaptation in bacteria (Tribelli and López, 2018). Guy et al. (2008) categorized properties that could contribute to stress tolerance into five groups of metabolites that function *"(1) as osmolytes to modify cellular water relations and reduce cellular dehydration; (2) as compatible solutes to stabilize enzymes, membranes, and other cellular components; (3) as chelating agents to neutralize or sequester potentially toxic levels of metals and inorganic ions; (4) in the retailoring of membrane lipid composition to optimize the liquid/crystalline physical structure […], and (5) as energy sources"* (Guy et al., 2008).

When grown in bacteria-free (axenic) conditions or in the absence of the necessary microbiome, green seaweeds such as Ulvales lose their usual shape (Wichard, 2015). As a result, plantlets proliferate in an undifferentiated and callus-like morphotype. These malformations are partly or entirely rescued by complementing the culture medium with two marine bacteria, *Maribacter* sp. strain MS6 and *Roseovarius* sp. strain MS2, forming a tripartite community (Spoerner et al., 2012) (Fig. 1A). Consequently, algal growth and morphogenesis promoting factors (AGMPF) released by those bacteria (such as thallusin released by *Maribacter* sp.) can ensure algal development (Alsufyani et al., 2020; Ghaderiardakani et al., 2019; Grueneberg et al., 2016). These observations imply that the alga and its associated bacteria, i.e. the entire holobiont, need to react to a specific stressful situation in an orchestrated action (Dittami et al., 2016; Ghaderiardakani et al., 2020).

In this study, we compared the stress responses of the Mediterranean *U. mutabilis* and the Antarctic *U. bulbosa* (both cultured) using a warm-cold temperature shift experiment to identify potential LMWCs (< 200 Da) functioning as cold stress adaptation factors (CSAF). These potential cryoprotectants are often compatible solutes that accumulate to high intracellular concentrations either through *de novo* biosynthesis or uptake from the environment to mitigate thermal and osmotic stress. These solutes include amino acids and derivatives, polyols and sugars, methylamines, and methylsulfonium compounds with multiple functions, including stabilizing macromolecules (Yancey, 2005). For example, algae collected in polar regions often have higher compatible solutes levels than eulittoral species collected from tropical and temperate regions (Bischoff et al., 1994; Karsten et al., 1990a). The axenic Antarctic sea-ice diatom, *Nitzschia lecointei* produces different amounts of 2,3-dihydroxypropane-1-sulfonate (DHPS), glycine betaine (GBT), and dimethylsulfoniopropionate (DMSP) at −1 °C and 4 °C and at salinities of 32% and 41%, and the effect of temperature is stronger than that of salinity (Dawson et al., 2020). DMSP is of interest because it performs a variety of ecological and physiological functions. DMSP can be detected by marine bacteria and is used as a chemoattractant (Kessler et al., 2018; Seymour et al., 2010). Further, it is important in the

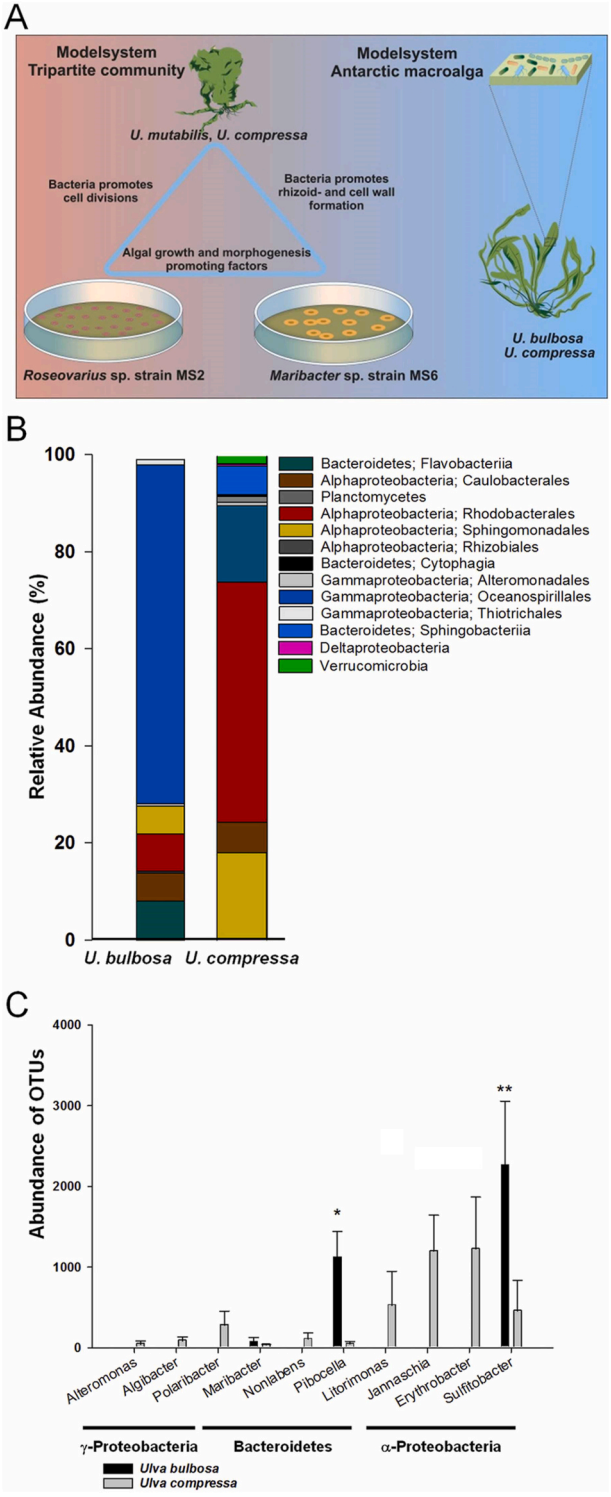

Fig. 1. (A) The reductionist holobiont model system *Ulva mutabilis–Roseovarius* sp.–*Maribacter* sp. (left side) and the Antarctic species *Ulva bulbosa* with its associated microbiome (right side) were used to identify changes in the metabolite profile under thermal stress. (B) The microbiological communities associated with *Ulva* on a taxonomic level are shown based on the abundant operational taxonomic units (OTUs) in 16S rRNA gene amplicon libraries from *U. compressa* and *U. bulbosa* samples. (C) The enrichment of selected bacterial genera in *U. compressa* and *U. bulbosa* was estimated based on the abundance of OTUs. Mean ± standard deviation is shown (n = 3). Significant differences between *Ulva* species are indicated by the asterisk (* $P \leq 0.05$, ** $P \leq 0.01$, n = 3).

pathogenesis of algae (Garren et al., 2014) and bacterial-algal symbiosis (Kessler et al., 2018), and it functions as an antifreeze osmolyte in several algae exposed to salinity variations (Edwards et al., 1987). Since DMSP released by *U. mutabilis* attracts *Roseovarius* sp., which provides AGMPF for the growth of *Ulva*, DMSP production is a key factor in cross-kingdom interactions under stress conditions. Overall, both AGMPF and compatible solutes are indispensable for facilitating communication between macroalgae and bacteria within the chemosphere where the organisms interact (definition by Alsufyani et al., 2017).

To investigate the cold adaptability of the green seaweeds, two experimental designs were used with two different long-time cultured *Ulva* species—one collected from Antarctica (*U. bulbosa*) and the other from the Mediterranean Sea (*U. mutabilis* recently reclassified in *U. compressa*)—to elucidate their metabolic responses to cold stress.

First, we tested the hypothesis that a cold-adapted (i.e., high cold acclimation capacity) holobiont and a warm-adapted (i.e., low cold acclimation capacity) holobiont produce different LMWCs when exposed to low temperatures.

Second, the well-established reductionist, tripartite model system of *U. mutabilis* and its two associated essential bacteria (Wichard, 2022), *Roseovarius* sp. and *Maribacter* sp., was investigated to determine the LMWCs when the tripartite community was exposed to low temperatures and to distinguish the production of compatible solutes between *Ulva* and its bacteria (Fig. 1A).

For metabolomic analysis, hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry (HILIC-ESI-MS) was applied. In addition, the microbiome of *U. bulbosa* was analyzed for AGMPF producing bacteria and compared with the non-cultured strain of *U. compressa*.

2. Methods

2.1. Algae and bacteria

The developmental mutant "slender" (mating type mt+) strain of *U. mutabilis* was propagated from unmated gametes derived from lab-grown parthenogenetic gametophytes. It has been demonstrated that *U. mutabilis*, collected initially by B. Føyn in the Ria Formosa (Portugal) in 1952 (Føyn, 1958), and *U. compressa* are conspecific (Steinhagen et al., 2019). The cultivar is referred to as *U. mutabilis* throughout this publication.

The cold-adapted strain *U. bulbosa* (strain AWI #1002) was collected in Antarctica in 1986 and cultivated at 5 °C at the Alfred Wegener Institute (AWI, Bremerhaven, Germany). In the case of *U. mutabilis*, the identity of the strain was confirmed using PCR with *tuf*A (forward: GGNGCNGCNCAAATGGAYGG, reverse: CCTTCNCGAATMG-CRAAWCGC; (Famà et al., 2002) and *pet*A primers (forward: TGAA-CACGAGCTGGGTTTTG, reverse: TCCACGTGAACCAAATGGAC (Cai et al., 2021)). Further phylogenetic analysis is necessary to taxonomize *U. bulbosa* (strain AWI #1002).

U. bulbosa and *U. mutabilis* gametophytes were cultured in sterile Nunc™ cell culture plastic flasks with gas-permeable screw caps (Thermo Fisher Scientific, Dreieich, Germany) containing 100 mL *Ulva* Culture Medium (UCM) under standard growth conditions, including a 17/7 h light/dark cycle with the illumination of approximately 80 µmol photons m^{-2} s^{-1} provided using 50% GroLux and 50% day-light fluorescent tubes (Stratmann et al., 1996). Both species can grow efficiently at 18 °C. Depending on the experiment, the species were shifted to 5 °C or 2 °C for further cultivation.

Axenic cultures of *U. mutabilis* were prepared according to the approach described by Califano et al. (2018) and subsequently inoculated with the morphogenesis-inducing bacterial strains *Roseovarius* sp. (GenBank EU359909) and *Maribacter* sp. (GenBank EU359911) (Alsufyani et al., 2017; Califano and Wichard, 2018; Spoerner et al., 2012). After inoculation, the final calculated optical density of bacteria was $OD_{600} = 1.0 \times 10^{-4}$. The bacteria were cultured in marine broth at 18 ± 1.0 °C (Roth, Karlsruhe, Germany).

2.2. Microbiome analysis of Ulva bulbosa and Ulva compressa

The DNeasy PowerSoil Pro Kit (Qiagen, Germany) was used to extract the microbial metagenomic DNA, following the manufacturer's protocol without damaging the thallus of *Ulva*. Next, 16S V4 amplicon sequencing of the microbial community was performed on the Illumina MiSeq (2 × 300 bp) using the 515 F-Y "new EMP" (GTGY-CAGCMGCCGCGGTAA) and 806RB "new EMP" (GGAC-TACNVGGGTWTCTAAT) primer pair (LGC Genomics GmbH, Germany). After inline barcode demultiplexing and clipping of sequencing adapters from the 3' ends of reads, the data was processed using the Mothur software package (read pair joining, quality filtering, alignment against Silva 16S reference, denoising, chimera removal) (Schloss et al., 2009). Operational taxonomic units (OTUs) were picked using Mothur (clustering of aligned sequences at the 97% identity level and taxonomy classification against the Silva reference database), and a BLAST search of OTUs against the Silva database of the 16S domain was performed (Califano et al., 2020). Different to the cultivated *U. bulbosa*, *U. compressa* (strain RFU #81) was collected in the Ria Formosa (Faro, Portugal) for microbiome analysis, and a portion of the thallus, including the rhizoid, was frozen without further cultivation (Alsufyani et al., 2014; Grueneberg et al., 2016). Microbiome analyses were performed in triplicate. The entire dataset can be retrieved from NCBI (National Center for Biotechnology Information) through the BioProject ID PRJNA828511 (BioSample Accession Numbers, *U. compressa*: SAMN27670879-SAMN27670881; *U. bulbosa*: AWI #1002: SAMN27670882-SAMN27670884).

2.3. Temperature treatment

Two experimental designs were carried out with a (i) complete and a (ii) designed microbiome:

(i) To compare *U. bulbosa* and *U. mutabilis,* strains were grown with their microbiomes, which had evolved over decades (> 30 years for *U. bulbosa* and > 70 years for U. mutabilis), and cultivated for 14 days at 5 °C ± 1.0 °C and 18 °C ± 1.0 °C. *U. bulbosa* was cultivated at 18 °C for three life cycles prior to starting the temperature shift.

(ii) Axenic gametes of *U. mutabilis* were cultivated together with *Roseovarius* sp. and *Maribacter* sp. in UCM at 18 ± 1.0 °C. Individuals of ~1 cm in length (n = 40) were transferred to bottles with UCM and maintained at 2 ± 0.5 °C and 18 ± 1.0 °C. Algae were harvested during their growing phase (Wichard and Oertel, 2010). All experiments were performed in triplicate.

2.4. Extraction protocols

The algal tissues were quickly dried with a paper towel, collected in plastic tubes (Sarstedt, Germany), and frozen with liquid nitrogen. The tissues were lyophilized (Lyophilizer: Christ Martin Alpha 1–2 LO Plus) for approximately 20 h at − 50 °C and 0.001 mbar. The freeze-dried biomass (weighing 13–21 mg) was transferred to 1.5 mL plastic tubes (Eppendorf, Germany). Two metal beads were added to each tube, and the cells were disrupted for 1 min using a TissueLyser II (Qiagen). Cold methanol (1 mL) was added to extract the metabolites after the addition of internal standards [4 µL of 100 µM D_6DMSP (dimethylsulfoniopropionate) and 4 µL of 4 µM D_3-ectoine ((4*S*)−2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid)] (Fenizia et al., 2020; Gebser and Pohnert, 2013). The mixture was treated in an ultrasonic bath for 1 min and subsequently centrifuged at 1500×*g* for 5 min. The extraction was repeated once to verify that the first extraction was exhaustive.

2.5. Mass spectrometry analysis

2.5.1. Liquid chromatography coupled to mass spectrometry

High performance liquid chromatography (HPLC) was performed using a SeQuant ZIC©-HILIC analytical peek column (5 μm, 150 × 2.1 mm, SeQuant, Umeå, Sweden) equipped with a SeQuant ZIC©-HILIC guard column (5 μm, 2.1 × 20 mm). Analytical separation and quantification of LMWCs (≤ 200 Da) were conducted on a Dionex Ultimate 3000 HPLC system linked to Q Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Mass measurements were performed using the electron ionization-positive mode. Mass/charge ratios ranging from 70 to 200 *m/z* were recorded with a mass resolution of R = 70,000 at *m/z* 200.

For high-resolution mass spectrometry (HRMS) coupled to a HILIC column, 50 μL of the methanol extract was diluted with 150 μL acetonitrile/water (9:1, v/v). The injection volume was 2 μL. For separation, the eluent contained high-purity water spiked with 2% acetonitrile and 0.1% formic acid (solvent A) and 90% acetonitrile with 10% water and 5 mM ammonium acetate (solvent B) (Fenizia et al., 2020; Spielmeyer et al., 2011). The flow rate was set to 0.6 mL min^{-1}, and a linear gradient was used for separation with 100% solvent B (2 min), 60% B (11 min), 20% B (11.8 min), 20% B (14.9 min), 100% B (15 min), and 100% B (18 min). The column was maintained at 25 °C. Before running the samples, the HPLC was conditioned by repeatedly running blanks.

2.5.2. Data processing and metabolite profiling

Peak picking, deconvolution, and metabolite identification was achieved using Compound Discoverer 3.2 (Thermo Fisher Scientific). Peak detection was conducted using a tolerance of 30% for intensity, a signal-to-noise threshold of 5, and a mass tolerance of 5 ppm. The lowest possible peak intensity was set at 100,000. For all tools used for spectrum similarity searches, the mass tolerance was set to 5 ppm. Features that were also identified in blank samples were removed. Pooled quality control samples (QC) were used to compensate for time-dependent batch effects during analysis (Dunn et al., 2011). Raw data were compared with open data repositories (ChemSpider, KEGG) within Compound Discoverer (CD) using the program's default settings and an untargeted metabolomics workflow.

To discover further significant changes between features (*m/z*) observed during the warm-cold temperature shift experiment, a canonical analysis of principal coordinates (CAP) was used to conduct multivariate data analysis to visualize differences in the feature assemblages (i.e., compounds) (Alsufyani et al., 2017; Anderson and Willis, 2003; Anderson and Robinson, 2003). The main coordinates (PCo) were computed from the Bray–Curtis similarity matrix, and potential over parameterization was avoided by selecting the number of PCo axes (m) that maximized the groups' leave-one-out allocation success. The square root was used to transform the data, and column (variable) sums were applied to normalize it.

To identify important metabolites from the mass spectrometric data, a false-positive rate (FDR) correction was performed, coupled with a two-tailed t-test ($P < 0.05$). Following this analysis, univariate statistical tests were completed using MetaboAnalyst 5.0 (Chong et al., 2019) to explore significant differences in intensities of metabolites in the raw data. Treatments were compared by analysis of variance (one-way ANOVA) at $\alpha = 0.05$ after verifying homoscedasticity and normal distribution of the data. A post-hoc Fisher's LSD test ($P < 0.05$) revealed the treatments between which the metabolites exhibited significant differences. Furthermore, volcano plots were utilized to display the univariate analysis results for each LMWC with adjusted $P < 0.05$ and a fold-change larger than two. Statistical analyses in MetaboAnalyst were repeated by Tukey's multiple comparisons test calculating adjusted P values using Prism v.7.00 (GraphPad Software, USA) to ensure accuracy.

2.5.3. Identification and quantification of selected compounds

Candidate compounds were detected by comparison with commercially accessible reference standards and co-injections. Ectoine and DMSP were quantified by adding synthesized labelled standards (Fenizia et al., 2020). An external calibration curve with five calibration standards was performed in triplicate (Deicke et al., 2013) to measure the concentration of the metabolites normalized to the dry weight (DW) of the extracted thallus. A linear regression model was calculated, plotted, and validated with SigmaPlot ver. 14 (Systat, Germany) and GraphPad Prism v.7.00. According to the calibration method, the limit of detection and quantification was determined for all candidate compounds (Reichenbächer and Einax, 2011) with a relative standard deviation (RSD) < 8.2% (Table 2). Repeated extraction with methanol did not increase the amount of any analyte.

3. Results and discussion

3.1. Algal growth and morphogenesis promoting bacteria found independently of Ulvá́s origin

Unlike *U. mutabilis*, the essential bacteria required for the growth and morphogenesis of *U. bulbosa* have yet to be identified (Wichard, 2015). Therefore, the microbiome analysis aimed to determine whether potential AGMPF releasing bacteria can be found independently of *Ulva*'s origin. The complete microbiome of *U. bulbosa* (strain AWI #1002) was first analyzed for the AGMPF producing bacteria and compared with the microbiome of *U. compressa* (strain RFU #81) (Alsufyani et al., 2014), collected in the Mediterranean Sea (Ria Formosa, Portugal), where *U. mutabilis* was originally found (Føyn, 1958). The microbiomes differed significantly from one another (Fig. 1B). While the Mediterranean species was primarily associated with α-Proteobacteria and Bacteroidites, as previously reported (Califano et al., 2020; Friedrich, 2012), the microbiome of *U. bulbosa* was dominated by γ-Proteobacteria and Bacteroidites. The number of OTUs (41) of the cultivated *U. bulbosa* was strongly reduced compared to the number of OTUs (251) obtained from the non-cultivated *U. compressa*, which is typical for the selection process in algal-bacteria interactions and has been described previously (Califano et al., 2020; van der Loos et al., 2021). Importantly, the genera *Maribacter* and *Sulfitobacter*, which are essential AGMPF producers, were associated with both species (Fig. 1C).

3.2. Determination of cryoprotectant compatible solutes

The systematic examination of zwitterionic substances and highly polar metabolites was accomplished using a ZIC-HILIC separation process combined with highly sensitivity MS analysis. Comparison with reference standards led to the detection of ten cryoprotectant candidates previously identified in studies involving both *Ulva* species (Fig. 2). Amino acids, sulfonates, zwitterions, and singly-charged ions were detected. Ectoine, DMSP, cysteinolic acid, and proline were the major compatible solutes. These are commonly employed by microbes and phytoplankton as stress protectants (Fenizia et al., 2020). Ectoine synthesis and uptake provides a considerable degree of osmotic stress tolerance which can provide protection against extreme temperatures. Compatible solutes improve cryopreservation in different organisms, e.g., a combination of proline and ectoine protects human endothelial cells against cryopreservation damage (Fenizia et al., 2020; Sun et al., 2012).

3.3. Different stress responses of Ulva holobionts from the Mediterranean Sea and Antarctica

To explore the cellular metabolic responses of a temperate and a cold-adapted species, cultures of *U. mutabilis* (Ria Formosa, Portugal) and *U. bulbosa* (Polar region, Antarctica) were subjected to a temperature shift from 18 °C to 5 °C simultaneously in the first experiment. After 14 days of incubation, *Ulva* biomass was harvested, extracted, and analyzed by HILIC-HR-ESI-MS. CD revealed 30 LMWCs with mass/

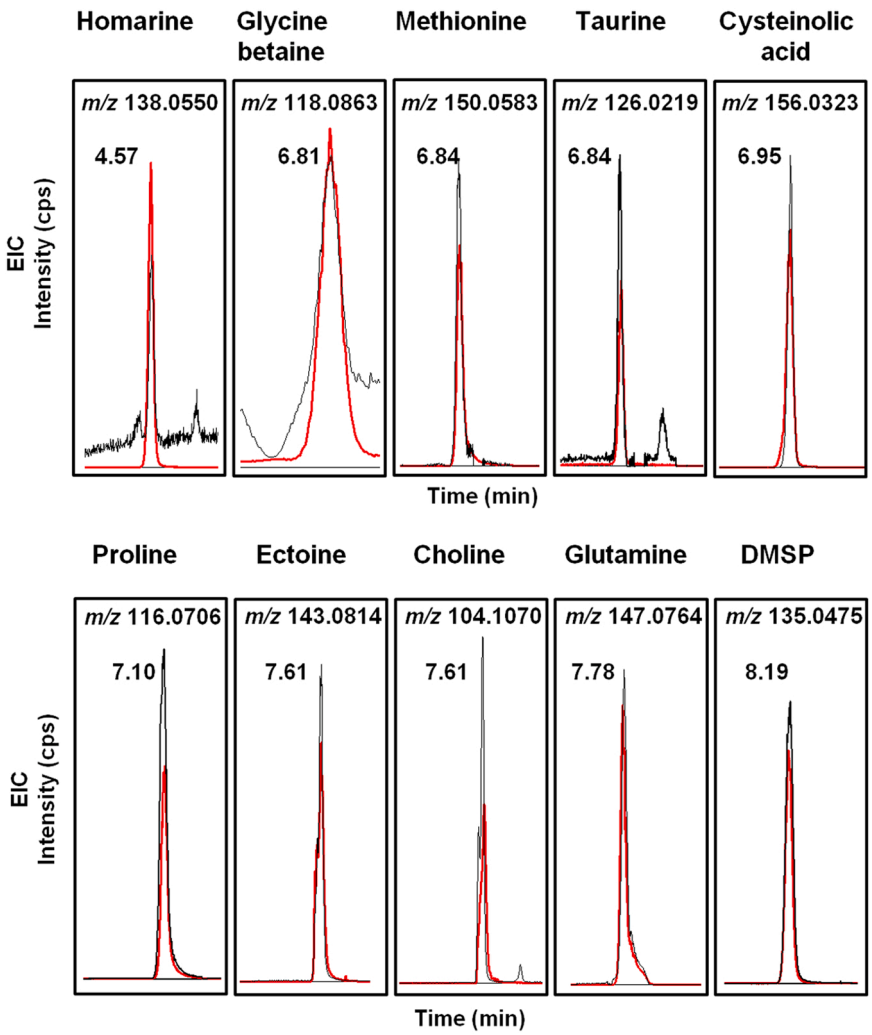

Fig. 2. Low molecular weight compounds identified by comparing the HILIC-ESI-MS chromatograms to the analytical reference standards (red lines). The extracted ion trace chromatograms (EIC, black line) of the *m/z* values ($[M + H]^+$ for all metabolites (except choline $[M]^+$)) detected in the methanol extract of the *Ulva mutabilis* tissue are shown. The EICs matched the reference standards (red line). *m/z*: Mass/charge ratios.

change ratios ranging from 70 to 200 *m/z* after applying background corrections (Table 1). Although CD identified many new features, only a few compounds, such as DMSP, taurine, and glycerol, were clearly characterized and confirmed by reference standards (Fig. 2, Table 1).

Principal analysis of coordinates (PCo) for the 30 identified features demonstrated two distinct groups under cold treatment. The plots indicate that *U. mutabilis* and *U. bulbosa* were separated in both PCo1 and PCo2 after cold treatment, suggesting differences in the metabolic profile of *Ulva* samples influenced by both temperature and species. In contrast, *U. mutabilis* and *U. bulbosa* were not distinctly separated on the PCo plot at 18 °C (Fig. 3A), which emphasizes their similar metabolic profiles in temperate conditions. For supervised discriminant analysis, three groups were defined: (i) *U. bulbosa* at 5 °C, (ii) *U. mutabilis* at 5 °C, and (iii) one combined group of algae grown at 18 °C. CAP analysis of the a priori groups revealed that metabolite profiles significantly differed between the two species depending on the temperature (Eigenvalues: 0.9309 for axis 1 and 0.9225 for axis 2) (Fig. 3B). Using the "leave-one-out" allocation approach, cross-validation between the a priori groups resulted in one misclassification. CAP calculated the correlation coefficient *r* (cutoff: $|r| > 0.5$) between each sample and the canonical axes to identify essential biomarkers.

Following multivariate analysis, a one-way ANOVA was used to prove whether the metabolites differed substantially across treatments. Eighteen metabolites out of 30 were identified as being significantly different, with an FDR-adjusted *P*-value < 0.05 (Table 1). Among the most significantly upregulated metabolites, some are well-known as compatible solutes, such as taurine (a sulfur-containing β-amino acid) and DMSP (Fig. 3C).

Although taurine concentrations (UM and UB in 18 °C) differ significantly between the two species (one-way ANOVA with Tukey posthoc test, $P < 0.05$), box plots reveal a species-specific increase in DMSP and taurine concentrations in *U. mutabilis* and *U. bulbosa*, respectively. Apart from DMSP and taurine, the upregulated features #20 and #15 under cold conditions in *U. mutabilis* and *U. bulbosa* were conspicuous (Fig. 3B, C). The basic concentration of #15 differs significantly ($P < 0.05$) between the two *Ulva* at 18 °C as well. Fisher's least significance difference test for post hoc comparisons revealed differences in metabolite abundance between the *Ulva* species at different temperatures. Future investigations will identify other substances that are only listed as sum formulas in Table 1.

In summary, the metabolic profiles of *U. mutabilis* and *U. bulbosa* in cold conditions are dissimilar, suggesting that these two holobionts respond to cold stress differently due to differences in algal metabolism (intrinsic) and microbiomes (extrinsic). It is still under discussion how the associated and stress-adapted microbiomes can help the host adapt to environmental stresses through the production and subsequent secretion of chemical metabolites (Ghaderiardakani et al., 2020).

Table 1
Identification of 30 features in *U. mutabilis* (UM) and *U. bulbosa* (UB) following a One-way ANOVA. Metabolites were considered significant if the FDR adjusted *P* value was < 0.05. Fisher's LSD post hoc test indicates significant differences ($P < 0.05$) between the comparisons (ns = not significantly different; n.d. not determined) (Note: retention times vary slightly from batch to batch within this study.).

No. #	*m/z* $[M+H]^+$ measured	RT [min]	Compound (identified)	Formula [M]	Significance *P*-value	FDR adjusted *P*-value	UB-5 °C vs UB-18 °C	UB-18 °C vs UM-18 °C	UM-5 °C vs UM-18 °C	UM-5 °C vs UB-5 °C
	Identification				One way ANOVA		Fisher's LSD post hoc comparison			
1	135.0472	8.40	DMSP	C5H10O2S	1.98E-02	3.30E-02	ns	ns	< 0.05	ns
2	156.0323	7.09	Cysteinolic acid	C3H9NO4S	5.89E-04	8.11E-03	< 0.05	ns	< 0.05	< 0.05
3	157.0293	8.58		n.d.						
4	145.0495	7.12		C6H8O4	2.93E-03	8.71E-03	< 0.05	ns	< 0.05	ns
5	145.0494	7.14		C6H10O5	3.55E-03	8.71E-03	< 0.05	ns	< 0.05	ns
6	85.0283	7.14		C4H4O2	1.89E-03	8.11E-03	< 0.05	ns	< 0.05	ns
7	127.0388	7.13		C6H6O3	3.59E-03	8.71E-03	< 0.05	ns	< 0.05	ns
8	138.0219	6.99		C3H7NO3S	1.22E-03	8.11E-03	ns	ns	< 0.05	< 0.05
9	97.0283	7.13		C5H4O2	1.81E-03	8.11E-03	< 0.05	ns	< 0.05	ns
10	180.0866	7.10		C6H15NO6	1.24E-02	2.32E-02	< 0.05	ns	< 0.05	ns
11	159.0252	8.51		n.d.						
12	158.0281	7.08		n.d.	1.48E-03	8.11E-03	ns	ns	< 0.05	< 0.05
13	180.0865	7.13		C6H13NO5	8.87E-03	1.78E-02	< 0.05	ns	< 0.05	ns
14	160.0344	8.01		C6H10NPS	7.67E-03	1.64E-02		ns	< 0.05	< 0.05
15	123.0553	3.84		C6H6N2O	2.38E-03	8.71E-03	< 0.05	< 0.05	ns	< 0.05
16	192.0866	7.98		C7H13NO5						
17	99.0439	7.13		C5H6O2	1.22E-03	8.11E-03	< 0.05	ns	< 0.05	ns
18	109.0283	7.09		C6H4O2	3.77E-03	8.71E-03	< 0.05	ns	< 0.05	ns
19	165.1134	8.01		C8H12N4						
20	189.087	2.68		C7H12N2O4	1.88E-02	3.30E-02	ns	ns	ns	< 0.05
21	93.0546	6.15	Glycerol	C3H8O3						
22	163.06	6.15		C6H10O5						
23	121.0318	7.70		C4H8O2S	3.02E-03	8.71E-03	< 0.05	ns	< 0.05	ns
24	178.1337	8.10		C10H15N3						
25	85.0284	6.15		C4H4O2						
26	151.0352	2.03		C3H6N2O5						
27	144.0477	8.13		C6H9NOS						
28	126.0218	6.91	Taurine	C2H7NO3S	3.50E-04	8.11E-03	< 0.05	< 0.05	ns	< 0.05
29	104.0706	8.04		C4H9NO2						
30	161.1283	8.02		C7H16N2O2						

3.4. Metabolite profiling of the tripartite community Ulva mutabilis under cold stress at 2 °C

To reduce the effect of non-essential bacteria for growth and development of *U. mutabilis*, the cold stress experiment was repeated with the tripartite community (*U. mutabilis–Roseovarius* sp.– *Maribacter* sp.; Fig. 1A) under more stringent conditions at 2 °C in the second experiment. Following cultivation at 18 ± 1 °C and 2 ± 0.5 °C for 14 days, the volcano plot of the identified features revealed significant differences between the metabolic profiles of *Ulva* grown under standard conditions and that grown in the cold (Fig. 4A). Among the 66 features detected in *U. mutabilis* (Table S1), 20 LMWCs were significantly different (18 upregulated and only 2 downregulated) between the two temperature treatments (Fig. 4A). As only three compounds, namely DMSP, proline, and glutamine, were identified by CD (Table S1), a targeted analysis was used to complement the exploratory approach (Fig. 4B) because additionally known candidate molecules were expected (Fig. 2).

3.5. Targeted analysis of cold stress-dependent changes in cryoprotectants

We performed a complementary experiment to determine the temperature stress-related production of the ten candidate compounds: DMSP, taurine, cysteinolic acid, ectoine, choline, homarine, glycine betaine, proline, glutamine, and methionine, under xenic and axenic conditions. The production of all compounds except homarine and methionine were upregulated following a temperature shift from 18 °C to 2 °C in the tripartite community of *U. mutabilis* (Fig. 4B). All substances were quantified in the low micromolar range with an operating range of one order of magnitude identified in one chromatographic run (Fig. 2) with a low relative standard deviation (RSD% from 2.5 to 8.2) and a low limit of quantification of the analytical process (Tables 2 and 3).

The concentrations of the analytes, normalized to dry weight (DW), ranged over several orders of magnitude from 7.4×10^{-4} mg g^{-1} (taurine) to 10.97 mg g^{-1} (DMSP; Table 3). The increases following cold treatment were highly statistically significant ($P < 0.001$, Fig. 4B) for DMSP (3.5-fold increase), cysteinolic acid (9.5-fold increase), choline (1.4-fold increase), glycine betaine (13.4-fold increase), and proline (24.9-fold increase). In contrast, the increases in taurine and ectoine were of lower significance ($P < 0.05$) due to the variance of the data. At 18 °C, proline, glutamine, and glycine betaine concentrations were below the quantification limit. Proline (6.55 ± 1.53 mg g^{-1} DW) and DMSP (10.97 ± 2.58 mg g^{-1} DW) may be the most potentially beneficial metabolites in *Ulva*'s cold adaption process, as reported for various bacteria and sea-ice algae under high-osmolarity and colder growth conditions (Brill et al., 2011; Dawson et al., 2020; Götz et al., 2018).

DMSP has been proposed as a cryoprotectant in several green macroalgae (Karsten et al., 1992). In our study, DMSP concentration increased from 3.07 ± 0.47–10.97 ± 2.58 mg g^{-1} DW following the shift to cold temperature, which is comparable to previously reported increases from approximately 1.3 to 37.5 mg g^{-1} DW in green macroalgae collected from various latitudes. Those collected from polar regions had the highest DMSP concentration (Van Alstyne and Puglisi, 2007), whereas DMSP concentration was low (0.04 mg g^{-1} DW) in the tropical chlorophyte *Ulva conglobata* (Bischoff et al., 1994). As the concentration of DMSP in *U. lactuca* increased significantly with latitude, Van Alstyne and Puglisi (2007) concluded that the concentration of compounds depends on environmental factors (e.g., light, temperature, day length) that change with latitudes. In this context, previous studies revealed that the cellular concentration of DMSP also increases with light intensity in various green macroalgal species (Karsten et al., 1990b, 1992).

Our findings support the hypothesis that DMSP production is

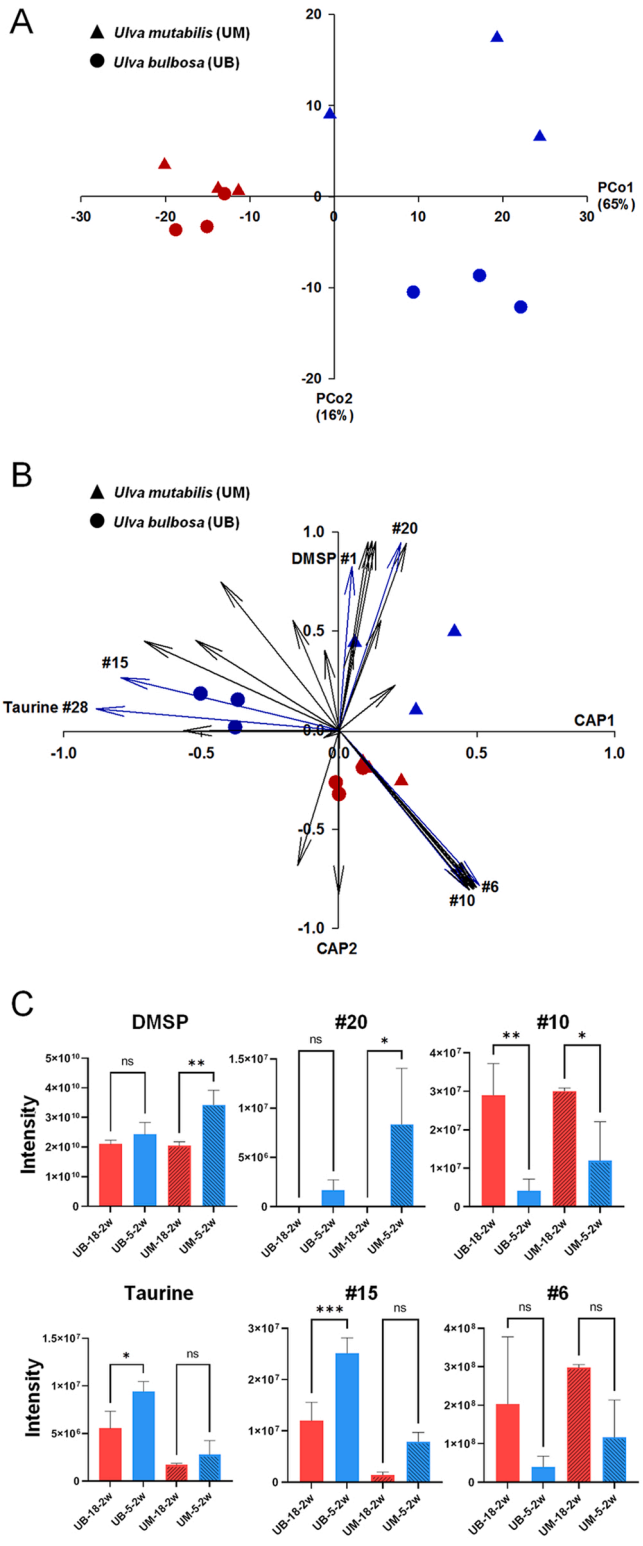

Fig. 3. Species-dependent cold stress response of the holobiont *Ulva*: (A) Multivariate data analysis of the mass spectrometric data upon HILIC-ESI-MS measurements. The score plot was obtained from principal coordinates analysis (PCoA) of the untargeted metabolite profiling of *Ulva mutabilis* (triangle) and *Ulva bulbosa* (circle) cultivated at 18 ± 1.0 °C (red color) and 5 ± 1.0 °C (blue color). (B) The first two canonical axes of the CAP analysis demonstrate the separation based on the *Ulva* species affected by the cold. Scaled vectors of the *m/z* features were significant, separating the groups for the correlation coefficient $|r| > 0.5$ (correlation of the *m/z* features with the two CAP axes). The numbers refer to the order of features identified using Compound Discoverer software (CD) listed in Table 1. (C) Bar graphs demonstrate the species-specific significant increase in DMSP and taurine in cold-stressed *U. mutabilis* (UM) and *U. bulbosa* (UB), respectively, after two weeks (2w) of temperature treatment. There are also several unknown features (e.g., #20 and #15 are increased, and #10 and #6 are decreased). One-way ANOVA was followed by Tukey's post-hoc to indicate the significant differences (ns $P > 0.05$, *$P \leq 0.05$, ***$P \leq 0.001$, n = 3).

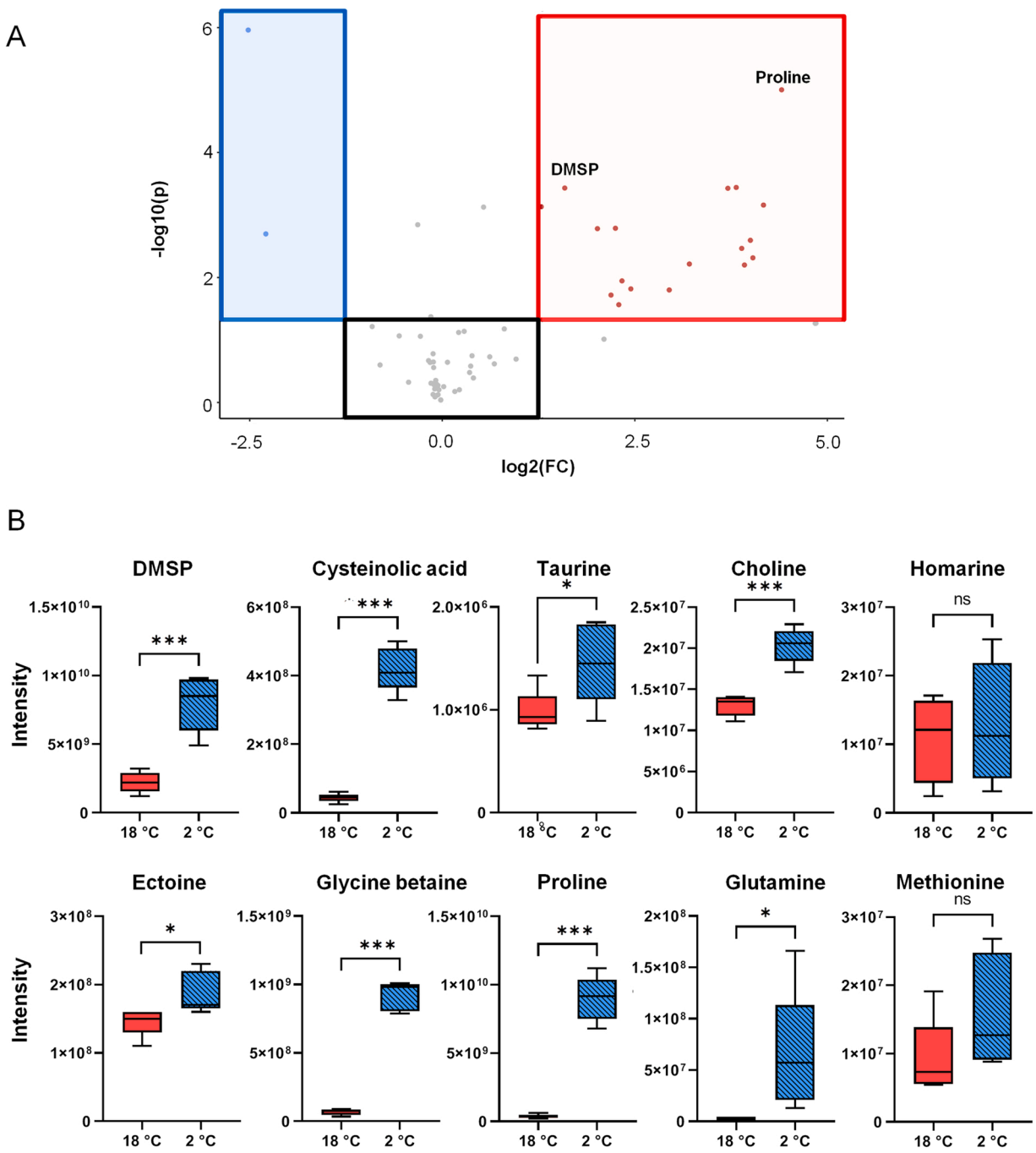

Fig. 4. Metabolite production in the tripartite community of *Ulva mutabilis* upon cold stress. (A) Volcano plot illustrates the 20 *m/z* features (i.e., metabolites) that were significantly altered in *U. mutabilis* due to a temperature shift from 18 ± 1.0 °C to 2 ± 0.5 °C. The rosa dots represent features with of statistical significance *P* less than 0.05 and a fold-change greater than 2-fold. Proline and DMSP are marked as examples. The greater the distance between the feature's coordinates and (0,0), the more significant the feature is. (B) Box plots illustrate the intensity changes in 10 selected cryoprotectants within the *U. mutabilis* metabolomic profile at 18 °C in comparison with their intensity at 2 °C. ANOVA followed by Tukey's post-hoc test was used to determine the significant differences (ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$, $n = 3$).

temperature-dependent. Interestingly, the increased DMSP concentration was not associated with an increased production of its precursor methionine, which did not vary in response to temperature changes (Fig. 3C). This observation is consistent with our previous findings that a temperature-sensitive methyltransferase regulates DMSP production (De Clerck et al., 2018). Care must be taken when comparing values that have been normalized to dry weight or fresh weight. It has been estimated that the drying process causes a loss of up to 75% of the total DMSP yield (Bischoff et al., 1994; Bucciarelli et al., 2021).

Taurine content almost doubled (1.8-fold) in *U. bulbosa* upon shifting the temperature from 18 °C to 5 °C. Taurine, a sulfur-containing β-amino acid, is produced from cysteine and released with no further degradation. Sulfonates are prevalent in marine algae, which may explain the extensive abundance of sulfonate-degrading bacteria in the marine ecosystem (Scholz et al., 2021). While only taurine traces were generally detected in plants, seaweeds can contain relatively high concentrations (Terriente-Palacios and Castellari, 2022). In Rhodophyta, the concentration of taurine was approximately 0.08% of DW (Scholz et al., 2021). Oxidative stress regulation was suggested to be the primary function of taurine. According to Jong et al. (2012), β-alanine-mediated

Table 2
Calibration parameters for those compatible solutes quantified in the thallus of *Ulva mutabilis* using the calibration method.

Compound	Working range	Limit of detection LOD	Limit of quantification LOQ	Rel. standard deviation RSD
Proline	5–50 μM	1.5 μM	5.4 μM	8.2%
Glutamine	1–16 μM	0.28 μM	1.01 μM	3.9%
DMSP	12.5–100 μM	2.6 μM	9.2 μM	3.5%
Taurine	6.25–50 nM	2.8 nM	9.4 nM	6.5%
Methionine	0.2–1 μM	0.1 μM	0.5 μM	2.5%
Glycine betaine	0.5–5 μM	0.14 μM	0.5 μM	5.3%
Ectoine	2.5–50 nM	2.9 nM	9.9 nM	5.0%

Table 3
Quantification of the compatible solutes per dry weight (DW) biomass of *Ulva mutabilis*.

Compound (mg/g DW)	18 °C	2 °C
Proline	< LOQ	6.55 ± 1.53
Glutamine	< LOQ	0.055 ± 0.002
Methionine	$1.5 \times 10^{-3} \pm 1.4 \times 10^{-4}$	$2.6 \times 10^{-3} \pm 9.2 \times 10^{-4}$
DMSP	3.07 ± 0.47	10.97 ± 2.58
Taurine	$7.4 \times 10^{-4} \pm 2.4 \times 10^{-4}$	$1.89 \times 10^{-3} \pm 4.5 \times 10^{-4}$
Glycine betaine	< LOQ	$1.78 \times 10^{-2} \pm 2.7 \times 10^{-3}$
Ectoine	$4.04 \times 10^{-2} \pm 6.9 \times 10^{-3}$	$5.4 \times 10^{-2} \pm 7.2 \times 10^{-3}$

taurine depletion reduces electron transport, resulting in increased mitochondrial superoxide production (Jong et al., 2012). This important observation could explain the role of taurine as a significant compound in stress regulation. In our experiments, taurine concentration was significantly affected by temperature in *U. bulbosa* but not in *U. mutabilis*, emphasizing the difference in responses of these two *Ulva* species to temperature stress. Moreover, taurine has the potential to be an important carbon source for heterotrophic bacterial growth. (Clifford et al., 2019). Therefore, algae-derived taurine may assist the metabolism of the algae-associated microbial community.

Cysteinolic acid, another sulfur amino acid, was elevated in *U. mutabilis* (9.5-fold) after the temperature shift, but the concentration of cysteinolic acid was below the limit of quantification. It has well recognized physiological antioxidant activity and osmoregulatory effects and is a member of the class of marine sulfonates that are often produced by algae and metabolized by bacteria. The high content of D-cysteinolic acid has been reported in *Ulva* sp. (Ito, 1963). The content of cysteinolic acid in the diatom *Thalassiosira weissflogii* rose dramatically in response to short- and long-term ambient salinity changes (Fenizia et al., 2021). Similarly, cysteinolic acid concentration changed significantly under cold stress in *U. mutabilis* (Fig. 4B).

Proline concentration increased sharply by 24.9% following the temperature shift, whereas methionine concentration did not change (Fig. 4B). The accumulation of proline in plants exposed to abiotic stress is a well-documented and conserved response in most vegetal species. In our experiments, proline concentration in *U. mutabilis* had the highest fold change of any metabolite studied. Similarly, proline accumulated (to 6.48 mg g^{-1} DW; 9.8-fold increase) in *U. fasciata* exposed to elevated salinity (Lee and Liu, 1999). Proline was also detected at high concentrations (4-fold increase) in response to decreased temperature (–1 °C vs. 4 °C) in the polar diatom, *N. lecointei* (Dawson et al., 2020). This change is similar to the 4.5-fold increase in proline concentration seen in *Fragilariopsis cylindrus*, a highly common psychrophilic diatom, while encased in newly formed sea ice and subjected to increasing external salinities and lower temperatures. In the face of these challenges, *F. cylindrus* growth is temporarily inhibited and only resumes after a prolonged period of acclimatization, during which proline accumulates in the cell (Krell et al., 2007). Proline, a cyclic amino acid, has thus emerged as a significant component of a typical algal physiological response to various abiotic stresses. Proline can stabilize sub-cellular structures, scavenge free radicals, and inhibit lipid peroxidation in the plant (Kaur and Asthir, 2015) and human cells (Sun et al., 2012). Free proline may be thus an indicator of cold-hardening and stress biomarkers in *Ulva*.

Under cold stress conditions the concentration of glutamine, also a proxy for its precursor glutamate, was increased by 20.6-fold in *U. mutabilis*. Glutamate is a precursor of several metabolites such as proline, arginine, and histidine, and it is involved in multiple responses such as those in response to pathogen resistance and abiotic stress (e.g., cold, heat, and drought) (Gawryluk et al., 2019; Qiu et al., 2020).

Glycine betaine, which increased 13.4-fold after exposure to cold stress, is widely considered an effective protectant to alleviate the negative effects of stressful environments on plants. Addition of

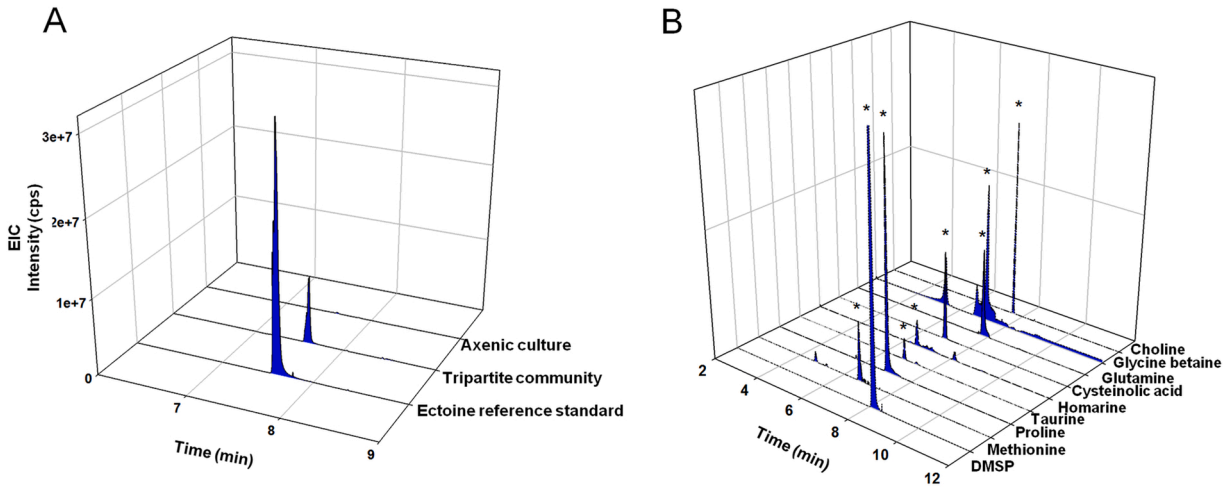

Fig. 5. Analysis of the tripartite community and axenic cultures of *Ulva mutabilis* for cold response candidate compounds. Extracted ion trace chromatograms (EIC) of the molecule ions $[M+H]^+$ of the candidate compounds obtained by HILIC-ESI-MS analysis are shown. (A) Detection and structural elucidation of ectoine (*m/z* 143.0814 for $[M + H]^+$) in *U. mutabilis* co-cultured with bacteria. Ectoine was not detected in the absence of bacteria, providing further support for the bacterial origin of ectoine. Samples were collected from *U. mutabilis* tissues cultured at 5 °C for 14 days. (B) Analysis of axenic cultures: Execpt for ectoine, all selected metabolites were detected in axenic cultures of *U. mutabilis* (asterisks indicate the relevant peaks).

exogenous glycine betaine to cultures of the microalga *Chlorella sorokiniana* at low suboptimal temperatures improved photosynthesis by increasing the expression of genes encoding RuBisCo (Wang et al., 2016). Consistent with the current study's findings, glycine betaine concentration doubled at 41% salinity compared to 32% salinity when grown at 4 °C in the Antarctic diatom *N. lecointei*.

Choline, the precursor to glycine betaine, rose in quantity in response to temperatures below zero, although at a significantly lower intracellular concentration than that of glycine betaine (Dawson et al., 2020). The concentration of homarine, the other nitrogen-content compatible solute, did not change in *U. mutabilis* under cold stress.

3.6. Source of the cryoprotectants and bacterial contribution to cold adaptation

The candidate compounds were examined in axenic algae to determine whether *Ulva* or the associated microbiome had produced the solutes within the holobiont. Ectoine was the only compound not detected in axenic cultures of *U. mutabilis* at 18 °C or 5 °C (Fig. 5A), showing that it is produced exclusively by the bacteria associated with the holobiont. It has previously been shown that ectoine could be used as a biomarker to identify *Roseovarius* sp. MS2 in the algal rhizoidal zone (Vallet et al., 2021). In contrast, axenic algae had large amounts of metabolites such as cysteinolic acid, DMSP, and choline (Fig. 5B) which indicates intrinsic production (Fenizia et al., 2021; Kessler et al., 2018). The low bacterial density within the tripartite community (Fig. 1A) also explains the low concentration of ectoine ($4.04 \times 10^{-2} \pm 6.9 \times 10^{-3}$ mg g^{-1} DW).

Bacteria possess properties that allow biofilm formation on various *Ulva* species and promote their growth and morphogenesis (Ghaderiardakani et al., 2017; Marshall et al., 2006; Wichard, 2015). *Ulva* development is thus entirely dependent on AGMPFs provided by specific bacteria such as *Roseovarius* sp. and *Maribacter* sp. (Ghaderiardakani et al., 2019; Spoerner et al., 2012). While xenic germlings survive at 2 °C, axenic gametes of *U. mutabilis* die at temperatures < 5 °C (data not shown). Thus, it is tempting to assume that *Roseovarius* sp. contributes not only AGMPFs but also cold stress adaptation factors for growth. However, the constant requirement for bacterial morphogens makes it challenging to distinguish the effects of bacterial and algal processes on cold adaptation. In future studies, quantification of bacterial morphogens and cryoprotectants in the *Ulva* environment, experiments on ectoine uptake, and fitness measurements of *Ulva* seedlings will shed light on the ecophysiological role of cryoprotectants.

4. Conclusion

Warm-cold temperature shifts affect various morphological, cytological, physiological, and biochemical traits in plants. Plants acclimated to harsh ecosystems such as polar regions should retain strategies to cope with severe stresses. Metabolic profiling of *Ulva* tissues of two different species collected from Antarctica and the Mediterranean Sea enabled the identification of unknown markers and specific cryoprotectants that alleviate temperature stresses. *U. bulbosa* and *U. mutabilis* reacted differently to the temperature shift from 18 °C to 5 °C by increasing the production of taurine and DMSP, respectively, among many other (unknown) metabolites. The production of compounds was triggered in the Mediterranean *U. mutabilis* due to the cold stress; however, in the case of DMSP, the level of production was lower than expected from species of cold regions (Van Alstyne and Puglisi, 2007). Future studies are required to investigate the correlation between DMSP, taurine, and proline production and changes in the growth rate of *U. mutabilis* during cold acclimation. Furthermore, when compared to freshly collected specimens, the long-term captivity of the studied cultures may have some effects on the biochemical inheritance.

Our study is limited to a small number of molecules that still need to be characterized to assign them to biosynthetic pathways and signal transduction chains. Future studies of *Ulva* species, which were freshly collected from the polar region, will help us further understand the cold adaptation process compared to the model system *U. mutabilis*.

Despite the several existing hypotheses regarding the microbiome's contribution to host response to environmental factors (e.g. their potential role in host resilience to stress), there is little evidence to support these hypotheses. Phycological experiments using the tripartite system and metabolomics approaches can be used to understand the contribution of microbiome composition to holobiont adaptation processes in future studies. Axenic cultures of *U. bulbosa* should be supplemented with morphogenetic compounds to reproduce algal development independent of the microbiome to distinguish the effects of morphogens on the metabolome and transcriptome from the innate cold stress response.

CRediT authorship contribution statement

Fatemeh Ghaderiardakani: Methodology, Investigation, Formal analysis, Validation, Visualization, Writing – original draft. **Linda Langhans:** Investigation. **Valentin B. Kurbel:** Investigation. **Simona Fenizia:** Investigation, Validation. **Thomas Wichard:** Conceptualization, Methodology, Supervision, Formal analysis, Validation, Data curation, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.104913.

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