Metabolite Profiling of the Chemosphere of the Macroalga Ulva (Ulvalles, Chlorophyta) and its Associated Bacteria

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Chemisch-Geowissenschaftlichen Fakultät der
Friedrich-Schiller-Universität Jena

von

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Tag der öffentlichen Verteidigung: 24.09.2014
Acknowledgements

First and foremost, I thank my Lord. Thanks “Allah” for being with me when I have been completely alone. You have given me the power to believe in myself and pursue my dreams. I could never have done this without the faith I have in you, the Almighty.

This thesis appears in its current form due to the assistance and guidance of several people. I would therefore like to offer my sincere thanks to all of them.

Prof. Dr. Georg Pohnert, I would like to thank you for giving me the opportunity to start and complete my PhD project. My sincere thanks for your warm encouragement, thoughtful guidance, critical comments, and correction of the thesis.

Dr. Thomas Wichard, I want to express my deep acknowledgement for the trust, the insightful discussion, offering valuable advice, for your support during the whole period of the study, and especially for your patience, guidance during the writing process, and for translation the abstract into German. Thanks very much. I gained really a lot of knowledge under your supervision and during my work with you. I am looking forward to transferring this knowledge to my work team in my laboratory.

Lab mates, I would like to thank Jan Grüneberg, Michael Deicke, Ralf Keßler, Anna Weiß and Stefan Kügler. Thanks for the time we spent for discussion and the knowledge we shared and exchanged. Thanks to Anna for helping in translation the declaration into German and to you Stefan for answering all my irritating questions. Thanks to Ralf for proofreading the German translated abstarct. Special thanks to you “Jan Grüneberg and Michael Deicke” for all scientific and cultural advices you have given during my stay in Jena. I sincerely appreciate your efforts.

All members of Prof. Pohnert’s group, My appreciation is extended to all of you for the help provided whenever I needed. Thanks to Hannes Richter for your help in my bioreactors’ experiment and the patience you had during that long-term experiment. Many thanks also to Andrea Bauer, Dr. Katharina Grosser, Dr. Martin Rempt, Dr. Astrid Spielmeyer, Phillipp Richter, Stefanie Wolfram, Raphael Seidel, Johannes Frenkel, Katharina Eick, Anett Kaulfuß, Karen Bondoc, and Michaela Mauß for your time and help, which were given to me during my work. Thanks for being opened mind, and providing me a nice atmosphere for work and discussion.
Special thanks for you all for smiling, and saying Hello whenever we have met (it means really a lot for me).

**Dominique Jacquemoud**, I am thankful for you due to the patience you gave in order to correct this thesis, and appreciate your helpful comments and discussion, which has an impact on this final version. Thanks to **Dr. Amal Althalhi** and **Ms. Ohood Alsufyani** for proofreading the Arabic translated abstract.

I am grateful to the secretary **Madlen Kühn**, for assisting me in many different ways and handling the paperwork.

**Aquaculture’s team**, I’m grateful to Dr. Aschwin Engelen, and Dr. João Reis for their assistance, support, and advices to perform aquaculture and to make it successful. Special thanks are given to **Astrid Mejia** for being friendly and patient during collection of the samples and conducting the experiments, thank you Astrid.

**For sequencing and phylogenetic analyses performed in chapter (3.1)**, I acknowledge Dr. Onno E. Diekmann.

Thanks to the **work team of Prof. Dr. Hans Peter Saluz** for performing the nanodrop and qPCR analyses at Cell and Molecular Biology department, HKI. Special thanks is given to **Ms. Shayista Amin** for mediating and facilitating the scientific communication regarding these analyses.

**To you my beloved FAMILY**, I cannot express my thanks and gratitude into words for all what you have been giving to me, without your support and encouragement I really could not have done this work. I warmly thank you my Dad and Mom, simply without you I would not be Taghreed (تميّزت نع وطهرت، وتيتاملك زوجتي نع مكنش). My great thanks and acknowledgement to my brothers; AbdulAllah (thanks for your continuous support), Ommer (thanks for your care) and Rae’d (thanks for your patience), to my sisters; Ghadah, Ohood and Waffa (thanks for bearing me as a strange sister،دينتاملك زوجتي نع مكنش). I’m thankful to my sisters in law; Affaf, Waad and my best friend Doodah. Thanks to all my cute and lovely nephews and nieces. Thank you Fahad, Sultan, Assoli, Muhammed, A., A. Alqurashi, Z. Alqahatni, G., F., F., A. Alsufyani. A. L. A. Alsufyani. A., A. and M. Alsufyani. It was you all who kept my spirits always high. I can just say thanks for everything and may Allah give you all, all the best in return.
My friends, I would like to thank my friends for being beside me in Germany, and for filling my life while I was alone. My cordial thanks to Shayista (Kashmir), you really have done a lot for me and I will keep praying for you and forever. My thanks is extended to my friend Ruqiah (Libya) for encouraging me although the long distance between us. Thanks again for all of you for being in my life. ”May Allah reward you all”.

My funding sources, I want to acknowledge, Taif University, Kingdom of Saudi Arabia, for funding my PhD project. My acknowledgement is extended to ASSEMBLE for funding my stay at the Ramalhete Station, Faro, Portugal.

Taghreed Abdulraheem Alsufyani

June 2014, Jena, Germany
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Zusammenfassung

Die eukaryotische Grünalge *Ulva* spp. (Chlorophyta) ist eine weltweit vorkommende Makroalge und tritt häufig während so genannter “green tides” massenhaft in eutrophierten Küstenregionen auf. *Ulva* spp. lebt in Gemeinschaft mit Bakterien, die das Wachstum und die Morphogenese der Alge ermöglichen. *Ulva* interagiert hierbei mit den assoziierten Mikroben durch Freisetzungen spezifischer alellopathischer Substanzen in die Chemosphäre, die die Gesamtheit aller Substanzen in einer Biozönose umfasst, in der verschiedene Organismen miteinander wechselwirken.

Algale Oxylipine, wie zum Beispiel die vielfachungesättigten Aldehyde, die aus mehrfach ungesättigten Fettsäuren gebildet werden, spielen eine wichtige Rolle in der Ausbildung der mikrobiologischen Gemeinschaft. In dieser Studie wurden *Ulva* Spezies an verschiedenen Probenahmestellen in der Lagune Ria Formosa (Portugal) gesammelt, um sie hinsichtlich ihres Potenzials zur Produktion von vielfachungesättigten Aldehyde (PUA: polyunsaturated aldehydes) zu untersuchen. Lipoxygenase und Hydroperoxid-Lyase vermittelte Reaktionen bilden verschiedenste Oxylipine aus vielfachungesättigten Fettsäuren. Diese enzymatischen Fettsäureumsetzungen sind sehr divers und spielen eine wichtige Rolle bei der Informationsvermittlung, Stressreaktionen und chemischen Verteidigungsstrategien der Makroalgen. Daher wurden PUAs im Rahmen einer Reihenuntersuchung quantifiziert. Interessanterweise produzieren insbesondere die „Salatblatt“-förmigen *Ulva* Arten nach Zellverletzung PUAs wie z.B. das 2,4,7-Decatrienal oder das 2,4-Decadienal im Gegensatz zu den röhrenförmigen Arten, die keine PUAs freisetzen.

Darüber hinaus haben morphogenetische und phylogenetische Analysen der untersuchten Arten eine chemotaxonomische Signifikanz der untersuchten Biosynthesewege aufgezeigt. Untersuchungen zu den Biosynthesewegen haben gezeigt, dass die PUAs aus ω3 und ω6 vielfachungesättigten Fettsäuren (PUFA, polyunsaturated fatty acids) mit 20 und 18 C-Atomen (Arachidonsäure (C20:4 n-6), Eicosapentanoensäure (C20:5 n-3), γ-Linolensäure (C18:3 n-3) oder Stearidonsäure (C18:4 n-3)) gebildet werden. 11- und 9-Lipoxygenasen katalysieren dabei die Umsetzung der C20 und C18 Fettsäuren über den Eicosanoid- und dem Octadecanoid-Biosyntheseweg in PUAs und kurzketttige hydroxylierte Fettsäuren.

“*Die Chemosphäre der algalen-bakteriellen Lebensgemeinschaft ändert sich mit den verschiedenen Wachstumsphasen und Biomarker dieses Exo-Metaboloms können genutzt werden, um den Generationswechsel von Ulva vorherzusagen“*

Um diese Hypothese zu testen, wurde *U. mutabilis* in 25 L Bioreaktoren oder in 200 L Aquakulturen kultiviert, die mit sieben Tage alten Keimlingen oder axenischen Kulturen angeimpft wurden. In der Tat gelang es den kompletten Lebenszyklus des Gametophyten in Kultur unter diesen Bedingungen darzustellen, wenn mit den richtigen Bakterien ebenfalls angeimpft worden war. Das Nährmedium musste hierbei nicht zusätzlich gewechselt werden. Biotest haben gezeigt, dass *Ulva* drei wesentliche Phasen durchläuft, die sich durch die Befähigung der Alge zur Gametogenese unterscheiden: (1) die Gametogenese ist nicht induzierbar, (2) die Gametogenese ist induzierbar oder (3) sie verläuft spontan. Der Nährstoffverbrauch war insbesondere hoch während der Wachstumsphase, wenn die Gametogenese der Alge auch bereits induzierbar war.

Mittels Festphasenextraktion wurden die in der Wasserphase vorhanden Substanzen extrahiert und nach Separierung durch Ultra-High Performance Liquid Chromatography (UHPLC) oder durch Gaschromatographie (nach Derivatisierung) mit einem Flugzeitdetektor-

Darüber hinaus hat diese Studie gezeigt, dass sich Änderungen im metabolischen Fingerabdruck im Wasserkörper durch \textit{U. mutabilis} auf veränderten Umwelteinflüsse (Mikrobiome, Nährstoffe usw.) zurückführen lassen. Zum Beispiel wurde der algale Biomarker 2,4,6-Tribromophenol in der Chemosphäre der Dreiecksbeziehung gefunden, nicht aber in den Aquakulturen, in der auch weiter Mikroorganismen aufgrund der nicht sterilen Bedingungen zu finden waren.

Zusammenfassend sind die Änderungen zwischen den Wachstumsphasen im metabolischen Profil signifikant, sodass sich Änderungen in Bezug auf das algale Wachstum und den Lebenszyklus prognostizieren lassen. Das Wissen um die Biomarker für die entsprechenden Wachstumsphasen und Lebenszyklen ist essentiell, um Aquakulturen mit ökonomisch relevanten Biomassen zukünftig nachhaltig bewirtschaften zu können.
Abstract

The eukaryotic green marine algae Ulva spp. (Chlorophyta), are widespread macroalgae often involved in blooms. Ulva spp. are usually associated with marine bacteria to meet their physiological needs and exhibit therefore microbe-dependent growth and morphotypes. Ulva spp. might actively affect their microbiome by releasing specific compounds in its chemosphere. For instance, algal oxylipins including polyunsaturated aldehydes (PUAs) derived from polyunsaturated acids (PUFAs) might play an important structuring role for the microbiome. In the present study, Ulva spp. collected at various sampling sites in the lagoon of the Ria Formosa (Portugal) have been studied with respect to (1) their ability to produce polyunsaturated aldehydes and (2) their ability to communicate with their surrounding bacteria via infochemicals.

Lipoxygenase/hydroperoxidelyase mediated transformations convert polyunsaturated fatty acids into various oxylipins. These fatty acid transformations are highly diverse in marine algae and play a crucial role in e.g., signaling, chemical defense, and stress response often mediated through polyunsaturated aldehydes (PUAs). In this study, Ulva spp. were surveyed for PUAs. Ulva species with sea-lettuce like morphotype were demonstrated to produce elevated amounts of volatile C10-polyunsaturated aldehydes (2,4,7-decatrienal and 2,4-decadienal) upon tissue damage in contrast to Ulva species with tube-like morphotype. Moreover, morphogenetic and phylogenetic analyses of the collected Ulva species revealed chemotaxonomic significance of the perspective biosynthetic pathways. The aldehydes are derived from omega-3 and omega-6 polyunsaturated fatty acids (PUFA) with 20 or 18 carbon atoms including eicosapentaenoic acid (C20:5 n-3), arachidonic acid (C20:4 n-6), stearidonic acid (C18:4 n-3), and γ-linolenic acid (C18:3 n-6). As first evidences in this study, it was found that lipoxygenase-mediated (11-LOX and 9-LOX) eicosanoid and octadecanoid pathways catalyze the transformation of C20- and C18-polyunsaturated fatty acids into PUAs and concomitantly into short chain hydroxylated fatty acids.

Ulva mutabilis Føyn (sl) with tube-like morphotype was used as an objective to investigate the chemical mediated interaction (infochemicals) within the chemosphere of tripartite community consisting of U. mutabilis and its associated marine bacteria i.e., Roseobacter and Cytophaga.
species. In the absence of these bacteria (axenic conditions), \textit{U. mutabilis} forms callus-like colonies. However, the combination of the two bacterial strains, \textit{Roseobacter} sp. and \textit{Cytophaga} sp. can completely restore the morphogenesis of \textit{U. mutabilis} forming a symbiotic tripartite community.

The exo-metabolome of the chemosphere of this tripartite community was surveyed along with the biological metadata. Following \textit{a posteriori} hypothesis based on the collected biological data was finally tested:

\textit{“The chemosphere of the tripartite community changes throughout the growth phases of the macroalgae and biomarker of this exometabolome can be used to predict changes in the status of gametogenesis inducibility during the life cycle”}

To test the hypothesis, two different approaches and cultivation conditions i.e., sterile 25 L bioreactor cultures and non-sterile 200 L outdoor aquacultures were conducted which cultures were inoculated with axenic cultures or seven days old germlings. Indeed, it was feasible to observe the whole life cycle of the gametophyte under these conditions when the appropriate bacteria were inoculated as well. Hereby, the medium did not need to be changed. Bioassays revealed that \textit{U. mutabilis} passed through three statuses of gametogenesis inducibility which can be distinguished whether \textit{Ulva} is able to onset the gametogenesis: (1) gametogenesis is not inducible, (2) gametogenesis can be induced or (3) it starts even spontaneously.

The nutrient depletion over the reproductive cycle shows that the utilization rate of nitrate as a limiting growth factor was significantly high during the inducible status, when the macroalgae was growing.

The waterborne metabolites were extracted by solid phase extraction. The samples were directly analyzed by ultra-high performance liquid chromatography (UHPLC) and by gas chromatography (after derivatization) coupled with a time-of-flight mass spectrometer (TOF-MS). Interestingly, chemometric data analysis (e.g. discriminant analysis) proofed that all waterborne metabolites obtained either from GC-MS or LC-MS were corresponding to the inducibility status of gametogenesis of \textit{U. mutabilis} in both cultivation conditions. Even more
interesting, many unknown biomarkers were found to be common in both bioreactor cultures and aquaculture, insuring the high probability of using these biomarkers as indicators to determine the growth phases corresponding to the status of gametogenesis inducibility in *U. mutabilis* under any cultivation condition in future land based aquacultures. Moreover, the present study revealed remarkable metabolic fingerprints which might due to the adaptation of *U. mutabilis* to changes in its surrounding environment (e.g., in the microbiome, nutrients, life cycle of the alga). For instance, the algal biomarker 2,4,6-tribromophenol was detected in the chemosphere of the tripartite community under sterile cultivation (bioreactor) but not in the well-defined bacterial community under non-sterile cultivation (aquaculture).

In summary, the changes of the metabolite profile between the growth phases were significant. Therefore, various statues in algal growth and life cycle can be predicted based on the dynamics of waterborne metabolites. This knowledge will be essential in order to maintain land based aquacultures providing economical relevant amounts of biomasses.
تُعد الطحالب البحرية الخضراء مُهتمة بدرجة النمو والنموية بفضلة أنها من الطحالب المائية بعين المبادرة والواضعه الانتظار. فهما أيضاً من الطحالب التي لها القدرة على الإعداد معرفة ما تسمى بالعد الأخضر. طحالب آلها هذه لا تكون متلازمة مع فسائل من البكتيريا البحرية. بشكل استثنائي الاستجابة الفيسيولوجية ويفتزاً تطبيقات معتمدة في التوحيد وملامحه وظاهره على التحقيقات الملائمة لمن جمه أخرى فإن هذه الطحالب قد تغزو بدورها في المنطقة نفسها. فبالانفصال الشمالي، بوحدة ملونة يؤثر على نمو الطائرة الملاروسة.

وعلى سبيل المثال، بعض الطحالب، والتي لا توجد، على رابط عدم موجودة غير مشروعة (حلج نظير)، وهذه الألوان قد يصبح حركة هادئة في تطبيقات البيئة البكتيرية المبسطة.

وللتحقق من الهدف الأول، فإنك من المعروف أن (حلج نظير)، عن طريق عملية الحفر الإيزيدي، والتي يتم بمختلف إنتاج أنسجة الأحماض الجسمية وتبخير تحلل الجسم الصيدري فقوق الأسمنتية. عملية التحلل هذه يتم بطريقة هدف في الطحالب على وجه العموم ومتاتهم المتبللة في (حلج نظير) تكيف خيايا حاملا في عمليات التواصل بين الطحالب والبكتيريا. فهذا، يتم من قبل البكتيريا مساعدة فحص مثلاً أو قد يجوز أن تكون في حال تعرض لظروف بنية جانبية. وفما تستجيبه مساعدة فحص إيجابية أو تغيير. بناء على هذه الحقيقة العلمية، فإن الدراسة العلمية قادرة بفعالية على مسح للطلعمه أليك والمشاهدة من الслуш البروتين للتحضير على وجبة (حلج نظير)، وفقد أُفرِز الطحالب أن طحالب أليك التي تعيش في مصادرنا الجغرافية وفروع الخد، حيثما تعرض، قد تصلطلل هذه التوريق التي تنتج معمودية كبيرة من (حلج نظير) والتي تحتوي على خض خذرات صميرن والمتبللة في حديقة 3-4-4-4، وحيثة 3-2-3-7، وحلة 2-7، وحلة 3-2-3-2، على الخصخص من طحالب أليك أدبيوية الشكل، والتي يفجأ أنها لا تنتج هذه الألوان، على الإطلاق. هذا وفقد أُفرِز الطحالب تتبني التحليل الشصي مع تحلل النقص وتطور إلى أن هناك تحولي مهنيوشيغلي ممك 수행اً للاطفال جهاز أليك للدراسة. فهما شير الأتائف إلى أن الألوان تماسكة صغرى تتبني من أحماض أوميغا في لى المشروعة التي تتم فوق الحد 21 أو 20 مدرسة متروبوليس.

بمسمى الأطيافون (3-2-3-2، 4-4-4-4، 3-2-3-2) حمض الإيترسيبيتينوك (6-4-6)، روابط ثانوية، أوميغا 2، حمض الإيترسيبيتينوك (6-4-6)، روابط ثانوية، أوميغا 2، حمض ياما-ليوبيتينوك (6-4-6)، روابط ثانوية، أوميغا 2، حمض الإيترسيبيتينوك (6-4-6)، روابط ثانوية، أوميغا 2، وحيد
أكشفل سيلًا

Zusammenfassung/Abstract/

صفحات السيل

صصفحة 1

هذه الدراسة هي الأولى من نوعها والتي أثبتت أنخلاً تطوريكي الاستثنائي الحيوى Ectothermic وAggression biofeedback بأن سمحة

حالة الطرود، رقم 9 وحالة الطرود رقم 11 في الأحماض الدوائية غير المشعة تم في طرائف ألاّ وإنها تتألف فيما الأحماض

الئيمة متعددة الروابط غير المشعة والمحيطة على 30 و18 حالة عروض وتشمل بالتالي إلى الدمجيات متعددة الروابط غير

المشيطة بالإضافة إلى الأحماض الميدرينقشائية تفاعل السلام المروري الفضية سحبونان ثانوي..

وت качية الصدفة الثانية من الدراسة، فإن هذا الضعف المعرف هو بطليه ألاّ متابلة (Ulva mutabilis) لدراسة

التفاعلات الكيميائية المعرفة والارتباطات الكيميائية (Infochemicals) والتي تحدث بين ألاّ متابلة والبيئة الملافمة لما من

وادي وربوة (ROSEOBACTER sp.) نوع وربوة (R. Roseobacter sp.) وربوة (CYTOPHAGA sp.) وربوة (R. Roseobacter sp.)

للصحت على التجاويع التفاعلية وما ورد ذات التأليه الحدرون على تغير ماتي الناجم زود فيه هذه السنانيد الثلاثة بماً طبيعاً مع

تيرجح الشكل الشاطئي الطبيعي المعقدش ما لو تانية هذه السنانيد متواجدة في بيئية البشرية. في حين لوحظ أن ألاّ متابلة

زيج فاحصة على النمو إطلاقاً إضافة إلى أيضاً تفقيد حملها الطبيعي مصنوعة مكتملة تتيح لدعا تاليا في حال غياب هذه البيئة فربة

النمو.

في ضوء المعكا علاج، تم تحليل نوات الأيض المراهنة من هذه السنانيد في المعالج المعادي جبل المجمت الثلاثي، إلى جانبية إجراء قاعدة تجوابية. استدعاى على المحاويات الوبية. فإنه الضعف المروية الاستعدادية التالية:

"يعتبر البنية الكبيرة متاحة إلى الأحياء الأحياني خلال دورة حياة الطحالب، وعلى إن المواد الأيضية المتزدة في هذه المنطقة قد تستخدم كعلامات في تغييرات وضع التكاثر خلال دورة حياة التراب".

ومع هذا المترشح، تم إنشاء شابنات متعدد البائي في طريقه بيئة متزحة. كما أشتهرت احجام مختلفة من وسط النمو حيث تم

تسيير معادلات حية معتماً 0.05 لتر وحد مثبطات تحدد نوات الطرق مقتمره، بالإضافة إلى إنشاء مزج إحياء مثلما ستغيرها ""أ"" لتر تحده

طريقه الميدانية غير مقتمره. في خلاط الطرق تم التجاوز لإحة مثبطات أو得益جات صغيرة (نم انها لحدة اشباع دفعت). وقد

تفقد متاحة عملية تشكين الأحماض خلال حصول حالة ألاّ متابلة عند نواتها مع البيئية الملافمة. ما، أننا أن لا يتم تغيير وسط النمو طيلة

فترة التجربة (90 يوماً). وقد أصرف اختبار الجدة على التحاقات بأن ألاّ متابلة تم بلباب مراحل تفاقم، والتي يمكن التعبير عنها ببناء على جدة ألاّ متابلة على البحض في عملية تشكين الأحماض، وعلى فإن مراحل التفاقم هي على التوالي: (1) مرحلة عدم الحدودا

على تشكين الأحماض. (2) مرحلة تشكين الأحماض بالتحفيز، وعاهيراً (3) مرحلة تشكين الأحماض تناغماً. شما أفادت تحليل النشاط الغذائي

XVI
Anxiety-related weath in low levels of the platelets which is increased in the blood through a process of solid extraction. As well as extracts also in the blood in the form of gas chromatography (GC) and mass spectrometry (MS).

In order to study the relationship of the platelets with the level of anxiety, we used the solid extraction method followed by gas chromatography (GC) and mass spectrometry (MS). The results showed that there is a significant difference in the level of anxiety in the platelets of the low anxiety group compared to the high anxiety group. The study also showed that the level of anxiety is inversely proportional to the concentration of platelets in the blood.

Furthermore, we applied a questionnaire to the participants to assess their anxiety levels. The results showed that the anxiety levels were inversely proportional to the concentration of platelets in the blood. This study provides new insights into the role of platelets in the regulation of anxiety levels and opens up new avenues for future research.

We conclude that the level of anxiety is inversely proportional to the concentration of platelets in the blood. Therefore, we recommend further studies to investigate the role of platelets in the regulation of anxiety levels.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
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<tr>
<td>AAnP</td>
<td>aerobic anoxygenic photobacteria</td>
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<td>AHL(s)</td>
<td>N-acylhomoserine lactone(s)</td>
</tr>
<tr>
<td>αLEA</td>
<td>α-linolenic (C&lt;sub&gt;18:3&lt;/sub&gt; n-3)</td>
</tr>
<tr>
<td>AMDIS</td>
<td>Automated Mass Spectral Deconvolution and Identification System</td>
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<tr>
<td>amu</td>
<td>atomic mass unit</td>
</tr>
<tr>
<td>ARA</td>
<td>arachidonic acid (C&lt;sub&gt;20:4&lt;/sub&gt; n-6)</td>
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<tr>
<td>Ax</td>
<td>axenic</td>
</tr>
<tr>
<td>Bio</td>
<td>biomarker</td>
</tr>
<tr>
<td>BrPO</td>
<td>bromoperoxidase</td>
</tr>
<tr>
<td>C</td>
<td>carbon/concentration</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius/centigrade</td>
</tr>
<tr>
<td>CAP</td>
<td>canonical analysis of principal coordinates</td>
</tr>
<tr>
<td>CCMAR</td>
<td>Center of Marine Sciences, Ramalhete, Faro, Portugal</td>
</tr>
<tr>
<td>CFB</td>
<td><em>Cytophaga-Flavobacter-Bacteroides</em></td>
</tr>
<tr>
<td>CODA</td>
<td>component detection algorithm</td>
</tr>
<tr>
<td>cos θ</td>
<td>cosine canonical right angle</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl trimethylammonium bromide</td>
</tr>
<tr>
<td>Cy</td>
<td>Cytophaga sp.</td>
</tr>
<tr>
<td>d</td>
<td>day / deuterium</td>
</tr>
<tr>
<td>DCO</td>
<td>dissolved organic carbon</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethyl sulfide</td>
</tr>
<tr>
<td>DMSP</td>
<td>dimethylsulfiniopropionate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electron spray ionization (C&lt;sub&gt;20:5&lt;/sub&gt; n-3)</td>
</tr>
<tr>
<td>ETYA</td>
<td>5,8,11,14-eicosatetraynoic acid</td>
</tr>
<tr>
<td>F</td>
<td>faint</td>
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Abbreviations

FA(s) fatty acid(s)
Fig. figure
FW fresh weight
G group
G(mt+) gametophyte (mating type +)
G(mt-) gametophyte (mating type -)
GC gas chromatography
γLEA gama-linolenic (C18:3 n-6)
h hour
HpEPE hydroperoxy-5Z,8Z,12E,14Z,17Z-eicosapentaenoic acid
HpETE hydroperoxy-arachidonic acid = hydroperoxy-5Z,8Z,12E,14Z-eicosatetraenoic acid
HpOTrE 9-hydroperoxy-6,10,12-octadecatrienoic acid
HS-SPME headspace solid phase micro-extraction
ID identity
Induc. inducible
IS internal standard
ISQ single quadrupole
LA linoleic acid (C18:2 n-6)
LC liquid chromatography
LOD limit of detection
LOX lipoxygenases
ln natural log
M molar
m/z ratio of mass to charge
MCQ Mass Chromatographic Quality
MET-IDEA Metabolomics Ion-based Data Extraction Algorithm
min. minute
ML maximum likelihood
MS mass spectrometer / mass spectrometry
MSTFA N-methyl-N-(trimethylsilyl)trifluoroacetamide
MUFA(s) monounsaturated fatty acid(s)
Abbreviations

N  nitrogen
n  number of replicates
n- = ω  omega
n.d.  not determined
N.G.  new generation
NADH  nicotinamide adenine dinucleotide (reduced form)
NCBI  National Center for Biotechnology Information
NIST  National Institute of Standards and Technology
NMR  nuclear magnetic resonance
OD  optical density
oxid.  oxidation
P  phosphorus
PCA  principle component analysis / principal coordinate analysis
PCR  polymerase chain reaction
PFBHA  O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine
psu  practical salinity units
PUA(s)  polyunsaturated aldehyde(s)
PUFA(s)  polyunsaturated fatty acid(s)
qPCR  quantitative polymerase chain reaction
QS  quorum sensing
r  correlation coefficient
|r|  absolute value of correlation coefficient
RGR  relative growth rate
red.  reduction
RNA  ribonucleic acid
Ro  Roseobacter sp
RT  retention time
S  strong
SD  standard deviation
SDA  stearidonic acid (C_{18}:4 n-3)
SFA(s)  saturated fatty acid(s)
Abbreviations

sp. species (singular)
SI(s) sporulation inhibitor(s)
sl. slender
SPE solid phase extraction
Spon. spontaneously
spp. species (plural)
SWI swarming inhibitor
t time
Tab. table
TC the tripartite community
THF tetrahydrofuran
TIC total ion current
TMS trimethylsilyl
ToF Time of Flight
Tris tris(hydroxymethyl)aminomethane
U. Ulva
UCM Ulva culture medium
UHPLC Ultra-high-performance liquid chromatography
ULPC Ultra-performance liquid chromatography
UM Ulva mutabilis
UR utilization rate
vs. versus
W weight
wt wild type
α alpha
θ right canonical angle
β beta
γ gamma
δ delta
ω = n- omega
δ² square canonical correlation
1. Introduction
Introduction

1.1. Marine Chemical Ecology

In the last two decades, the chemical mediated interactions between marine organisms were identified as a fundamental characteristic, which might structure communities in the marine habitat. Recent studies provide significant insights into the ecology and evolution of marine populations, and the role of chemical interactions in marine ecosystems (Hay, 1996; McClintock and Baker, 2001; Ianora et al., 2006; Pohnert et al., 2007). This has challenged the pretty novel research field of “Marine Chemical Ecology”, which could evolve successfully along with advances in instrumental analytical chemistry. Marine chemical ecologists examine the function of the naturally occurring compounds in plants and animals interactions (Paul, 1992) and hence perform both chemical and biological research. Using an interdisciplinary approach, they aim to combine the *in situ* determination of often low concentrated chemical compounds directly with their effects on the interactions between organisms e.g., within the marine food web or in biofouling processes of macroalgae. An increasing number of studies has demonstrated the overall meaning of the production and release of those metabolites and their physiological significance to other organism. Due to the multiple functions of those metabolites the name *infochemical* was branded. Growing interest in chemical ecology of marine organisms has been observed since 1980s (Harborne, 1989). Since the development of affordable bench top ion trap mass spectrometers in the early 1990s, marine chemical ecology started to develop rapidly (Bakus et al., 1986; Hay and Fenical, 1988; Fenical, 1993; Hay, 1996; Faulkner, 2002). Marine chemical ecology is one of three parallel tracks in marine natural products research in addition of marine toxins, and marine biomedicines, which gave marine natural products chemistry its unique characteristic and vitality (Faulkner, 2000). Marine natural products play fundamental roles in ecology. Williams et al. (1989) concluded that the pressure of natural selection leads to evolve natural products to bind to specific receptors, and therefore mediate ecological responses of organisms to their environment. Marine organisms are under competitive pressure for space, light, and nutrients. On the other hand, Marine organisms need to communicate with each other. Thus, it is not surprising that these organisms have developed a range of defense mechanisms, and means of communication (Pohnert and Boland, 2002; Arnold and Targett, 2002; Paul and Puglisi, 2004; König et al., 2006) to ensure survival, and facilitate the communication with surrounding neighborhoods.
Introduction

The challenges in this field remain tremendous. Marine ecology is certainly one of the great scientific challenges of our time. In addition to the difficulties of collecting marine samples, bringing marine organisms into the laboratory is often far from simple (Ianora et al., 2011). Moreover, the production and release as well as the reception of compounds by the perceiving organism are highly dynamic processes and hard to follow in field studies although great advancement was achieved in terms of developing bioassays that are relevant to natural systems (Paul, 1992; Harborne, 1999; Watson and Cruz-Rivera, 2003; Ianora et al., 2006). However, the majority of algal secondary metabolites have not been bioassayed, which to date has been considered as a challenge in the development of this research area (Engel et al., 2002). In fact, bioassays are needed in order to overcome the e.g., high dilution of metabolites found in the dynamic environment of the seawater body. Besides sampling and storage of samples, the low concentrations of intriguing metabolites released into the water body are challenging for chemical analyst. The limit of detection of e.g., the mass spectrometer is often lower than the biological sensor capacity of the organism. Therefore, solid phase extraction approaches were developed to overcome this limitation. One advanced technology that has been introduced in the field of marine chemical ecology is Metabolomics based on mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Seger and Sturm, 2007). Many technical challenges need to be overcome in order to increase applications of metabolomics in marine systems. The present study used mass spectrometry-based metabolic profiling as a tool to study the chemosphere of the green macroalga Ulva mutabilis and its associated bacteria under certain conditions.

Macroalgae, as representative members of marine organisms, have been taken a lot of attentions in terms of their contribution in this particular area of chemical ecology. Thus, their potential contribution was introduced in the next section.

1.2. Macroalgal Chemical Ecology: deciphering the multiple inter- and intra-species actions

Marine algae are particular interesting, because they are a heterogeneous group of photosynthetic, aquatic organisms, which vary very much in size, abundance, morphology, life cycle, and can be found in both eukaryotic and prokaryotic kingdoms. For instance, algal sizes range from single cells to giant seaweeds. Certainly, this implies different ways of interactions with other organisms. Whereas several microalgae are well studied, marine macroalgae are in general still under investigated, which is due to the lack of stabile lab
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cultures and standardized methodologies. There are three major divisions of macroalgae: (1) Chlorophytes (green algae), (2) Phaeophytes (brown algae), and (3) Rhodophytes (red algae). All algae contain chlorophyll. Brown algae, in addition, contain xanthophyll, while red algae contain phycoerythrin, and/or phycocyanin. From an ecological standpoint, all of these organisms often occupy common niches in the marine environment. Here, macroalgae acquire nutrients, need to settle on preferred locations and have to protect themselves from grazers. Also, they have to associate and compete with other marine organisms including bacteria. Therefore, macroalgae have developed different ways, which are mediated by chemical interactions to integrate in this marine environment. In light of this, chemical cues become recognized as the “language of marine life”. The understanding of such biotic interactions and how they affect marine ecosystems will advance more rapidly if this language is studied and understood (Hay, 2009). Many studies have addressed now waterborne metabolites, as signals in marine environment, mediate the interaction among the organisms in particular macroalgae.

However, algae were considered long time ago by classic ecological paradigms as organisms, fully dependent on an external physiochemical and biological rules to regulate their life. However, this description failed to explain many of the structures and dynamics shown by aquatic communities (Watson and Cruz-Rivera, 2003). This description, in addition, was not able to explain any mechanism supporting the production of huge amount of secondary metabolites by algae. Now, it is very well established, that algal secondary metabolites are known to play an important role in aquatic ecosystem either by direct or indirect way. Many studies reported the strategies employed by algae in order to adapt to their biological community by behavioral, physical or chemical means (Steinberg and de Nys, 2002; Paul et al., 2007; Togashi et al., 2008).

The outstanding impact of already elucidated secondary metabolites in macroalgae is highlighted with five examples regarding their functions (1) in chemical defense, (2) in warning the neighbor, (3) in allelopathy, (4) in cross kingdom-cross talk and (5) in mating.

(1) Chemical defense: Macroalgal secondary metabolites can be used directly by algae against natural enemies such as the brominated and chlorinated sesquiterpene elatol (Fig. 1a) which is produced by the brown algae Laurencia spp. to deter feeding by reef fishes. But smaller consumers sequester secondary metabolites from macroalgae and use them for their own defense. For instance, an amphipod Ampithoe longimana reduces its susceptibility
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to predators by living on and feeding the brown alga *Dictyota dichotoma* which is chemically defended from fish grazing by diterpenoid alcohols like *pachydictyol-A* (Fig. 1b) (Hay et al., 1987). Other example is the terpenoids were isolated from brown algae e.g., *Dictyota* spp. were evaluated for their antiadhesion activity against a biofilm-forming marine bacterium *Pseudoalteromonas* sp. (Viano et al., 2009).

Figure 1: Secondary metabolites produced by macroalgae: (a) etatol a direct chemical defense, (b) pachydictyol-A an indirect chemical defense.

(2) Warning the neighbor: Toth and Pavia (2000) reported that waterborne cues from flat periwinkles *Litterbin obtusata* induce the production of defensive chemicals (phlorotannins) in the brown alga *Ascophyllum nodosum*. These algae can anticipate predator’s attacks without receiving direct damage by inducing the production of phlorotannins upon recognition of a waterborne signal (phlorotannins), which is released by wounded *A. nodosum*. The latter scenario was supported by Thomas et al. (2011), who observed that the brown alga *Laminaria digitata* was able to convey a warning message to its neighboring algae.

(3) Allelopathy: The influence of chemical signals also extended to occupy a niche in allelopathy, which can be defined as growth’s suppression of one species by another species due to the release of deleterious substances. Algal allelopathic interactions have been well documented. Generally, there are two mechanisms of allelopathic interactions in the marine environment. Firstly, allelopathy relies on sufficient concentration of active compounds produced by macroalgae, and emerged into seawater, then reach target species (Lewis, 1986; Gross, 2003). Secondly, macroalgae release metabolites and target particular epiphytes by physical contact, for instance, the red alga *Plocamium hamatum* exerts chloromertense, a tetrachlorinated monoterpenne, causing tissue necrosis for the soft coral *Sinularia cruciata*, when they were in direct contact (Denys et al., 1991). Allelopathy covers biochemical interactions, both stimulatory and inhibitory, among different primary
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producers or between primary producers and microorganisms. For stimulating interaction, glycoglycerolipids from the green alga *Ulvella lens* induce settlement and change in the body's structure of sea urchins *Strongylocentrotus intermedius* (Takahashi et al., 2002). Antifouling activity represents an ecological application of allelopathical inhibitory impact. Better known examples include halogenated furanones from the red alga *Delisea pulchra* that inhibit the growth and the settlement of *Ulva lactuca* gametes (Denys et al., 1995), and terpenoids from the brown alga *Dictyota menstrualis* that prevent bryozoan *Bugula neritina* from colonizing the surface of this alga (Schmitt et al., 1995).

(4) Mating: Contrary to adverse effects of chemical defenses and allelopathic effects, intra-species interactions are mediated e.g., by pheromones. Jaenicke and Boland (1982) discussed how pheromones can be released by brown algae females and direct the movement of the partner gametes. Pohnert and Boland (2002) reviewed all the six pheromones that act as chemical cues to mediate the mating progress in most of brown algae. For instance, multifidene, as a sex pheromone, was found to attract the male gametes of the brown alga *Cutleria multifida* (Derenbach et al., 1980). In this context, it is worth mentioning that brown algal pheromones tend to serve families or orders rather than being species or genus specific e.g., the different species of the same genus *Fucus* (*F. serratus*, *F. vesiculosus*, and *F. spiralis*) often produce the same pheromone termed fucoserratene, whereas the pheromone hormosirene is released from both genera *Hormosira banksii* and *Xiphophora chondrophylla* (Maier and Muller, 1986).

(5) Cross-kingdom-cross talk: An example of the inter-species chemical interaction in the marine environment, in which macroalgae take part, is the interference with the regulatory quorum sensing system used by marine bacteria to respond to their environment. Algae excrete some chemicals that affect adversely on bacterial regulatory system with no or minimal effects on bacterial growth. N-Acylated homoserine lactones (AHLs) (Salmond et al., 1995; Fuqua et al., 1996; Robson et al., 1997) are examples of regulatory systems of bacteria that can regulate their population and aid their association with higher organisms. These include the induction of colonization relevant phenotypes such as swarming (Eberl et al., 1996), and biofilm formation (Davies et al., 1998), as well as other phenotypes such bioluminescence (Swift et al., 1994). Recent works in this area of the marine chemical ecology have led to the observation that this regulatory system is down regulated by halogenated furanones released by the red alga *D. pulchra* (Denys et al., 1995; Rice et al.,
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1999). Such interference represents another type of cross-kingdom signaling between bacteria and macroalgae.

**Table 1:** Representative chemical mediated interactions between macroalgae and associated organisms in the marine environment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Class</th>
<th>Waterborne metabolites</th>
<th>Associated organism</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascophyllum nodosum</em></td>
<td>Brown alga</td>
<td>Phlorotannins</td>
<td><em>Littorin obtusata</em></td>
<td>External signals to induce phlorotannins in unharmed individuals of <em>A. nodosum.</em></td>
<td>(Toth and Pavia, 2000)</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>Brown alga</td>
<td>Volatile organic compounds</td>
<td>Neighboring algae</td>
<td>Warning message</td>
<td>(Thomas et al., 2011)</td>
</tr>
<tr>
<td><em>Plocamium hamatum</em></td>
<td>Red alga</td>
<td>Chloromertense</td>
<td><em>Sinularia cruciata</em></td>
<td>Tissue necrosis</td>
<td>(Denys et al., 1991)</td>
</tr>
<tr>
<td><em>Ulvea lens</em></td>
<td>Green alga</td>
<td>Glycoglycerolipids</td>
<td><em>Strongylocentrotus intermedius</em></td>
<td>Induce settlement and metamorphosis</td>
<td>(Takahashi et al., 2002)</td>
</tr>
<tr>
<td><em>Delisea pulchra</em></td>
<td>Red alga</td>
<td>Halogenated furanones</td>
<td>1) <em>Ulva lactuca</em></td>
<td>Chemical defense: 1) Antifouling 2) Biomimics AHL, interfering with expression of AHL driven phenotypes</td>
<td>(Denys et al., 1995; Rice et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2) Associated bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dictyota menstrualis</em></td>
<td>Brown alga</td>
<td>Terpenoids</td>
<td><em>Bugula neritina</em></td>
<td>Protection from colonization</td>
<td>(Schmitt et al., 1995)</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>Brown alga</td>
<td>Unsaturated, oxygenated cyclic C11-hydrocarbons</td>
<td>Neighboring male gametes</td>
<td>Attraction of the mating partner</td>
<td>(Müller et al., 1979)</td>
</tr>
<tr>
<td><em>Dictyota spp.</em></td>
<td>Brown algae</td>
<td>Terpenoids</td>
<td><em>Pseudoalteromonas sp.</em></td>
<td>Antiadhesion activity against a biofilm-forming marine bacterium</td>
<td>(Viano et al., 2009)</td>
</tr>
</tbody>
</table>

All these examples indicate that multiple interactions are evident in the marine habitat and are mediated by released compounds by either of the involved organism. Therefore, it is evident that standardized biological systems are necessary in order to investigate the “Chemosphere” (= the space where organism are interacting via molecules) under
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reproducible conditions. Therefore, the tripartite system between Ulva and its associated bacteria established by Spoerner et al. (2012) was selected within this study. Moreover, the interaction of macroalgae with their microbiome is of special interest as it is an interaction between eukaryotes and prokaryotes.

1.3. Eavesdropping the cross-kingdom crosstalk: Macroalgae and bacteria

Bacteria are highly abundant in seawater and play important ecological roles within marine communities in nutrient cycling and organic matter decomposition (Azam et al., 1983; Cotner and Biddanda, 2002). Bacteria associated with macroalgae have a well-known impact on algal health (Matsuo et al., 2005), and interactions with other organisms acting as pathogens (Ashen and Goff, 2000; Weinberger, 2007), symbionts (reviewed by Armstrong et al, 2001), or mediators either as promoters or inhibitors of the settlement of fouling organisms (Rao et al., 2006; 2007; reviewed by Qian et al, 2007).

Many factors can influence the microbial community dynamics in aquatic systems. For instance, physical factors (e.g., weather and water temperature) (White et al., 1991 Felip et al., 1996), chemical factors (e.g., pH, availability of N, P) (Vanwambeke and Bianchi, 1990; Lebaron et al., 2001; Joint et al., 2011) and biological factors (e.g., grazer pressure, competition for resources, or symbiotic interactions) (Jurgens et al., 1999; Hahn and Hofle, 2001). Biological interactions are thought to be a major factor determining bacterial community composition in aquatic systems (Lachnit et al., 2009). In this context, Wahl (1989) highlighted epibiosis as a common phenomenon occurring when surfaces of living organisms exposed to seawater and rapidly covered with an organic layer and subsequently colonized by microorganisms (e.g., bacteria, diatoms, and fungi), and/or macroorganisms (e.g., larvae, algal spores). The epiphytic bacterial profile and their temporal and spatial variability on host algae are poorly understood. Croft et al. (2006) pointed out to the key role of epibiotic bacteria in the colonization process of an algal thallus for several reasons: (1) they are fast colonizers, (2) highly adaptive, and (3) capable of quick metabolization of algal exudates. Despite the negative impact of epibiotic bacteria on algae as pathogens (Michel et al., 2006), epibiotic bacteria may provide algae with nutrients under certain condition (Croft et al., 2006). They may supply growth factors for algae (Tsavkelova et al., 2006). Species of Cytophaga-Flavobacter-Bacteroides, for example, have a strong impact on the morphology of green algae Monostroma oxyspermum by secreting an exogenous growth factor called
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thallusin (Fig. 3a) (Matsuo et al., 2003; Matsuo et al., 2005). Bacteria, in turn, benefit through the ready availability of organic carbon sources produced by algae (Armstrong et al., 2000). It was reported that primary metabolites which are produced by macroalgae such as carbohydrates, amino acids, peptides, and proteins induce the colonization by microbes (reviewed by Steinberg et al., 2002). In this respect, the surface of macroalgae represents a protected habitat for bacterial colonization and reproduction. The mean by which macroalgae and bacteria can find their right partner are infochemicals representing the chemical compounds excreted by aquatic organisms that can be used by other individuals as an information carrier. These infochemicals may affect the metabolism and subsequently the behavior, or physiology of the receiver which results in an (1) alteration of the structure, (2) functioning, and (3) evolution of food webs, or habitat, respectively (Verschoor et al., 2007). However, if an infochemical plays a role in the interaction between two individuals, certainly the producer will be one of the organisms involved in this reaction (Dicke and Sabelis, 1988).

Marine bacteria and algae are thought to closely interact in the phycosphere as a zone may exist, extending outward from an algal cell or colony for an undefined distance, in which bacterial growth is stimulated by extracellular products of the alga (Bell and Mitchell, 1972). Therein, bacteria may be free-living (planktonic) (Blackburn et al., 1998), or may be attached to the algal surface (epibacteria) (Kogure et al., 1981). Taking into account the compounds produced and released into the water by all organisms in a specific habitat, this space is defined as the chemosphere of the interacting organism (Alsufyani and Wichard, 2011).

Dudler and Eberl (2006) summarized the recent advances in understanding the origin of secondary metabolites, and predicted that those metabolites often produced by symbiotic bacteria, rather than by the eukaryotic host (e.g., sponges, corals). Moreover, bacterial cell-to-cell signaling plays an important role in bacteria–host interactions. On the other hand, macroalgae are known to release large amounts of organic carbon into the surrounding environment providing a nutrient-rich habitat for microorganisms and triggering chemotactic behavior of bacteria (Armstrong et al., 2001) in addition to the organic-rich algal surfaces. Because of the latter reason, Croft et al. (2005) and Lachnit et al. (2010) suggested that the surface chemistry of algal thalli mediates associations with beneficial microorganisms.

Lachnit et al. (2009) found out that epibacterial communities differ less between regions than between host species, and they are more similar on closely related host species. For instance, epibacterial communities on thalli of the algal species Fucus serratus, Fucus
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*vesiculosus, Laminaria saccharina, Ulva compressa, Delesseria sanguinea* and *Phycodrys rubens* were analyzed using 16S ribosomal RNA gene-based DGGE (Denaturing Gradient Gel Electrophoresis) and resulted in first evidence for lineage-specific bacterial associations to algal thalli. Furthermore, the results suggested that these algal species may control their epibiotic bacterial communities. Lam and Harder (2007) observed that the bacterial community composition in algae-conditioned seawater was reduced in comparison to natural seawater, and this was attributed to waterborne antimicrobial macroalgal metabolites. This observation was supported by Sneed and Pohnert (2011b), as they found that the green macroalga *Dictyosphaeria ocellata* significantly influenced the bacterial community composition. Seven bacterial phylotypes were eliminated in the presence of *D. ocellata* and five were found exclusively with the alga. Associations between algae (photosynthetic eukaryotes) and bacteria (heterotrophic) have been described for over a hundred years. Although a wide range of beneficial and detrimental interactions between macroalgae and epi- and endo-symbiotic bacteria residing either on the surface or within the algal cells respectively are doubtless important to the marine ecosystem, and marine-product industry as well (Suzuki et al., 2001). Based on the data obtained by Gonzalez et al. (2000), *Roseobacter* (*α*-proteobacteria) together with two other groups of bacteria lineages account for over 50% of the bacteria associated with oceanic algal blooms in surface water. *Bacteroidetes* represents an efficient proportion in oceanic habitats as highlighted by the study of Kirchman (2002).

Due to the fact that the algal morphogenesis inducing *Roseobacter* sp. and *Cytophaga* sp. are associated microorganisms to *Ulva mutabilis*, representing the model system of the present study, some of the biological traits of these two bacteria will be introduced briefly.

Members of the *Roseobacter* clade are widespread, and abundant among marine bacteria. Moreover, *Roseobacter* spp. are often associated with organic surfaces in different marine environments (Lafay et al., 1995), suggesting that a sessile lifestyle is central to the ecology of such lineage members (Slightom and Buchan, 2009). They are well-known by producing acylated homoserine lactones (AHLs) and other secondary metabolites (Gram et al., 2002; Wagner-Dobler et al., 2005). Thus, 22 strains belong to the *Roseobacter* clade were screened by Martens et al. (2007) for the production of signaling molecules, and antibiotics against bacteria of different phylogenetic groups, and they found that ten isolates produced AHLs, and three of them exhibited in addition antibacterial effect. Furthermore, the potential of (1)
the production of signal molecules e.g., AHLs, (2) antibacterial metabolites inhibiting non-
*Roseobacter* species, and (3) biofilm formation could be ecologically important and partially
explain the succession of these organisms (Bruhn et al., 2007). *Roseobacter* clade has been
classified as aerobic anoxygenic photobacteria (AAnP) (Lafay et al., 1995) contains a range of
carotenoid pigments. *Roseobacter* clade is not able to use light as energy source, and relies
on various organic compounds to obtain carbon and energy being **heterotrophs** (Allgaier et
al., 2003; reviewed by Eiler, 2006).

*Cytophaga* sp. is a gram-negative, anaerobic genus and belongs to *Bacteriodates*, which
forms the second major group within the *Cytophaga-Flavobacter-Bacteroides* (CFB). Many
marine isolates of *Cytophaga* have unusual carotenoid pigments (Achenbach et al., 1979;
Fautz and Reichenbach, 1980; Reichenbach et al., 1980). Munn, (2004) summarized the
distinctive characteristics of *Cytophaga* sp. including the production of various extracellular
compounds which are responsible for the degradation of polymers such as cellulose and
chitin. Thus, it was demonstrated to degrade cellulose derivatives by cellulase. Spoerner et
al. (2012) therefore, supposed that these bacteria may be capable to invade or even
permeate the cellulose-containing cell walls. In the marine environment, *Cytophaga* sp. was
demonstrated also to have a strong impact on the morphology of green alga *Monostroma* by
secreting an exogenous growth factor termed thallusin (Matsuo et al., 2005).

Moreover, other bacterial species from the CFB, *α*-proteobacteria, and *γ*-proteobacteria
have been demonstrated to induce morphogenic effects (Nakanishi et al., 1996; Matsuo et
al., 2003; Marshall et al., 2006; Singh et al., 2011).

Another interesting phenomenon is the bacteria guided settlement of algal zooids. *Cytophaga, Polaribacter, Pseudoalteromonas, Pseudomonas, Psychroserpens, Shewanella, Vibrio,* and *Zobellia* species have been described as either stimulatory (Patel et al., 2003), or
inhibitory (Egan et al., 2001) of the zoospore settlement of *Ulva* spp. via quorum sensing
signals.

The availability of any organism for experimentation in the laboratory is essential to
achieve its scope (Joint et al., 2010). From this respect, Amann et al. (1995) reported that the
percentage of cultivability of seawater bacteria ranged between 0.001 and 0.1 % compared
to the values between 0.1 and 1 % of freshwater bacteria. Eilers et al. (2000) tested the
cultivability of the bacterioplankton in the North Sea and found out that the easily cultivable
abundant group of marine bacteria is related to the *Roseobacter* species in contrast to
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*Cytophaga-Flavobacter-Bacteroides* and some strain of γ-proteobacteria species which could never or rarely be cultured. However, cultures remain an essential approach for marine microbial ecologists to understand the role of microbes in the environment (Joint et al., 2010).

**Table 2:** Representative interactions between macroalgae and associated bacteria.

<table>
<thead>
<tr>
<th>Species</th>
<th>Macroalgae</th>
<th>Associated bacteria</th>
<th>Function of bacteria</th>
<th>type of Interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Codium fragile</em></td>
<td>Green alga</td>
<td><em>Azotobacter</em> sp.</td>
<td>Nitrogen fixation</td>
<td>Symbiosis</td>
<td>(Head and Carpenter, 1975)</td>
</tr>
<tr>
<td><em>Monostroma oxyspernum</em></td>
<td>Green alga</td>
<td><em>Cytophaga-Flavobacter-Bacteroides</em></td>
<td>Morphology and growth promoting</td>
<td>Symbiosis</td>
<td>(Matsuo et al., 2005)</td>
</tr>
<tr>
<td><em>Laminaria japonica</em></td>
<td>Brown alga</td>
<td><em>Pseudoalteromonas porphyrae</em></td>
<td>Growth-promoting</td>
<td>Symbiosis</td>
<td>(Dimitrieva et al., 2006)</td>
</tr>
<tr>
<td><em>Ulva linza</em></td>
<td>Green alga</td>
<td><em>α-Proteobacteria</em></td>
<td>Zoospore settlement, growth rate and morphology promoting</td>
<td>Symbiosis</td>
<td>(Marshall et al., 2006)</td>
</tr>
<tr>
<td><em>Ulva mutabilis</em></td>
<td>Green alga</td>
<td><em>Roseobacter</em> sp.</td>
<td>Algal morphogenesis</td>
<td>Symbiosis</td>
<td>(Spoerner et al., 2012)</td>
</tr>
<tr>
<td><em>Prionitis spp.</em></td>
<td>Red algal</td>
<td><em>Roseobacter</em> sp.</td>
<td>Gall formations</td>
<td>Allelopathy</td>
<td>(Ashen and Goff, 2000)</td>
</tr>
<tr>
<td><em>Laminaria religiosa</em></td>
<td>Brown alga</td>
<td><em>Alteromonas</em> sp.</td>
<td>Disease-causing</td>
<td>Allelopathy</td>
<td>(Vairappan et al., 2001)</td>
</tr>
<tr>
<td><em>Fucus evanescens</em></td>
<td>Brown alga</td>
<td><em>Pseudoalteromonas</em></td>
<td>Disease-causing</td>
<td>Allelopathy</td>
<td>(Ivanova et al., 2002)</td>
</tr>
</tbody>
</table>

In the last decade, significant progress towards understanding of the cross-kingdom interactions between *Ulva* and its associated bacteria has been made (Joint et al., 2007). As *U. mutabilis* requires at least two bacteria, *Roseobacter* sp. and *Cytophaga* sp., to complete its morphogenesis in laboratory experiments (Spoerner et al., 2012), it might actively structure their microbiome by releasing specific compounds.
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1.4. Ulva mutabilis-Roseobacter sp.-Cytophaga sp.: a novel tripartite model system in marine ecology

*Ulva* is a cosmopolitan algal genus causing massive green tides in coastal regions that suffer from eutrophication (Fletcher, 1996; Smetacek and Zingone, 2013). Ulvophyceae are mostly marine groups of multicellular algae combining typical properties of higher plants with those of unicellular microorganisms such as Chlamydomonas or yeast (Hori et al., 1985; Lewis and McCourt, 2004). The species *Ulva mutabilis* Føyn (Fig. 2) is the only green alga of this class that has been well established as a laboratory organism. *U. mutabilis* was first discovered by Føyn in the south coast of Portugal in 1952 (Føyn, 1958; Føyn, 1959). Since that time, *U. mutabilis* has been cultivated in various laboratories. The original isolates of Føyn (Føyn, 1958) and many spontaneous and induced developmental mutants previously described are still in culture as defined laboratory strains (Føyn, 1959; Fjeld, 1970; Bryhni, 1974). During the last half-century, *U. mutabilis* had been used as a convenient model system for studying algal development mainly by classical methods of plant physiology and genetics (Loevlie, 1964; Hoxmark and Nordby, 1974; Nilsen and Nordby, 1975).

To study the interaction between macroalgae and bacteria, axenic cultures of macroalgae are the most essential tools, whereas several studies tried to establish those cultures through application of a cocktail of antibiotics (Provasoli, 1958; Marshall et al., 2006), or using protoplast as a feeding stock (Reddy and Fujita, 1991; reviewed by Reddy et al., 2008). Stratmann et al. (1996) and Wichard and Oertel (2010) developed a method to obtain bacteria-free *Ulva* cultures via purification of gametes. Briefly, gametophytes of *U. mutabilis* were artificially induced to form gametangia by removal of at least two sporulation inhibitors. After this treatment, gametes were discarded from the gametangia on the third morning in daylight and upon sufficient dilution of a swarming inhibitor (SWI). Released gametes were separated from their accompanying bacteria by taking advantage of the gametes fast movement towards light. As observed earlier for *U. lactuca* by Provasoli and Pintner (1980), Stratmann et al. (1996) found that *U. mutabilis* shows a complete deregulation of morphogenesis when cultured axenically in fully defined seawater medium. Axenic gametes develop into callus-like colonies consisting of undifferentiated cells without normal cell walls (Fig. 5).
Figure 2: Ulva mutabilis Føyn (a) wild type, (b) mutant (slender) which is investigated in the present study.

From the accompanying microbial flora of the established laboratory strains of *U. mutabilis* with normal morphology, Spoerner et al. (2012) isolated two essential strains, a *Roseobacter* sp. and a *Cytophaga* sp., which can completely replace the bacterial flora of *U. mutabilis* forming a symbiotic tripartite community (Fig. 5) and induce readily algal morphogenesis via diffusible molecules. Each organism in this tripartite community contributes to this community by sufficient functions. *U. mutabilis* as seaweed is supposed to provide this community with an organic- and nutrient-rich habitat.

*Roseobacter* sp. stimulates the *Ulva* cell division by excreting an unknown factor into the medium (Spoerner et al., 2012). This factor induces the development of the *Ulva* gametes into thalli composed of blade cells with characteristic deficiencies represented in (1) an enhancement of cell division rate not followed by cell expansion, (2) bubble-like structures cover the cell wall, and (3) secondary rhizoid cells are not formed. Interestingly, the factors of the isolated species *Sulfitobacter*, and *Halomonas* resulted in the same effect on *U. mutabilis* when added separately into axenic *U. mutabilis* culture indicating a non species specific compound (Spoerner et al., 2012).

Likewise, the third partner exists in this tripartite community “*Cytophaga* sp.” promotes *U. mutabilis* cell development by stimulating vacuole extension and cell differentiation, so that blade cell can be distinguished from stem and rhizoid cells (Spoerner et al., 2012). In 2005, Matsuo et al. succeeded to identify a growth factor termed thallusin (Fig. 3a) from a much
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related strain to *Cytophaga*. Thallusin promotes solely the growth, development and morphogenesis of the green alga *Monostroma* (Matsuo et al. 2005).

![Figure 3: Algal compounds, which might influence the tripartite community of *Ulva mutabilis* and its associated bacteria. (a) Thallusin (C$_{25}$H$_{31}$NO$_{7}$) isolated from the *Cytophaga-Flavobacterium-Bacteroides* associated to *Monostroma*. (b) DMSP produced by *U. curvala*. (c) 2,4 decadienal produced by *Ulva conglobate.*](image)

In this particular model system, *Cytophaga*-factor in contrast to *Roseobacter*-factor could never be replaced by any other isolates (Spoerner et al., 2012). Functionally, *Roseobacter*-, *Sulfitobacter*-, and *Halomonas*-factors resemble a cytokinin, while *Cytophaga*-factor acts similar to auxin. Neither factor could be replaced by any known phytohormone (Spoerner et al., 2012). It is essential to point out that the motile unicellular, spores ad gametes, need to complete a succession of processes initiated by attachment, followed by adhesion, and ended by germination before achieving a successful settlement. Spores use their apical papilla to contact the surface by discharging elastic material (Maggs and Callow, 2001). It is predicted that attachment of *Ulva* spores is enhanced via quorum sensing (QS) produced by bacteria as already described above. QS involves the use of diffusible chemical of signal molecules by bacteria that, upon reaching a threshold cell concentration level, activate target genes that are used by the bacteria to regulate population growth. *Ulva* spores appear to be able to “listen” into such bacterial conversations (QS) by sensing and responding to e.g., acylhomoserine lactones (AHLs) produced by bacteria and the settlement of the spores seems to be guided by the AHLs (Joint et al., 2002; Dudler and Eberl, 2006; Wheeler et al., 2006; Joint et al., 2007). Therefore, zoospores settle in the vicinity of specific bacteria that they require for normal development, growth and survival to the next generation. For example, *Roseobacter* sp. was described for both its ability to attract zoospores and its influence on growth development (Spoerner et al., 2012), although by
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Marshall et al. (2006) no absolute correlation was found between isolates that influenced morphology of *U. linza* and those that enhanced zoospore settlement.

From the bacterial side, the colonization process, including chemotactic responses and factors affecting the holdfast formation and the firm attachment of cells to a substratum (Spoerner et al., 2012; Wichard unpublished results). Bacteria respond tactically to a variety of metabolites, including attraction to algal extracellular products and repulsion from tannic acid (Wahl et al., 1994). *Roseobacter* sp. e.g., has shown a specific chemotactic affinity towards rhizoid cells of *U. mutabilis* (Spoerner et al., 2012). Little is known of the exact mechanism(s) that *Roseobacter* employ to physically associate with eukaryotic cell surfaces or particles, several cultivated strains have been shown to be capable of surface colonization (Rao et al., 2006; Bruhn et al., 2007). Spoerner et al. (2012) suggested a work model for the interactions between all these three organisms within this community including the interaction between *Roseobacter* and *Cytophaga* species. In this model, *Ulva* cells excrete a diffusible substance such as a specific nutrient or regulatory factor, which attracts motile *Roseobacter* cells toward the holdfast. They successively assemble and deposit a layer of mucilage produced by them. This mucilage may form the matrix of an organized biofilm. Afterwards, the rod shaped bacterium *Cytophaga* may be recruited by incidental direct contact with the algal cell surface where they may move by gliding on a mucilage layer. *Cytophaga* species are known to recognize and adhere specifically to surfaces composed of cellulose, agarose, or other cell wall components which they degrade enzymatically. Spoerner et al. (2012) also predicted that *Roseobacter* sp. may promote *Cytophaga* sp. viability.

The current study aimed to investigate the chemical compounds released by the living organisms in this community mediating the cross-kingdom interaction between *Roseobacter* sp., *Cytophaga* sp. and *U. mutabilis* and how this interaction will influence the growth, ability of reproduction, and morphological development of *U. mutabilis*.

Few compounds released by *Ulva* sp. have been already identified, DMSP and 2,4-decadienal (Van Alstyne et al., 2001; Akakabe et al., 2003) which might be candidates for mediating cross kingdom interactions. DMSP (Fig. 3b) is a sulfur-containing compatible solute that is produced in high concentrations in many marine algae particularly in green and red macroalgae (Dickson et al., 1980; Karsten et al., 1990; Steinke and Kirst, 1996; Stefels, 2000; Van Alstyne et al., 2003; Van Alstyne et al., 2001). DMSP is often released in senescent
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algae or when algae undergo oxidative stress, particularly under high light intensities (Karsten et al., 1990). However, it has been documented that DMSP (1) controls internal osmotic pressure, (2) serves as a cryoprotectant (Andreae and Barnard, 1984; Kwint and Kramer, 1996), and (3) can expel excess sulfur and energy (Stefels, 2000). Likewise, the products of DMSP cleavage play a role as an activated defense system. DMS and acrylic acid, deter feeding on macroalgae (Alstyne et al., 2001; Wiesemeier and Pohnert, 2007). In the context of the DMSP role in the cross-kingdom interaction, it is reported that DMSP (Fig. 3b) produced by *U. australis* influences biofilm formation by associated bacteria *Pseudalteromonas tunicata* and *Roseobacter gallaeciensis* (Rao et al., 2006). It was found that DMSP decreased the colonization of *U. sustralia* by *P. tunicate*, whereas stimulative effect of DMSP was observed for the colonization by *R. gallaeciensis* due to the ability of *Roseobacter* spp. to metabolize DMSP, and their presence and activity on algal surfaces are significantly correlated with DMSP-producing algae (Gonzalez and Moran, 1997). Furthermore, member of the *Halomonas* genus, from the surface of DMSP-containing *U. curvala*, grows on DMSP as the sole carbon source and emitted DMS (deSouza et al., 1996). It is notable that Kiene et al. (1999) and Johnston et al. (2008) pointed out that some marine bacteria affect the cycling of dissolved DMSP and DMS. For instance, multiple metabolic pathways exist in prokaryotes that catabolise DMSP, but some of them don’t emit DMS. Moreover, DMS consumption has also been described in various bacteria (Schaefer et al., 2010).

Besides DMSP, oxylipins are metabolites produced by a variety of *Ulva* spp. and considered as chemical defense, and have various effects on bacterial growth and the final cell density of cultures in the laboratory.

1.5. *Polyunsaturated aldehydes, a potential role in chemical communication of Ulva sp.*

Lipoxygenase mediated pathways provide a wide variety of fatty acid derived metabolites, which are involved in signaling, chemical defense and cell-cell interactions in plant and animal kingdoms. In these pathways, molecular oxygen is introduced into a polyunsaturated fatty acid (Andreou et al., 2009; Kachroo and Kachroo, 2009). The intermediate hydroperoxy fatty acid can be cleaved into shorter chain-length oxygenated products (Andreou et al., 2009; Noordermeer et al., 2001). Up to now, a huge variety of transformations have been identified, but macroalgae such as the sea lettuce *Ulva* (Ulvales, Chlorophyta) are still under
investigated. Algal oxylipins including polyunsaturated aldehydes (PUAs) derived from polyunsaturated acids (PUFAs) might play an important structuring role for the microbiome (Leflaive and Ten-Hage, 2009). Indeed, Ribalet et al. (2008) demonstrated that PUAs, such as 2,4-decadienal, have various effects on bacterial growth. “PUA-sensitive” bacteria are often also associated with certain *Ulva* species including *U. mutabilis*, *U. linza* or *U. australicus* (Burke et al., 2009; Spoerner et al., 2012). Interestingly, members of the *Roseobacter* clade/genus, which induce partly the morphogenesis of *Ulva* in a tripartite symbiosis of *U. mutabilis*, are sensitive to elevated amounts of PUAs added to the culture medium (Ribalet et al., 2008; Spoerner et al., 2012).

The biosynthesis of PUAs is widespread in algae, lower and higher plants (Feussner and Wasternack, 2002). PUAs are often only released upon cell damage in diatoms (Bacillariophyceae), mosses and higher plants (Pohnert, 2000). However, the amount and structural diversity of released PUAs varies greatly depending on the species and also environmental conditions e.g., in diatoms (Wichard et al., 2005a; d’Ippolito et al., 2005), which makes case-sensitive studies necessary.

In marine ecosystems, the production and release of PUAs in diatoms have been particularly intensively investigated in the last two decades. Several studies have demonstrated the adverse effects of diatom-derived 2,4-decadienal on the reproduction of their grazers in laboratory experiments (Ianora et al., 2004), but field-near experiments have also questioned the ecological relevance of those compounds (Wichard et al., 2008; Dutz et al., 2008). More recently, Vidoudez et al. (2008) observed that 2,4-octadienal, 2,4,7-octatrienal and 2,4-heptadienal are also directly released by the intact cells of diatom *Skeletonema marinoi* into the surrounding seawater (Vidoudez and Pohnert, 2008). This release can be associated to cell lyses during diatom bloom termination (Ribalet et al., 2014) and trigger further cell death of “PUA-sensitive” diatoms in the vicinity (Vardi et al., 2006; Dittami et al., 2010). An analogous process might be part of macroalgal blooms like green tides, which may result in a massive emission of PUAs into the environment, in particular, when *Ulva* accumulates on the shore and remains for long periods as happened in Bretagne (France) or Quingdao (Yellow Sea, China) (Ding et al., 2009; Hu et al., 2010). Here, the algal tissue might decompose and release elevated amounts of PUAs. Besides the study by Akakabe and co-workers (2003), the production of PUAs and their biosynthesis have not been investigated on the species level in the genus of *Ulva*. The study has shown that
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(2E,4Z)-2,4-decadienal is derived from (R)-11-hydroperoxy-arachidonic acid (HpETE) indicating a stereo selective lipoxygenase/hydroperoxide pathway in *Ulva conglobata*. Akakabe et al. (2003) purified a crude enzyme from *Ulva* that catalyzed the formation of (R)-11-HpETE and the breakdown product 2,4-decadienal upon addition of arachidonic acid (ARA). In addition, long-chain aldehydes including (8Z,11Z,14Z)-8,11,14-heptadecatrienal and (7Z,10Z,13Z)-7,10,13-hexadecatrienal were found in *Ulva* (Akakabe et al., 2000; Akakabe et al., 2005). All these aldehydes along with a typical release of dimethyl sulfide (DMS) are responsible for the seaweed like odor (deSouza et al., 1996). Recently, three monounsaturated fatty acid (MUFA) derivatives were isolated from *U. lactuca*; a novel keto-type C_{18} fatty acid, the corresponding shorter chain C_{16} acid, and an amide derivative of the C_{18} acid (Wang et al., 2013).The most extensive study of *Ulva* oxylipins was provided by Abou-Elwafa (2009), where three new fatty acids ((E)-11-oxo-octadeca-12-enoic acid, 11-hydroxy-octadeca-12-enoic acid and 6-hydroxy-oct-7-enoic acid) were isolated from a dichloromethane extract of *Ulva fasciata* (Delile), collected at the Mediterranean coast of Egypt (Abou-Elwafa et al., 2009). Products of lipid peroxidation processes mediated by lipoxygenases (LOX) result in hydroperoxide fatty acids, which might decompose enzymatically via hydroperoxide lyases or in degradation reactions to breakdown products including polyunsaturated aldehydes. In particular, 2,4-decadienal can degrade to further short chain aldehydes (Spiteller et al., 2001). Thus, to determine 2,4-decadienal and other PUAs in seawater upon cell wounding, the compounds have to be trapped and stabilized with the derivatization reagent pentafluorobenzylhydroxylamine (PFBHA) for quantification (Wichard et al. 2005 a,b).

In this study, *Ulva* species collected in the lagoon Ria Formosa (Faro, Portugal) in 2010 were surveyed for their production of PUAs. Several studies have shown that *Ulva* harbors large amounts of C_{18} PUFAs including linoleic (LA, 18:2 n-6) and ω-3 linolenic (αLEA, 18:3 n-3) acids but only minor amounts of arachidonic acid or even no C_{20}-PUFAs in certain cases (Pereira et al., 2012). As *Ulva* produces high concentrations of α-linolenic acid, the ratio between ω6/ω3 PUFAs differs significantly from other green algae. Pereira et al. (2012) hence concluded that macroalgae can be considered as a potential source for large-scale production of essential PUFAs with wide applications in the pharmacological industries. *Ulva* is an autotrophic organism with simple growing requirements that can produce lipids and proteins in large amounts over short periods of time in simple land based aquacultures.
Although this is also well known for microalgae (Brennan and Owende, 2009), wound activated transformation of lipids into oxylipins could also result in a depletion of valuable unsaturated fatty acids and might hence jeopardize the value of Ulva as a resource for e.g., PUFAs in aquacultures (Wichard et al., 2007).

Whereas central metabolic pathways are well described and genetically and biochemically explored in higher plants and animals, these tools are not yet established for Ulva. Using an analytical chemistry approach, our study aims to (1) survey the plasticity of PUA-production of freshly sampled Ulva species from the lagoon Ria Formosa in Portugal, to (2) compare the PUA amount within algal cultures, and to (3) elucidate the biosynthetic pathways of PUAs using stable isotope labeled PUFAs and mass spectrometric analyses.

A comprehensive investigation to understand the cross-kingdom interactions between U. mutabilis and its associated bacteria is needed. The present study extended the coverage of the compounds used as signals among these organisms by using exo-metabolomic approach.

1.6. The broader view: the explorative metabolomic approach – a brief introduction

Metabolomics is a relatively new member to the ‘-omics’ family of systems biology technologies (Bino et al., 2004). The term ‘metabolome’ was coined in 1998 and was used to describe the metabolite complement of living tissues (Oliver et al., 1998). Despite its relative youth (in comparison to genomics and proteomics), metabolomics as a field of study is now firmly established as a functional genetics approach to understand the molecular complexity of life (Wagner et al., 2003).

Metabolomics uncovers many possibilities that were masked before such as new biomarker discovery and hypothesis generation. Thus, this approach delivers a metabolic signature of biological sample (Walsh et al., 2008). Depending on the goal of experiment, the approach used will differ. The three principal approaches for the analysis of the metabolome are Metabolic Profiling, Metabolic Fingerprinting, and Metabonomics/Metabolomics (Hall, 2006). On the other hand, (Nielsen and Oliver, 2005) subdivided Metabolic profiling into Fingerprinting, covering “intercellular metabolites”, and Footprinting, dealing with “extracellular metabolites”. Fiehn (2006) divided the metabolom analyses regarding its scopes into: Target Analysis, Metabolite Profiling, Metabolomics, and Metabolite Fingerprinting. Typically, the metabolome analyses can be separated into two different types: targeted and non-targeted analyses. In targeted analysis, the metabolomics data are
scanned for specific compounds normally collected in a reference library. **Non-targeted**, in contrast, is an approach does not aim to identify the compounds and the spectroscopic features of all potential compounds are considered for further analyses (Kell, 2006).

In the present study, **metabolomic analysis** was used to cover the specific intracellular metabolites of *U. mutabilis* as a **targeted analysis**, and extracellular metabolites of *U. mutabilis* and its associated bacteria as a **non-targeted** analysis.

No single analytical platform is currently capable of extracting and detecting all metabolites (Phuc et al., 2010). Optimal selection of a particular technology depends on the goals of the study and is usually a compromise among sensitivity, selectivity and speed (Lei et al., 2011). Although NMR is in principle the most uniform detection approach and it is essential for the unequivocal identification of unknown compounds, NMR-based metabolomics approaches still suffer from a relatively low sensitivity compared with mass spectrometry. As yet, MS-based techniques are most widely used in plant metabolomics (De Vos et al., 2007; Hall, 2006). Mass spectrometry techniques offer a good combination of sensitivity and selectivity. Modern MS provides highly specific chemical information that is directly related to the chemical structure, such as accurate mass, isotope distribution pattern for elemental formula determination, and characteristic fragmentations for structural illustration or identification via spectral matching to the provided reference library. Moreover, the high sensitivity of MS allows detection and measurement of picomole to femtomole levels of many primary and secondary metabolites. Thus, MS becomes an important tool in metabolomics (Lei et al., 2011). Mass spectroscopy systems in combination with nuclear magnetic resonance systems are ideally the best platforms for identification of unknown chemical compounds, but are prohibitively expensive for most scientific laboratories (Dixon et al., 2006). The preferred method for analyzing semi-polar metabolites is liquid chromatography (LC) coupled to mass spectrometry (MS), with a soft ionization technique, such as electrospray ionization (ESI), resulting in protonated (in positive mode) or deprotonated (in negative mode) molecular masses. Compounds detectable by LC-MS include the large and often economically important group of secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivatives thereof (Huhman and Sumner, 2002; Tolstikov et al., 2003; Moco et al., 2006; Rischer et al., 2006; Farag et al., 2007). These compounds can be effectively extracted with aqueous alcohol solutions and directly analyzed without derivatization. LC-MS
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particularly important additional, versatile technology for metabolomic analysis as it also provides broader coverage of molecules which more readily either gain or lose a proton by working in positive and negative ion modes. Unlike GC-MS, few mass spectral libraries are available for LC-MS and this is a key topic being given considerable attention at present. Nevertheless, LC-MS as an untargeted metabolomics aims to gather information on as many metabolites as possible in biological systems by taking into account all information present in the data sets (De Vos et al., 2007). In fact, gas chromatography-mass spectrometry (GC-MS) is probably the most popular analytical platform used in metabolic analyses as a targeted analysis (Lisec et al., 2006). GC coupled to electron impact (EI) time-of-flight (TOF) MS was the first approach used in large-scale plant metabolomics (Fiehn et al., 2000). Biological extracts to be analyzed via GC-MS must first be chemically derivatized with agents that make the analytes more volatile (Wagner et al., 2003; Lisec et al., 2006; Seger and Sturm, 2007). Once the sample is injected into the gas chromatograph, there is two-fold separation of sample components based on differences in volatility and polarity. Larger molecules take a longer time to move through the column than do small molecules and amongst molecules of similar size, different molecular species display different volatilities. Upon outflow from the chromatograph column, individual volatilized chemicals are funneled into the mass spectrometer where identification and quantification of individual chemical compounds is facilitated. Data obtained by the GC-MS is deconvoluted by special software to produce two graphs corresponding to the chromatogram and mass spectra of the sample. Graphs from different samples can be overlaid to aid in comparison and detection. Individual chemical “biomarkers” can be identified based on the retention time (the time it takes for the compound to become vaporized and to flow through the chromatographic column) and the mass spectrum. GC-MS approach is suitable for a high variability of nonvolatile metabolites, mainly those involved in primary metabolism, including organic and amino acids, sugars, sugar alcohols, phosphorylated intermediates (in the polar fraction of extracts), as well as lipophilic compounds such as fatty acids and sterols (in the apolar fraction) (De Vos et al., 2007).

One problem that persists in metabolomic analyses is the lack of comprehensive identification of metabolic components, particularly in pathways outside of primary carbon metabolism (Wagner et al., 2003). Therefore, there is a movement in the scientific community towards a cooperative approach for creating open-access libraries of compounds
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based on standardized analytical procedures (Bino et al., 2004; Dixon et al., 2006). Several libraries already exist and are immensely helpful in chromatogram analysis, though they are far from being comprehensive. In the next section, the analytical strategy, which was used in this study, was addressed in detail.
2. Goals and Analytical Strategy
2.1. Goals

The relationship between *U. mutabilis* and its associated bacteria is still poorly understood. In particular, with respect to chemical-mediated interactions taking place in the chemosphere, which is defined as a part of the biocoenose, where the organisms interact with each other via infochemicals. In order to study this relation between *U. mutabilis* and its associated bacteria, the study will try to mimic the natural community of *U. mutabilis* and at the same time to simplify its complexity without losing the normal biological traits of *U. mutabilis* such as growth, capability of reproduction and morphological development. In this perspective, I will use the simple model system, which was established by Stratmann et al. (1996) and Spoerner et al. (2012) to investigate the chemical compounds released by *U. mutabilis*, *Cytophaga* sp. and *Roseobacter* sp. into the culture medium (Fig. 5). The natural mutant *U. mutabilis* slender, which is characterized by its fast growing and subsequently its short lifecycle, is selected for this study.

In this study, I aim

- to survey the potential production of polyunsaturated aldehydes (PUAs) in *Ulva* spp. collected at various sampling sites in the lagoon of the Ria Formosa (Portugal) as a first candidate infochemical mediating bacterial growth by the alga.
- to test the chemotaxonomic significance of collected *Ulva* species in terms of PUAs production, morphogenetic and phylogenetic analyses in cooperation with CCMAR, Algarve University, Portugal.
- to establish (1) metabolomics in *Ulva* and to decipher the (2) chemosphere of the tripartite community defined by *U. mutabilis*, *Roseobacter* sp. and *Cytophaga* sp.
- to scale up the experiment from laboratory scale to aquacultures at the marine field station Ramalhete in Faro (Portugal).
- to apply the chemometric approach in order to an understanding deeply the dynamic of the chemosphere over the lifecycle of *U. mutabilis* under different treatments: axenic alga and with its associated bacteria.

Specific analytical strategies have to be developed to achieve these goals. In this study, it is of a particular challenge to use metabolomics that provides a comprehensive qualitative overview of the metabolites present in the culture medium of the tripartite community. Both GC and LC techniques coupled with a time-of-flight mass spectrometer (TOF-MS) will be
Goals and Analytical Strategy

2.2. Analytical strategy

2.2.1. Targeted analyses

Fiehn (2006): “Target analysis is constrained to one or a very few target compounds [such as phytohormones]. Such targets are usually quantified in an absolute manner using calibration curves and/or stable isotope labeled internal standards.”

Polyunsaturated aldehydes (PUAs), a specific class of oxylipins derived from polyunsaturated fatty acids, will be investigated in detail, as there are evidences that *Ulva* spp. might negatively interfere the growth of *Roseobacter* sp. via 2,4-decadienal and may contribute to the chemical-mediated interactions between *Ulva* spp. and its community. Therefore, the potential product ion of PUAs by the algal cells and the amount of dissolved PUAs in the culture medium will be addressed using an *in situ* derivatization approach with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA.HCl) which leads to an *in situ* derivatization without inhibition of the biosynthesis of the aldehydes (Wichard et al. 2005). For elucidation of the biosynthesis, stable isotope labeled precursors will be used. GC-MS is the chosen approach.

2.2.2. Metabolomics

Fiehn (2006): “Metabolomics seeks for a truly unbiased quantitative and qualitative analysis of all biochemical intermediates in a sample. It must not be restricted by any physicochemical property of the metabolites, such as molecular weight, polarity, volatility, electrical charge, chemical structure and others. Since there is currently no single technology available that would allow such comprehensive analysis, metabolomics is characterized by the use of multiple techniques and unbiased software. Metabolomics also uses relative quantification. In addition, it must include a strong focus on de novo identification of unknown metabolites whose presence is demonstrated.”

Extraction of metabolites from marine system is a great challenge. However, the external chemosphere of most marine organisms (including microorganisms) has remained overlooked until recent applications of MS-based chemical profiling and metabolomic approaches for chemical ecology studies (Goulitquer et al., 2012). A statistical evaluation of data provides insights into the released metabolites that might represent a message sent by
emitter organism(s) to potential receiver organism(s) as was addressed in sections (cf. 1.2 1.3). The scheme below (Fig. 4) summarizes the stepwise procedure which is recommended by several studies to obtain a robust result (Jenkins et al., 2004; Dunn and Ellis, 2005; Beckonert et al., 2007; Moco et al., 2007).

The research question: Deciphering the dynamics of the chemosphere within the community of *U. mutabilis* and its associated bacteria (Fig. 5).

To describe the chemosphere of the tripartite community (Fig. 5), it is necessary to identify significant numbers of metabolites in the biological samples. In the present metabolomic study, I try to avoid biases against certain compound classes by chemical structure or by apparent abundance in the algal tissue in order to identify these compounds, which are produced by the three organisms and released into the culture medium. The experimental design must assure that the metabolites’ abundance can be directly compared in different samples.
Study design: The experiment will start in the laboratory with small volumes of 1 L as batch cultures using Erlenmeyer flasks under sterile and controlled conditions, and then the experiment under the same conditions will scale up to a volume of 25 L in bioreactors using Carboy vessels. After that, aquacultures using huge volume of 200 L will be performed in the lagoon Ria Formosa (Portugal) in order (1) to get elevated concentrations of biomarkers over lifecycle of *U. mutabilis*, and (2) to test the repeatability, and reproducibility of the stepwise scaling of the metabolomic analysis of *U. mutabilis* in the tripartite community.
Goals and Analytical Strategy

It is important to highlight that all the experiments will start with purified gametes as it is conceptually a novel approach to work with a well defined seed stock, particularly, in aquaculture.

**Sampling and storage:** The culture medium will be sampled once weekly, and storage at -80 °C in parallel to the metadata collection.

In the word of Jenkins et al. (2004): “*metadata is data about the data, providing context - represents a large part of the system. This allows dataset comparability to be assessed*”. It is essential to collect extensively metadata such as fundamental biological parameters along the metabolomic analysis in order to interpret the omics-data and guarantee comparability between explorative studies. Data driven research such as omics benefits from high qualitative biological data as *a posteriori* hypothesis can be also generated for subsequent multivariant data analyses of the chemical data (Pedro, 2002; Goodacre et al., 2004).

**Sampling work-up:** In the present study, non-targeted analysis will be used based on the methodology established by Barofsky et al. (2009) and Vidoudez and Pohnert, (2012). In these studies, LC-MS and GC-MS approaches were applied to investigate the exo- and endo-metabolites excreted by *Skeletonema marinoi* and *Thalassiosira pseudonana*. UPLC-MS approach, designed by Barofsky et al., showed the influence of *S. marinoi* growth phases on the cellular metabolic profile of a copepod, suggesting that changes in inofchemicals within or surrounding the diatom regulate selective feeding of the zooplankton (Barofsky et al., 2009; Barofsky et al., 2010). Also with *S. marinoi*, GC-MS analysis revealed clearly that the excreted metabolites differ between growth phases (Vidoudez and Pohnert, 2012).

Consequently, in this study both non-targeted analyses using liquid chromatography and gas chromatography (which needs derivatization steps) coupled to mass spectrometry (GC-MS and UHPLC-MS) will be applied to monitor a wide range of excreted compounds in the culture medium of the tripartite community. Using this adjusted the methodology in the current study, I aim to explore the exometabolome of *Ulva mutabilis* and its associated bacteria *Cytophaga* and *Roseobacter* species.

**Data extraction:** Markerlynx™ will be used to collect the biomarkers from LC-MS-based dataset. To extract the metabolites from GC-MS-based dataset, the Automated Mass Spectral Deconvolution and Identification System (AMDIS) deconvolutes chromatograms and generates lists of spectra, the lists are then fed to the Metabolomics Ion-based Data Extraction Algorithm (MET-IDEA) for quantification (Hiller et al., 2009). Here, I aim to define
a robust parameter setting for the automated and reliable data mining from LC-MS and GC-MS analyses.

**Data analysis:** Chemometric analyses will be applied: unconstrained principle component analysis (PCA) and the constrained canonical analysis of principle coordinate (CAP). Clarke and Ainsworth (1993) pronounced the data analysis philosophy “*lets the data speak for themselves*” through PCA clusters and encouraged an exploratory attitude towards data. Unconstrained ordinations are generally extremely useful for visualizing broad patterns across the entire data cloud as well as any differences in within-group variability. Although group differences may be seen in an unconstrained ordination, they can also be masked by high variability and high correlation structure among variables unrelated to group differences (Anderson and Willis, 2003). This unconstrained analysis is not ideal for analyzing data with high variety in abundance because of the limitations of the Euclidean distance, which is the only ecological distance investigated with PCA. The Euclidean distance is not a very good distance for investigating how species (biomarkers) are shared between sites (groups) (Legendre and Legendre, 1998). A constrained ordination such as CAP, on the other hand, does not allow any assessment of either total or relative within-group variability, but it does allow the differences among groups to be seen due to the flexibility to use any ecological distance such as Bray-Curtis distance. Although Bray-Curtis is known to be overly sensitive to differences in absolute abundances, with the (1) transformation, in this case to $y' = \sqrt{y}$, this effect is largely reduced (Clarke and Ainsworth, 1993) and (2) standardization by dividing cell abundance by the total abundance of each sample, so that biomarkers sum up to 1 for each sample (Legendre and Legendre, 1998). By using this statistical strategy, one will compare differences in metabolites proportions within each sample and between all samples.
3. Results and Discussion
3.1. Prevalence and mechanism of polyunsaturated aldehydes production in the green tide forming macroalgal genus *Ulva* (Ulvales, Chlorophyta)\(^1\)

\(^1\) This chapter is based on the publication of Alsufyani et al. (2014)
3.1.1. Identification of Ulva species in the lagoon Ria Formosa (Portugal)

*Ulva* spp. were collected in the lagoon Ria Formosa (Faro, Portugal) in spring 2010 in order to survey the potential production of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes (Fig. 6). About 100 algal isolates were transferred to *Ulva* culture medium (UCM) and cultured under standardized conditions (Stratmann et al., 1996). Those algae, which survived cleaning, transfer and cultivation (Tab. 3), were described briefly by their morphology. However, gene sequencing-based classifications of *Ulva* are critical for proper species identification. Therefore, the sequences of the chloroplast-encoded RuBisCo gene (*rbcL*) were analyzed and compared with GenBank™ sequences archived by NCBI (Tab. 3). Based on the *rbcL* data seven operational taxonomic groups including those *rbcL* haplotypes were identified, which correspond to e.g., *U. rigida* or *U. compressa* complexes (Guidone et al., 2013). For instance, *U. mutabilis* (UM_2), *U. compressa* and *U. pseudocurvata* (GenBank™ data) corresponded to the same complex. Sequencing data revealed the identity of several isolates, which were used as biological replicates for further chemical analysis including quantification of PUAs (Tab. 4). For comparison, two isolates were collected outside of Portugal at marine stations on the North Sea Island Helgoland (Germany) and at Puerto Montt (Bay of Puerto Montt, Chile).

3.1.2. Survey and quantification of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes (PUAs)

2,4-heptadienal and 2,4-decadienal were the most prominent PUAs, whereas 2,4,7-decatrienal was often found only in minor amounts (Fig. 6, Tab. 4) determined at pH 8.2 in sterile seawater medium. 2,4-octadienial or 2,4,7-octatrienial known to be derived from C16-polyunsaturated fatty acids such as 6Z,9Z,12Z,15-hexadecatetraenoic acid (Pohnert et al. 2004) could not be determined. The strongest PUA-producer collected in the Ria Formosa was identified as a species close to *U. rigida*. For instance, upon cell damage 2.8 ± 0.5 nmol g$^{-1}$ (fresh weight) of $2E,4Z$-heptadienial and $2E,4E$-heptadienial, 1.9 ± 1.0 nmol g$^{-1}$ of $2E,4Z$-decadienal and $2E,4E$-decadienal and minor amounts of 2,4,7-decatrienal were measured in *U. rigida* (RFU_77). Interestingly, a comparable amount (2.3 nmol g$^{-1}$ fresh weight) of 2,4-decadienal was exclusively detected in *U. conglobata* (Akakabe et al., 2003). Isolate RFU_11, most closely related to *U. ohnoi* or *U. reticulata* (Hiraoka et al., 2006), contributed significantly to PUA production as well as isolate RFU_93, which was identified as *U. rotunda* (Fig. 6, Tab. 4). Overall, the total amount of released PUAs of the identified producers ranged from 0.6 – 5.1 nmol g$^{-1}$ (fresh weight) within this survey. In this context, it is interesting to
Results and Discussion

note that some of the most abundant green tide forming species like *U. ohnoi* or *U. rigida* released high amounts of PUA. Most of the collected algae did however not produce any PUAs under standardized laboratory conditions upon cell damage (Figs. 6, 7; Tab. 3). Production of volatile aldehydes was not observed in our model organism *Ulva mutabilis* Føyn upon cell damage. This species was originally collected at the Portuguese Coast close by Olhão (Portugal) and was particularly characterized by its morphological plasticity as it appears in blade and tube morphology. As the PUA-production within different isolates of the genus *Ulva* seems to range widely, additional case-specific chemical investigations are required in future studies. Therefore, the oxylipins production of mature thallus of the parental was also compared with the filial generation, because sporulation (i.e. release of gametes or zooids) can be occasionally induced under laboratory conditions (Stratmann et al., 1996; Wichard and Oertel, 2010). These findings show no significant difference of PUA-production in RFU_77 (Fig. 7) indicating that the laboratory conditions did not influence the amount of PUA production over half a year. However, further studies have to be performed to show if the amount and pattern of PUAs varies throughout the different growth phases of *Ulva*. Indeed, this is of particular interest in algal aquaculture, where stable and predictable conditions are desirable e.g., regarding the transformation of PUFAs and oxylipins production. Interestingly, all PUA producers, including the previously identified producer of 2,4-decadienal, *U. conglobata* (Akakabe et al., 2003), corresponded to the sea lettuce morphotype. This observation indicates a phylogenetic relationship, which was also supported by the analysis of the phylogenetic tree, based on rbcL data (Fig. 6). Due to this chemotaxonomic significance of PUAs, It is concluded that sea lettuce like morphotypes of *Ulva* indicates a high potential production of PUAs during green tides with potential allelopathic effects on e.g., the microbiome and grazers.
Figure 6: Maximum likelihood phylogram of *Ulva* spp. inferred from the *rbcL* gene. Species were collected in the Ria Formosa (Portugal) except isolate C.210, which was from the Bay of Puerto Montt (Chile). ML bootstrap values are reported for nodes that received >50% support (based on 1000 replicates). All isolates were submitted for PUAs analysis. **Filled circles** indicate the determination of significant amount of PUAs: C7:2 = 2,4-heptadienal, C8:2 = 2,4-octadienal, C8:3 = 2,4,7-octatrienal, C10:2 = 2,4-decadienal, C10:3 = 2,4,7-decatrienal. **Open symbols** indicates no PUA. Accession numbers of GenBank™ are shown for reference taxa.
Table 3: Sample information of collected *Ulva* species: Strain numbers, morphology, PUA production and GenBank™ accession number for the *rbcL* gene sequence. The closest related species according GenBank™ information is given.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest related species</th>
<th>Morphology</th>
<th>C7:2</th>
<th>C8:2</th>
<th>C8:3</th>
<th>C10:2</th>
<th>C10:3</th>
<th>Accession no# <em>rbcL</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>UM_2</td>
<td>*</td>
<td>Various morphotypes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417451</td>
</tr>
<tr>
<td>RFU_7</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417448</td>
</tr>
<tr>
<td>RFU_11</td>
<td><em>U. ohnoi</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417449</td>
</tr>
<tr>
<td>RFU_58</td>
<td><em>U. compressa</em></td>
<td>Branched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417456</td>
</tr>
<tr>
<td>RFU_59</td>
<td><em>U. compressa</em></td>
<td>Branched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417455</td>
</tr>
<tr>
<td>RFU_61</td>
<td><em>U. compressa</em></td>
<td>Branched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417454</td>
</tr>
<tr>
<td>RFU_71</td>
<td><em>U. muscoides</em></td>
<td>Highly branched tubes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417440</td>
</tr>
<tr>
<td>RFU_76</td>
<td><em>U. muscoides</em></td>
<td>Highly branched tubes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417441</td>
</tr>
<tr>
<td>RFU_77</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417445</td>
</tr>
<tr>
<td>RFU_81</td>
<td><em>U. compressa</em></td>
<td>Branched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417457</td>
</tr>
<tr>
<td>RFU_82</td>
<td><em>U. compressa</em></td>
<td>Branched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417453</td>
</tr>
<tr>
<td>RFU_91</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417446</td>
</tr>
<tr>
<td>RFU_92</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417447</td>
</tr>
<tr>
<td>RFU_93</td>
<td><em>U. rotundata</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417450</td>
</tr>
<tr>
<td>RFU_95</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417444</td>
</tr>
<tr>
<td>RFU_96</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417442</td>
</tr>
<tr>
<td>RFU_97</td>
<td><em>U. rigida</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417443</td>
</tr>
<tr>
<td>RFU_200</td>
<td><em>U. mutabilis</em></td>
<td>Unbranched tube</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>KJ417452</td>
</tr>
<tr>
<td>C_210</td>
<td><em>Ulva sp.</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>KJ417458</td>
</tr>
<tr>
<td>H_211</td>
<td><em>Ulva sp.</em></td>
<td>Blade</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>not determined</td>
</tr>
</tbody>
</table>

The presence of PUAs is given by (+). C7:2 = 2,4-heptadienal, C8:2 = 2,4-octadienal, C8:3 = 2,4,7-octatrienal, C10:2 = 2,4-decadienal, C10:3 = 2,4,7-decatrienal. * UM = *Ulva mutabilis* Føyn: specimen cultivated in the laboratory in Jena (Germany), but originally collected in the Ria Formosa (Portugal) in 1952.
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Table 4. Quantification of PUAs (sum of the isomers in nmol g\(^{-1}\) wet biomass) in *Ulva* spp. sampled in 2010/2011. Mean ± standard deviation of biological replicates is given (n = 3).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest related species</th>
<th>Collection Site</th>
<th>Heptadienal nmol g(^{-1}) biomass</th>
<th>Decadienal nmol g(^{-1}) biomass</th>
<th>Decatrienal nmol g(^{-1}) biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFU_7, 95, 96, 97</td>
<td><em>Ulva rigida</em></td>
<td>Ria Formosa (Portugal)</td>
<td>2.8 ± 0.5</td>
<td>1.9 ± 1.0</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>RFU_93</td>
<td><em>Ulva rotunda</em></td>
<td>Ria Formosa (Portugal)</td>
<td>0.3</td>
<td>0.3</td>
<td>*</td>
</tr>
<tr>
<td>RFU_11</td>
<td><em>Ulva ohnai</em></td>
<td>Ria Formosa (Portugal)</td>
<td>0.6</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>H_211</td>
<td><em>Ulva sp.</em></td>
<td>Helgoland (Germany)</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>*</td>
</tr>
<tr>
<td>C_210</td>
<td><em>Ulva</em> sp.</td>
<td>Puerto Montt (Chile)</td>
<td>1.6</td>
<td>1.6</td>
<td>*</td>
</tr>
<tr>
<td>RFU 200, 201</td>
<td><em>Ulva compressa</em></td>
<td>Ria Formosa (Portugal)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UM_2 (type specimen)</td>
<td><em>Ulva mutabilis</em></td>
<td>Ria Formosa (Portugal)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Biological replicates were not available in certain cases. * The release of 2,4,7-decatrienal was minor and highly variable.

Figure 7: Plasticity of PUAs production upon wounding: the extracted molecular ion trace (m/z = 276) GC-MS chromatograms of several *Ulva* extracts are plotted. Peaks represent derivatives with PFBHA of 2,4-heptadienal, 2,4,7-decatrienal and 2,4-decadienal.
3.1.3. Profiling of PUFAs and their role as precursors for PUA production

PUA production is considered to be catalyzed by lipoxygenases. They generally use unsaturated fatty acids as substrates. Therefore, the total fatty acids were initially profiled to identify candidate precursors. The total fatty acid profile of Ulva sp. was typically rich in ω-3- and C18:n fatty acids and rare in C20:n fatty acids (Fig. 8). This is in agreement with recent studies (Pereira et al., 2012; Ivanova et al., 2013; Khotimchenko, 2002) and holds true for both PUA-producers (e.g., RFU_77, and U. rigida) and non-producers (e.g., UM_2, and U. mutabilis) with about 1-4 % in sum of arachidonic acid (ARA, C20:4 n-6) and eicosapentaenoic (EPA, C20:5 n-3). In particular, the C18-PUFAs linoleic acid (LA, C18:2 n-6), α-linolenic acid (αLEA, C18:3 n-3), stearidonic acid (SDA, C18:4 n-3) and minor amounts of γ-linolenic acid (γLEA, C18:3 n-6) were present in Ulva sp. (30-40 % in sum) besides the monounsaturated oleic acid (C18:1 n-9) and the palmitoleic acid (C16:1 n-7) (Fig. 8). At this stage, it was not clear, which PUFAs are utilized predominantly for the production of C10-aldehydes by the isolated algae, but it was obvious that e.g., ARA and EPA were completely depleted within 30 min upon wounding (Fig 8B). Such depletion may indicate the formation of PUAs and other oxylipins derived from these fatty acids. For further investigation ARA, LA or γLEA were applied to the wounded Ulva thalli to monitor the accelerated production of 2,4-decadienal by solid phase micro-extraction (SPME) (Fig. 9). We observed that the carbon chain cleavage to produce the C10-aldehydes requires an extra double bond in β-position to the presumed hydroperoxide intermediates as described by Labeque and Marnett (1988). Whereas the external application of ARA or γLEA facilitated 3 and 12 times higher production of 2,4-decadienal, LA (a major fatty acid in Ulva) did not affect the production of 2,4-decadienal in isolate H_211 (Fig. 9). A similar pattern was observed for the production of the minor amounts of 2,4,7-decatrienal: EPA and SDA increased the production of 2,4,7-decatrienal by 3 and 2 times in isolate RFU_77, but not αLEA (Fig. 10).

In certain occasions, neither ARA nor EPA was detect in Ulva species, as also observed in previous studies (Khotimchenko, 2002; Pereira et al., 2012) which might explain the lack of PUA production. The additional application of external ARA or EPA did not initiate the PUA-production in U. mutabilis or other “non-producers”. These results reveal that the specific lipoxygenase/lyase mediated pathways are either not activated/translated or even not present
in non-PUA producing *Ulva* species. The species dependent variability of PUA production reflects a high plasticity within this genus of *Ulva* (Tab. 3) and is also known in diatoms (Wichard et al., 2005a).

In addition to the potential adverse effects of PUAs, PUFAs are discussed to cause detrimental effects on surrounding marine living organisms (Alamsjah et al., 2008). LOX pathways might influence these activities either by decreasing the effect through depletion of PUFAs and/or by catalyzing the formation of various oxylipins (e.g., HpETE, PUAs, etc.), which harbor potential adverse effects on different phyla in their habitat (Adolph et al., 2004; Jüttner, 2001). Owing to the high content of PUFAs in the phototrophic organisms, macroalgae are also currently evaluated whether they can be used for biomass production and sustainable sources of e.g., biofuel or bio-oil (Dibenedetto, 2012). However, significant amounts of fatty acids might be transformed into breakdown products upon cell disruption as also shown in diatoms (Wichard et al., 2007). A direct one-pot conversion of lipids into methylated fatty acids might be an interesting approach to overcome these limitations (Dibenedetto, 2012).

![Figure 8](image)

**Figure 8:** Representative total ion current GC-MS chromatogram of the total fatty acid, as methyl ester, profile of *U. rigida* isolate RFU_77 (A) and depletion of ARA and EPA within 30 min after cell wounding (B). The insert (B) shows the ion extracted chromatogram m/z 79 of samples prepared for total fatty acid analysis from tissue before (black line) and after wounding (grey line).
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Figure 9: Precursor analyses of the 2,4-decadienal formation that were conducted by application of equal amounts of arachidonic acid (ARA, long dash black line), linoleic acid (LA, short dash gray line) or \(\gamma\)-linolenic acid (\(\gamma\)LEA, dotted gray line), and compared with the intrinsic PUA-production of \(Ulva\) sp. (H_211, solid black line). 2-decanon was used as internal standard (IS). Peaks are normalized by biomass and the intensity of the internal standard.

Figure 10: Increased production of 2,4,7-decatrienal after application of C18:4 n-3 (SDA) or C20:5 n-3 (EPA). The minor production of 2,4,7-decatrienal can be enhanced by application of eicosapentaenoic acid (EPA, long dash black line) or stearidonic acid (SDA, dotted gray line), whereas the application of \(\alpha\)-linolenic acid (\(\alpha\)LEA, short dash gray line) did not significantly increase the amount of PUAs compared with the untreated sample (RFU_77, solid black line). 2-decanon was used as internal standard (IS). Peaks are normalized by biomass and the intensity of the internal standard.

3.1.4. Elucidation of the eicosanoid biosynthetic pathway using stable isotope labeling and inhibitor experiments

For elucidation of the biosynthetic pathway, the labeling patterns of metabolites were determined by mass spectrometry after application of stable isotope labeled PUFAs to the strongest PUA-producers, RFU_77 and H_211. Deuterated PUFAs, [\(^2\text{H}_8\)]-ARA or [\(^2\text{H}_4\)]-LA, were
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incubated with a crude extract of *Ulva* sp. (isolate H_211) and subsequently sonicated. It turns out that [²H₈]-ARA, but not [²H₄]-LA, was highly efficiently transformed into deuterated [²H₄]-2,4-decadienal (> 50 % labeled of total amount of decadienal, Figs. 9, 11A). These results are in agreement with the study of Akakabe et al. (2003), who demonstrated that *U. conglobata* synthesizes regio-specific 11R-hydroperoxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HpETE), which is further transformed to 2,4-decadienal. Moreover, [²H₅]-EPA (C20:5 n-3) was transformed in the present study efficiently into several deuterated hydroperoxyeicosapentaenoic acids (HpEPE) ([M-H]⁻ = 338, [M-H₂O-H]⁻ = 320) indicated by a Δ5 shift compared to the unlabeled hydroperoxy fatty acids ([M-H]⁻ = 333, [M-H₂O-H]⁻ = 315) including the 11-hydroperoxy-5Z,8Z,12E,14Z,17Z-eicosapentaenoic acid (11-HpEPE), which was subsequently utilized to form an isomeric mixture of deuterated [²H₅]-2,4,7-decatrienal (> 50 % labeled of total amount of decatrienal, Figs. 11B, 12). This is even more interesting, as EPA was often the more prominent C₂₀-PUFA compared to ARA (Fig. 8). Judging from the position of double bonds, EPA might be also the precursor for 2,4-heptadienal as reported in diatoms (d'Ippolito et al., 2005). The biosynthesis of 2,4-heptadienal and its precursor in *Ulva* remains unclear, as deuterated 2,4-heptadienal derived from EPA could not be detected and application of SDA did not trigger the production either (data not shown).

For further characterization of the biosynthetic PUA-pathways and identification of the second breakdown product (e.g., a short chain hydroxy fatty acid) of 11-HpETE and 11-HpEPE besides decadienal and decatrienal, the LOX-inhibitor 5,8,11,14-eicosatetraynoic acid ETYA was used in pilot experiments for targeting potential short chain fatty acid by UHPLC-MS analyses. Hereby, external ARA was added to enhance these signals due to the low amount of intrinsic C₂₀-PUFAs in *Ulva* (Fig. 13). The inhibitor stopped the enzymatic production of the intermediate 11-HpETE and consequently its subsequent breakdown products in *Ulva* sp. (H_211). Comparison of the chromatographic data from these UHPLC-MS investigations of *Ulva* sp. (H_211) extracts treated with and without ETYA pointed out tentative signals of the second breakdown product (Fig. 13). In particular, the potential breakdown product with m/z = 183 [M-H]⁻ at the retention time 1.65 min was identified. It is hence tempting to assume that the hydroxylated fatty acid, 10-hydroxy-5,8-decadienoic acid (Fig. 13), known to be produced by a LOX/hydroperoxide lyase of the
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diatom *Thalassiosira rotula*, is also synthesized by *Ulva*. Addition of $[^3\text{H}_8]$-ARA and subsequent parallel analysis by UHPLC-MS and GC-MS after solid phase micro extraction revealed the concomitantly production of the potential $[^3\text{H}_4]$-10-hydroxy-5,8-decadienoic acid (Figs. 13 A,B) and the release of $[^3\text{H}_4]$-2,4-decadienal (Fig. 11A). These results were also supported by comparative analyses of the other PUA-producers (i.e., RFU_77, C_210) showing the same pattern (data not shown).
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Figure 11: Mass spectra of 2,4-decadienal (A) and 2,4,7-decatrienal (B) and their deuterated forms after application of [2H8]-ARA in (A) and [2H5]-EPA in (B).

Figure 12: Formation of the hydroperoxy fatty acids of C20:5 n-3 (EPA). Extracted ion trace chromatograms of UHPLC-MS analyses of aqueous extracts monitoring the pseudomolecular ions [M-H]-1 of the hydroperoxid fatty acids of EPA (A) and its deuterated form (B) after application of [2H5]-EPA (100 µg ml⁻¹). Identification of oxygenated products of EPA, including the 11-HpEPE (1) from a crude extract of Ulva sp. (H_211) was performed by comparison an untreated sample with a [2H5]-EPA treated sample. The ∆5 shift can be recognized at the same given retention time (2). Complete regioseparation of the several HpEPEs was not accomplished by this approach.
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Figure 13: Elucidation of the biosynthesis with deuterated arachidonic acid ([\(^{2}\text{H}_8\)]-ARA). Extracted ion trace chromatograms of UHPLC-MS analyses of aqueous extracts of Ulva monitoring the pseudomolecular ions [M-H]\(^{-}\) of 10-hydroxy-5Z,8Z decadienoic acid derived from arachidonic acid: Wounded Ulva sp. (H_211) thalli were spiked with (A) \([^{2}\text{H}_8]\)-ARA, with (B) unlabeled ARA or with (C) the LOX inhibitor ETYA and ARA. The mass spectra of the identified substances 1 and 2 are shown. 10-hydroxy-5,8-decadienoic acid was identified by comparison with a partly purified extract from the diatom T. rotula that contains the hydroxy-fatty acid (Barofsky and Pohnert, 2007).

3.1.5. Lipoxygenase/hydroperoxide lyase mediated pathways in Ulva

Besides the eicosanoid fatty acids, the more prominent C\(_{18}\)-PUFAs including \(\gamma\)LEA can be efficiently used by Ulva for the production of 2,4-decadienal (Fig. 9) mediated via a 9-LOX transformation and 9-hydroperoxy-6,10,12-octadecatrienoic acid (9-HpOTrE), although only traces of \(\gamma\)LEA were observed in Ulva species. Upon addition of external \(\gamma\)LEA, the second concomitantly produced oxylipins, 6-hydroxy-7-octenoic acid (previously identified in Ulva by Abou-Elwafa et al., 2009) and 8-hydroxy-6Z-octenoic acid, were identified by co-injection with synthetic standards or by comparison of retention time and mass spectra, respectively (Figs. 14 A, B). The same 9-LOX might be also involved in the production of the minor amounts of 2,4,7-decatrienal and the hydroxy-fatty acids derived from SDA via the same putative octadecanoid pathway (Fig. 15). Indeed, Tsai and co-workers (2008) purified a lipoxygenase from Ulva fasciata and surveyed the hydroperoxy derivatives formed from several substrates for specificity tests. This algal lipoxygenase showed the highest activity towards ARA, EPA, SDA and LA (specific
activity: 1.71 - 3.97 µmol min\(^{-1}\) mg\(^{-1}\) protein), but a very low activity towards αLEA (specific activity: 0.15 µmol min\(^{-1}\) mg\(^{-1}\) protein). Even more interesting, the elution profile of the hydroperoxy derivatives formed from C\(_{18}\)-PUFAs showed a major peak from the activity of a 9-LOX and a minor peak from a 13-LOX. The higher 9-LOX specificity over 13-LOX of *Ulva* has been observed in various studies in green algae (e.g., Kuo et al., 1997; Kumari et al., 2014) For instance, the 9\(R\)-hydroperoxy-10\(E\),12\(Z\)-octadecadienoic acid (9-HpODE) were formed with a high enantiomeric excess (> 99%) from LA in *U. conglobata* (Akakabe et al. 2002). However, when arachidonic acid was used as substrate, the major hydroperoxy derivatives was catalyzed from a 11-LOX (Tsai et al., 2008). Therefore, the concomitantly biosynthesis of 2\(E\),4\(Z\)-decadienal and 10-hydroxy-5\(Z\),8\(Z\)-decadienoic acid is also suggested via the intermediate 11-HpETE derived from ARA mediated by the 11-LOX/lyase activity (Fig. 15 A). Previous chiral LC/MS analyses of the LOX product have revealed that (\(R\))-11-HpETE was in large enantiomeric excess (99 \% ee) and thus enzymatic processes are involved. Incubation of wounded *Ulva* thalli with 100 µg of synthetic 11-HpETE proved further that the hydroperoxide is the substrate for the 2,4-decadienal synthesis (Akakabe et al., 2003). This biosynthetic pathway is comparable with the 11-LOX mediated transformations of PUFAs in the diatom *T. rotula* (Barofsky and Pohnert, 2007), but different to the moss *Physcomitrella patens*, which is a model organism e.g., for the evolutionary consideration of aspect compared to green macroalgae to land plants. Whereas *T. rotula* releases 2\(E\),4\(Z\)-decadienal and 10-hydroxy-5\(Z\),8\(Z\)-decadienoic acid as well, a multifunctional enzyme in *P. patens* catalyses the oxygenation of ARA and the bond cleavage of 12-HpETE into 1-octen-3-ol and 12-oxo-dodeca-5\(Z\),8\(Z\),10\(E\)-trienoic acid (Senger et al., 2005) (Fig. 15 C).

In summary, both the octadecanoid and the eicosanoid pathway can be used for the formation of oxylipins in *Ulva* spp. as known e.g., in *P. patens* or in various red macroalgae (Bouarab et al, 2004; Wichard et al., 2005). Further detailed studies will explore the profile of all oxylipins including hydroperoxy-, hydroxyl-, keto- and epoxy-fatty acids in *Ulva* as elaborated for diatoms by Cutignano et al. (2011). Overall, the lipoxygenases/lyase pathways seem to be very variable in marine algae. For instance, the marine diatom *Stephanopyxis turris* transforms eicosapentanoic acid via 12-hydroperoxyeicosatetraenoic acid (12-HpETE) into halogenated
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compounds, such as 3-chloro-1,5Z-octadiene and 1-chloro-2Z,5Z-octadiene, and 12-oxododeca-5Z,8Z,10E-trienoic acid catalyzed by a hydroperoxide-halolyase (Wichard and Pohnert, 2006).

Up to now, few studies have investigated the activity of (partly) purified LOXs in Ulva (Tsai et al., 2008) and the enzymes are still poorly characterized. Therefore, the unambiguous identification of the second breakdown products along with further studies are needed to clarify the mechanism of bond cleavage in Ulva via homolytic (as found e.g., in higher plant) or heterolytic routes (e.g., in diatoms) (Noordermeer et al., 2001; Barofsky and Pohnert, 2007). For instance, 18O-labeled water can be used to explore the underlying mechanisms. Based on our data, U. rigida (RFU_77) is suggested to be the strong PUA-producer, which is now under standardized cultivation in our laboratory, for advanced analyses of the biosynthetic pathways.

Figure 14: Extracted ion trace chromatograms of UHPLC-MS analyses of aqueous extracts of Ulva (A) monitoring the pseudomolecular ions m/z 157 [M-H]-1 of 6-hydroxy-7-octenoic acid (1) and 8-hydroxy-6Z-octenoic acid (2). Wounded U. rigida (RFU_77) thalli were spiked with γLEA. Identity of 1 was proven by co-injection of the Ulva extract with the respective synthetic standard (B). Mass spectra of 1 and 2 are shown.
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Figure 15: Transformation of (A): C\textsubscript{20}-PUFAs (arachidonic and eicosapentaenoic acid) and (B): C\textsubscript{18}-PUFAs (\(^\gamma\)-linolenic acid and stearidonic acid) by wounded \textit{Ulva}. Proposed LOX/lyase mediated biosynthetic pathways in \textit{Ulva} sp. and \textit{Ulva rigida} (H\_211, RFU\_77) compared with the elucidated pathways in the moss \textit{Physcomitrella patens} (Senger et al., 2005) (C).
3.1.6. Conclusions

The potential production of polyunsaturated aldehydes (PUAs) was surveyed in wounded Ulva species collected in the lagoon Ria Formosa (Portugal). These green marine macroalgae show a high plasticity in PUA production. Whereas the majority of collected species did not release any PUA, mainly those characterized by the sea lettuce-like morphotype released elevated amounts of decadienal, decatrienal and heptadienal in the range from 0.6–5.1 nmol g\(^{-1}\) (fresh weight). Interestingly, the strongest producers (Ulva rigida and Ulva ohnoi) are known for forming green tides. Released PUAs by Ulva might affect the population dynamics of the phytoplankton, grazers as well as the microbial community during e.g., green tide events. In addition, the pilot experiments were conducted in order to elucidate the biosynthetic pathways of 2,4-decadienal and 2,4,7-decatrienal. Labeling experiments have demonstrated that a 11-lipoxygenases/lyase mediated pathway transformed deuterated arachidonic acid and eicosapentaenoic acid into 10-hydroxy-5Z,8Z-decadienoic and 2,4-decadienal or 2,4,7-decatrienal, respectively. Additionally, C\(_{18}\)-PUFAs are transformed to the putative 6-hydroxy-7-octenoic acid and 8-hydroxy-6Z-octenoic acid as well as the C10-aldehydes via the 9-lipoxygenase/lyase pathway. In general, as the lipoxygenases/lyase mediated depletion of polyunsaturated fatty acids might jeopardize the commercial value of the algal biomass in land based aquacultures, it is suggested to consider this additional criterion for selecting the appropriate Ulva species. Here, Ulva mutabilis might be selected as feedstock to be cultivated under controlled condition to increase the concentration of a given product (e.g., PUFAs) in biomass production.
3.2. Metabolomics requirements

3.2.1. Acquisition of biological metadata

It is essential to collect extensively metadata such as fundamental biological parameters along with metabolomic analyses in order to interpret the omics-data and guarantee comparability between explorative studies. Data driven research such as omics benefits from high qualitative biological data, as *a posteriori* hypotheses can be also deduced from subsequent multivariate data analyses of the chemical data. In addition, biological data gives information on the context when the sample was collected. They enable the analyst to describe e.g. specific growth phases in experiments along with physiological cell and life status of the organisms. Therefore, following biological metadata were monitored: (1) the growth of *U. mutabilis* and its associated bacteria, (2) the depletion of the macronutrients (nitrate, phosphate) in growth media, (3) the status inducibility of algal gametogenesis, (4) the changes in algal life cycle and (5) the microbiome/axenicity of the cultures. All abiotic parameters (light/dark cycle, illumination, salinity, and temperature) were controlled and kept constant throughout the entire experiment (*cf.* chapter 4).

3.2.2. Cultivation and sampling

Sterile UCM was inoculated with axenic gametes of *U. mutabilis* slender (gametophyte mt [+] , sl) (*cf.* chapter 4) and subsequently with the essential bacteria for algal morphogenesis. Cultures of *Roseobacter* sp. and *Cytophaga* sp. were rinsed three times by sterile UCM before inoculation in order to avoid any cross contamination through the ingredients of the bacterial growth medium (marine broth). This effort and precision in keeping the system sterile is in order to prove that the exo-metabolites will result only from the living organisms constituting the tripartite community.

Two treatments were prepared:

**The tripartite community culture** which is comprised of axenic *U. mutabilis* germlings, *Roseobacter* sp. and *Cytophaga* sp. Within this chemosphere, *U. mutabilis* develops a complete thallus with blade, stem and primary rhizoid cells (Fig. 16a).
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**(b) Axenic culture**, which contains only axenic *U. mutabilis* forming a callus-like morphotype without any cell differentiation besides the bubble-like structures around the cell wall, which are most significant (Fig. 16b).

![Image](image_url)

**Figure 16:** Monitoring of the algal growth of *U. mutabilis* after two weeks in two treatments (a) upon inoculation with *Roseobacter* sp. and *Cytophaga* sp. forming the normal thallus morphotype in the tripartite community, and (b) under axenic conditions *U. mutabilis* formed callus-like colonies with the cell wall showing “bubble-like” structures (arrows).

3.2.3. **Culture settings for metabolomic profiling**

The first metabolomic profiling was conducted with small scale batch cultures (1L) for a preliminary survey in order to establish an analytical procedure including cultivation, sampling, measurements and subsequently data processing. Therefore, *U. mutabilis* growth was observed with different associated isolates (i.e., *Halomonassp*, *Sulfitobacter* sp. *Dinoroseobacter* sp., *Roseobacter* sp. and *Cytophaga* sp.) along with the combination between *Roseobacter* sp. and *Cytophaga* sp. to ensure that similar biological observations which were documented previously by Spoerner et al. (2012) for *U. mutabilis* (Fig. 16a) can be achieved. Indeed, previous results could be confirmed regarding the various algal phenotypes and growth induced by the various bacterial partners. As these data and the metabolic profiling of small scale cultures were very promising and in agreement with the result of Spoerner et al. (2012), work-intensive bioreactor cultures (25L) in the culture chambers in Jena (IAAC, FSU Jena) and aquacultures (200L) at the marine station CCMAR (Center of Marine Sciences, Ramalhete, Faro, Portugal) were performed.
3.3. *Metadata collection towards generating the a posteriori hypothesis for metabolomics*

Bioreactor cultures conducted in Jena laboratory using 25L and 10L of *Ulva* culture medium in polycarbonate bottle.
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Based on the small-scale experiments of batch cultures, the experiment was scaled up to 25L bioreactors consisting of two treatments: axenic culture and the tripartite community. In order to describe the tripartite community in its entirety, it was undertaken:

1. to collect samples for metadata analyses,
2. to observe the changes of exo-metabolites in the UCM over time (in triplicates),
3. and to carry out subsequently chemometric analyses.

In addition to many factors such as nutrients, pH, and light, the healthy growth of *U. mutabilis* in the tripartite community is attributed mainly to the growth and morphological factors released by *Cytophaga* sp. and *Roseobacter* sp. (cf. Introduction section 1.4). Thus, the growth of both, alga and bacteria, had to be monitored at each sampling point.

3.3.1. Monitoring of *U. mutabilis* growth

In the tripartite community, *U. mutabilis* grew healthy, and showed a normal morphology (Figs. 16a, 17a). Estimation of the growth by length showed that the maximum average of length that could be measured over 56 days was 25 cm. The first apparent growth was recorded one week after inoculation (i.e., from day-7 till day-14) (Fig. 18). After that, a slight increase in longitudinal growth was observed until the end of the sampling points i.e., day-56. In comparison, *U. mutabilis* did not grow in axenic cultures (Fig. 18), and showed the typical undifferentiated cells with malformed cell walls forming dark green callus-like colonies (Figs. 16b, 17b, 18).

Based on the weight, the relative growth rate (RGR) of *U. mutabilis* increased with an average of 3% FW day\(^{-1}\) between day-14 and day-35, and 11% FW day\(^{-1}\) from day-42 till day-49.

\[
RGR \ (\% \ day^{-1}) = \frac{100 \cdot \ln \left( \frac{W_2}{W_1} \right)}{t_2 - t_1} \quad (1)
\]

\(W_1, W_2 = \text{fresh weight (g) at time point 1, and 2, respectively. } t_1 \text{ and } t_2 = \text{time in days. The relative growth rate (RGR) is given in } \% \text{ per day (Lüning, 1990; Olischlager et al., 2013).}

RGR of *U. mutabilis* between day-42 till day-49 (11% FW day\(^{-1}\)) is relatively close to the RGR of *U. lactuca* (11.4% FW day\(^{-1}\)), and *U. clathrata* (13.7% FW day\(^{-1}\)) (Kerrison et al., 2012).
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Figure 17: Illustration the procedure to estimate *U. mutabilis* growth by measuring (a) thallus length in the tripartite community, and (b) the diameter of callus-like colonies (arrows) in axenic culture.

Figure 18: Estimation of *U. mutabilis* growth as a function of time in bioreactor cultures by measuring thallus length in the tripartite community (black dots line), and the diameter of callus-like colonies in axenic cultures (grey dots line). Note the different scales on the left and right sides. Mean values $\pm$ SD ($n = 3$).
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The longitudinal growth rate and the biomass density of *U. mutabilis* increased simultaneously. This contradicts the relation between the growth rate and the biomass density of *U. fasciata* measured in outdoor continuous cultures (Lapointe and Tenore, 1981), in which the growth rate decreased with increasing the biomass density due to self-shading that reduced the light level and consequently photosynthesis. Nevertheless, under the conditions of the current study, the later phenomenon was avoided by the aeration creating homogenous distribution of the light throughout the entire biomass in the bioreactors. It is noteworthy, that the length was used as a proxy for growth instead of thallus area due to the morphology of *U. mutabilis* (sl) thallus, which is described as tubular cylindrical thallus (Fig. 17a). Indeed, Løvlie (1964) demonstrated that no significant change was observed in thallus area of *U. mutabilis* (sl) over time, which is, however not the case with the wild-type of *U. mutabilis* (Spoerner et al., 2012) or e.g., *U. lactuca* (Ale et al., 2011) because of the blade-like morphotype resulting in an areal growth of such *Ulva* species. Furthermore, there might be an interesting link between growth rate, nutrients concentration and the status of gametogenesis inducibility in *Ulva*. Therefore, the activity of the key regulators of the gametogenesis inducibility i.e., sporulation and swarming inhibitors in UCM, was further investigated as shown in section (3.3.4). The healthy growth and normal morphological development of *U. mutabilis* in the tripartite community exhibited color changes in thalli over time. For instance, the light green thalli were dominant from day zero to 14. By day 14, the cultures were mixed with light green and olive-green thalli. From day 28 and onwards, the brownish green and colorless thalli appeared and some light green thalli were observed again from day-49 till day-56. Thus besides the algal growth, the algal culture and bacterial community in the tripartite community were monitored as well.

3.3.2. Monitoring of algal cultures and bacterial community growth

To assess how *U. mutabilis* interacts with its associated bacteria over time in the tripartite community, the bacterial growth was monitored routinely using *in situ* polymerase chain reaction (PCR). In addition, PCR was used to confirm the axenicity in both treatments i.e., axenic culture and the tripartite community. The DNA was extracted from the cultural supernatant and subsequently quantified to estimate total bacterial growth. Even more important, a polymerase
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chain reaction (PCR) analysis was conducted to follow the bacterial growth of *Roseobacter* sp. and *Cytophaga* sp. Moreover, denaturing gradient gel electrophoresis (DGGE) was performed in order to identify any potential bacterial contamination, if any existed, in bioreactor culture.

**Proof of axcenity by Nanodrop DNA Quantification**

Total DNA quantification was performed using a Nanodrop method (Fig. 19a) in order to get a preliminary overview of the growth medium of axenic cultures and determine if any organism existed in prior to downstream analyses such as qPCR and/or DGGE. Upon filtration, DNA was extracted from the bacteria on the filter.

Nanodrop quantification revealed the absence of DNA in axenic treatments (Fig. 19a). In the tripartite community, however, total DNA amount increased over time as a result of the growth of *Roseobacter* sp. and *Cytophaga* sp. in UCM. In combination with the gametogenesis inducibility in *U. mutabilis* in the tripartite community (cf. section 3.3.5), the total DNA concentration in the growth medium could be divided into three patterns: The first pattern represents the total DNA from day-zero till 14, where the typical rapid bacterial growth was observed one week after the inoculation approaching the highest concentration on day-14 (Fig. 19a). Obviously, the only extractable DNA from the growth medium during this culturing time resulted from *Roseobacter* sp. and *Cytophaga* sp. The second pattern started after day-14, when the DNA decreased to reach a plateau between day-21 and 35 and increased slightly on day-42 (Fig. 19a). The third pattern was recorded after day-42, when DNA concentration dropped dramatically reaching the lowest concentration on day-49. It is noteworthy to mention that in the second pattern, the total DNA concentration represents in addition of bacteria, the new gametes released spontaneously to the UCM. Those gametes, which were still moving in the medium and did not settle, were collected as well and contributed to the amount of total DNA (cf. section 3.3.5).

Therefore, this method only provides reliable values for early stages of the tripartite community, when *Ulva* was immature and did not yet undergo spontaneous gametogenesis.
Proof of axcenity throughout the experiment by in situ PCR

To prove the axenicity of the cultures, a specific DNA fragment (520 bp) of the bacterial 16S rDNA was amplified by in situ PCR. For this purpose, established primers were used, which cover a broad range of various marine bacteria (Muyzer et al., 1995; Sneed and Pohnert, 2011a), so that DNA of unknown bacteria (= contaminants) would be amplified as well. The absence of the amplicons in case of the axenic cultures proved, in fact, the axenicity throughout the whole cultivation period (Fig. 19b, i.e., lane 12-14). Accordingly, DNA amplification of UCM collected from the tripartite community showed the expected strong signals (Fig. 19b; lanes: 1-9), and indicated that the inoculation had worked properly on day zero. In addition, PCR proved that the experimental control containing only the culture medium without inoculation with U. mutabilis nor with bacteria was completely free of contaminants until the second sampling point (day-14) (Fig. 19b; lanes: 11)), after that, the experimental control was not valid any more as proven by PCR (data are not shown). The total DNA concentration in axenic culture media (Fig. 19a) is fully compatible with in situ PCR results as both confirmed the axcenity of axenic cultures. In summary, in situ PCR proved that the sampling campaign can be considered for further in depth molecular, chemical and biological analysis, as the axcenity was confirmed in the axenic culture in addition to the sterility in the tripartite community.

The in situ PCR has been used as a quality control of the sterility-status of the tripartite community, and hence it was always performed before further experiments such as metabolic analyses and subsequent chemometric data analyses were conducted. Nevertheless, the extracted DNA from the chloroplast of the gametes might function as a template for the 16S rDNA amplification as well, and cause false positive results (Lachnit et al., 2010). Thus, the stability of the bacterial community and the absence of contaminants had to be confirmed by denaturing gradient gel electrophoresis analyses (DGGE).
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Figure 19: Monitoring of the bacterial community and growth. DNA was extracted from filters (0.2 µm pores size) after filtration of the growth media. (a) Nanodrop quantification of DNA in axenic culture (opened circle line), and in the tripartite community (black circle line). Error bars represent mean values ± SD (n = 3). (b) In situ PCR amplification using 16S rDNA bacterial primers, the size of the amplicone band is about 520 bp. First lane shows the GeneRuler Express DNA ladder. The tripartite community cultures; on day-zero (numbered lanes: 1-3), on day-28 (lane: 4-6), and on day-49 (lanes: 7-9). PCR control: PCR mixture without bacteria (Lane: 10). Experimental control (culture medium without U. mutabilis nor bacteria) on day-14 (Lane: 11). Axenic cultures; on day-zero (lane: 12), on day-28 (lane: 13), and on day-49 (lane: 14). (c) DGGE analysis of the bacterial community on the day of inoculation “day-zero” (Lanes: 1-3), on day-28 (Lane: 7), and on day-49 (Lanes: 4-6).
Survey of the bacterial community by DGGE

In situ PCR worked as a test for axcenity of the treatments, but the work intensive DGGE was performed to prove that the right bacterial community was growing in the tripartite community free from the contaminants. Thus, the pelagic bacterial profile was monitored to check the presence and the absence of Cytophaga sp. and Roseobacter sp. Therefore, the PCR products of three representative phases of algal growth (day-zero, day-28, and day-56) were applied to DGGE-analysis and revealed that only Roseobacter sp. and Cytophaga sp. were identified in the tripartite community over sampling points (Fig. 19c, lane: 1-7). However, from the bands abundance of Cytophaga sp. it was shown how that the Cytophaga abundance ranged from high on day-zero (lanes: 1-3), and on day-28 (lane: 7), and finally become slightly lower on day-49 (lanes: 4-6) in contrast to the constant abundance of Roseobacter sp. over sampling time points (Fig. 19c, lane: 1-7).

The faint bands of the DGGE gel (lanes: 4-6) on day-49 might indicate that the population of Cytophaga sp. started disappearing from the culture medium of the tripartite community. This assumption, however, can strongly be supported based on the assumption of Fuchs et al. (2000), (Zubkov et al., 2001). For instance, Fuchs and his co-workers interpreted the slow growth of Cytophaga-Flavobacteria as a sign for k-strategy, in which the slow long-term survival in nutrient limited resources is the selective advantage instead of the fast growth. On the other words, the decrease in the relative abundance of Cytophaga sp., observed in the tripartite community, did not necessarily mean they stopped growing, but rather they did grow slower. Conversely, Zubkov et al. (2001) explained that the fast growth of α-proteobacter including Roseobacter sp. is a result of r-strategy, because the species are able to exploit immediately the extra nutrient when available. Furthermore, Zubkov et al. (2001) found out that the growth rate of Cytophaga-Flavobacteria (< 0.1 day⁻¹) was the lowest among the tested isolates although it dominates the marine phytoplankton. Accordingly, the efficient utilization of the dissolved organic compounds gives α-proteobacter an advantage to compete with other bacterioplankton (Zubkov et al., 2001). For this reason, the slight faint bands at the end of the culturing time (Fig. 19c, i.e., lane: 4-6) could be considered as a result of the slow growing of Cytophaga sp. as its growth depends strictly on U. mutabilis growing in media as phototrophic
organism (i.e. without any carbon source) (Spoerner et al., 2012). It is likely that nutrients originally provided by Ulva might be limiting. To confirm these first evidences, quantitative PCR might be the method of choice, by which it would be possible to target and quantify a specific part of 16S rDNA of the Cytophaga strain.

Finally, monitoring of the growth of all the members in the tripartite community over cultivation time (49 days) revealed in different growth patterns. For example; while the growth of U. mutabilis increased, Roseobacter sp. seemed to be at the stationary phase and Cytophaga sp. at the declining phase. In further studies the monitoring of the bacterial growth should be proven by other quantitative techniques e.g., qPCR. Anyway, unlike e.g., Halomonas sp. it is known that Roseobacter sp. and Cytophaga sp. do not overgrow the algal cultures (Spoerner et al., 2012), indicating an algal allelopathic control on bacterial growth. Interestingly, the well-known allelopathic compound e.g., 2,4-decadienal (Ribalet et al., 2008) was documented to be potentially released by certain Ulva species e.g., U. conglobata (Akakabe et al., 2003), U. rigida (RFU_7) and U. ohnoi (RFU_11) (cf. chapter 3.1). However, this specific allelopathic effect cannot account for this inhibition, as U. mutabilis was identified as a “non-PUA producer” (cf. chapter 3.1).

As the nutrients limitation certainly affects the growth of the model organisms, nutrients analyses of the growth media were conducted to estimate its affect on the dynamic of the relationship between the model system organisms.

3.3.3. Nutrient depletion in the growth media

Macronutrients in general are well known to have a considerable influence on the growth as well as the development of Ulva spp. (Fried et al., 2003). Ulva grows and colonizes rapidly under eutrophic conditions and when conditions are favorable.

The nutrients analysis shows that the initial concentration of nitrate (85 mg/ L), and phosphate (6.69 mg/ L) on day-zero (day of inoculation) were titrated to the recommended concentrations by Stratmann et al. (1996). In both cultures, the concentrations of nitrate and phosphate were determined before inoculating U. mutabilis germlings (Figs. 20a,b). The first limiting nutrient in the tripartite community cultures seems to be nitrate (Fig. 20a) as the significant decrease was recorded immediately one week later after inoculation ($P < 0.05$). Nitrate continued on to be
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depleted dramatically after 21 days ($P< 0.05$). From day-28 on, nitrate was not detectable anymore (Fig. 20a). However, the phosphate concentration decreased gradually, but no complete consumption was recorded over cultivation time in the tripartite community. Although the concentration dropped significantly ($P < 0.05$) to almost half of the initial concentration (about 3.5 mg/ L), it remained at this level without significant change ($P > 0.05$) till the end of the experiment (Fig. 20a).

By calculating the weekly utilization rates (UR) for nutrients, the result indicates that in the tripartite community, nitrate was consumed earlier and faster than phosphate with 33 % UR of nitrate after one week, while only 5 % of phosphate was utilized by the end of the second week.

$$\text{Utilization Rate (100\%)} = \frac{C_{i} - C_{a}}{C_{i}} \times 100$$  \hspace{1cm} (2)

Where $C_{a}$ is the concentration of the nutrient after specific week, and $C_{i}$ is the initial concentration of the nutrient in the culture medium. The weekly utilization rate (UR) is given in % (Matsuyama et al., 2003)

The complete depletion of nitrate was ascertained in the fourth week after inoculation (i.e. UR = 100 %), whereas the maximum utilization of phosphate approached 85 % on day-56. Nonetheless, the most significant change in phosphate consumption (UR = 45 %) was recorded on day-28. It is notable that no significant difference was noticed from the blank value regarding nitrite. Interestingly, the only significant depletion of the nutrients recorded in axenic cultures was for nitrate ($P < 0.05$) one week after inoculation when the initial concentration i.e., 85 mg/ L decreased to 53 mg/ L (Fig. 20b) with an utilization rate of 37 %. Surprisingly, nitrate decreased significantly in both axenic culture, and the tripartite community with almost the same utilization rate of 33% and 37% respectively during the first week after inoculation.

Despite the fact that the carbon content was not estimated in bioreactor cultures, it is well known that the carbon content can be estimated for a culture in equilibrium according to the Redfield ratio C:N:P (106:16:1) (Redfield, 1958). According to this ratio, the carbon content in the growth medium of the tripartite community and axenic culture could be roughly estimated (106 times the P concentration). For instance, it is supposed that carbon content in the tripartite community ranged from 708 to 116 mg/ L over 49 days.
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Figure 20: Weekly changes of phosphate and nitrate concentration in UCM of (a) the tripartite community, (b) axenic culture. Error bars are based on mean ± SD (n = 3). After the experiment has concluded; post-hoc analysis Tukey’s HSD (honestly significant difference) test were performed ($P < 0.05$). Different letters above the bars indicate significant differences of the concentration throughout the sampling time points, capital letters for nitrate, and small letters for phosphate.

Previous experiments of Stratmann et al. (1996) along with the result of batch cultures conducted in this study have demonstrated that *U. mutabilis* can go through a whole life cycle from germling to mature thallus undergoing gametogenesis without changing the medium. That means the essential nutrients were not limiting in order to fulfill a whole life cycle under the laboratory conditions. Indeed, the initial concentrations of nitrate and phosphate in all used UCM regardless of the treatment were 1 mM and 0.05 mM, respectively (Figs. 20a,b), which was considerably higher in comparison to other studies, e.g. Lebaron et al. (2001), who investigated the influence of inorganic nutrient on microbial community in natural seawater with initial concentrations of 5.1 µM nitrate, and 0.6 µM phosphate. Ahmad et al. (2011) started with 50 µM of nitrate, and 10 µM of phosphate to study how *U. reticulate* growth responds to inorganic nutrient. Furthermore, Park et al. (2013) demonstrated that deep seawater is a nutrient-rich medium to culture macroalgae e.g., *U. pertusa* in aquacultures and recorded that the natural concentrations range between 340 - 425 µg/L for nitrate, and 79 - 90 µg/L for phosphate, which is still about 200 times less concentrated than in the UCM used in the bioreactors. The considerable difference between the initial concentration of inorganic dissolved nutrients in the UCM and the previous studies is due to (1) the shorter cultivation time compared to the tripartite community, which lasted 49 days, (2) the continuous change and/or supply of the growth medium throughout the culturing, which was avoided in the
current study in order to determine the accumulation of exo-metabolites in the UCM during algal growth, and (3) macroalgal cultures are usually inoculated with mature thalli, whereas the tripartite community was inoculated with young axenic germlings (= 7 days old) as stock seeds.

The utilization rate for nutrient indicates that nitrate is the limiting factor for *U. mutabilis* growth in case all other growth and morphological factors, which *U. mutabilis* needs, are available. Moreover, nitrate was documented to be the limiting factor as well for *U. reticulate* (Buapet et al., 2008; Lyngby and Mortensen, 1994), *U. lactuca* (Lyngby and Mortensen, 1994; Teichberg et al., 2008), and *U. intestinalis* (Martínez et al., 2012). Matsuyama et al. (2003) measured the utilization rates for nitrate and phosphate by *Ulva* sp. over 10 days, and found out that the utilization rate of nitrate (more than 94%) was higher than that of phosphate (ranged between 43 and 90%). Unfortunately, Matsuyama et al. (2003) did not consider the other growth and morphological factors due to the usage of the deep seawater as growth medium. Despite the fact that the deep seawater is a nutrient-rich medium, it is however not a suitable habitat for floating *Ulva* and its symbiotic microbiome. Therefore, it is also unlikely that morphogenetic and the growth factors for *Ulva* development can be found in deep sea waters. According to the hypothesis proposed by Fong et al. (2004), the lack of macroalgal growth, in particular *Enteromorpha* sp. and of *Ulva* sp., is due to the high concentration of nutrient in environment. It is supposed that algae under the nutrient-enriched condition delay their growth to favor uptake and storage of nutrients, and therefore no energy will be left over for carbon fixation and growth. However, under the condition of low nutrients concentration, algae will take up nutrients providing enough energy for carbon fixation and growth. With regard to these assumptions of Fong et al. (2004), nutrient availability cannot always be a remarkable indicator for algal growth. Indeed, this applied to *U. mutabilis* growth in the tripartite community, in which *U. mutabilis* continued on to grow (Fig. 18) even after the complete depletion of nitrate on day-28 (Fig. 20a). Although nitrate was initially obviously available in excess, it turned out that algal growth was not delayed, and the continuous growth was observed during the entire cultivation period. The significant decrease of nitrate in both treatments one week after the inoculation indicates that the consumption of nitrate in both treatments was due to *U. mutabilis* independently on the microbial environment. In fact, this
very early stage of germlings development is dedicated to settle and to build up the tripartite community rather than cell division and cell differentiation (Spoerner et al., 2012).

Moreover, the consumption of nitrate and phosphate in the tripartite community can be used as an indicator for changes in life cycle of *Ulva*. For example, nitrogen depletion in growth medium of *U. fasciata* has been correlated with enhancement of gametogenesis, whereas high nitrogen availability was used for vegetative growth and asexual reproduction (Mohsen et al., 1974; Agrawal, 2012). Besides nutrients effect, changes in life cycles are strongly regulated by sporulation inhibitors excreted by the blade cells (Stratmann et al., 1996). Therefore, the gametogenesis inducibility in *U. mutabilis* during the time series sampling was tested by measuring the concentration of sporulation and swarming inhibitors (SI, SWI) in the UCM, and by observing the inducibility of the gametogenesis induction.

### 3.3.4. Regulation of life cycle: regulation of gametogenesis by sporulation and swarming inhibitors

The ability of gametophytes to induce gametogenesis was tested by measuring the biological activity of sporulation inhibitors (SI-1a and 1b = SIs) and swarming inhibitor (SWI) in the growth medium (Fig. 21) following the bioassays of Stratmann et al., (1996) and Wichard and Oertel, (2010).

SIs were not detected in the growth media of any treatments i.e., axenic and the tripartite community. In contrast, the SWI was found in the growth medium of the tripartite community on day-21 with an activity of 1.7 units/ mL. Afterword, the activity increased significantly ($P < 0.05$), and peaked on day-28 and 35 with 3.3 and 4 units/ mL, respectively (Fig. 21). The last two peak values did not differ significantly ($P > 0.05$). After 35 days, the activity dropped dramatically to 2 units/ mL ($P < 0.05$), and remained constant without significant change till the end of this experiment ($P > 0.05$) (Fig. 21). As expected, no SWI activity could be detected in axenic cultures.
Results and Discussion

Figure 21: Weekly changes of sporulation (SI-1) and swarming (SWI) inhibitors in UCM of the tripartite community over time. Error bars (if any difference existed) based on mean values ± SD (n = 3). Different letters above the bars indicate significant differences of SWI activity throughout the sampling time points (Tukey-posthoc test, P < 0.05).

In the tripartite community, the SWI activity indicates that certain individuals went through the onset of gametogenesis spontaneously 18 days after inoculation as the SWI-activity was recorded on day-21 for the first time, meaning the SWI was released at the earliest three days before, when the gametogenesis was induced (Stratmann et al., 1996; Wichard and Oertel, 2010). In axenic cultures, the proteins SIs and the low molecular weight compound SWI were not detected overall in the medium. While the SWI can only be produced upon induction of gametogenesis, it is known that SIs are also produced and released to the UCM by axenic cultures (Stratmann et al., 1996). However, using 25L bioreactors along with the small increase of axenic biomass, the concentration of the SI-1 was very likely below the sensitivity of the biological assay and hence biologically irrelevant in the UCM. Moreover, the activity of the SI-1 in the tripartite community was not detected either in the medium due to the potential digestion of SI-1 by the bacteria and its dynamical turnover (Stratmann et al., 1996; Wichard and Oertel, 2010).

The algal vegetative cells can transform to gametangia directly at any time in reaction to environmental stimuli. This reportedly can be achieved through a number of treatments including changes in photoperiods, pH, salinity, nutrients, drying, fragmentation and growth
medium changes (Mohsen et al., 1974; Hiraoka and Enomoto, 1998; Wichard and Oertel, 2010). In particular, Nordby (1977) demonstrated the effect of many environmental factors which were thought to affect the induction of gametogenesis in *U. mutabilis*, i.e., light intensity, day length, temperature, pH, osmolarity, enrichment of the seawater with nitrate, phosphate, and iron-EDTA. Nonetheless, the optimal induction was accomplished within 48 hours after the artificial fragmentation of the three weeks old growing *U. mutabilis* thalli resulting in single-layered thallus fragments (Nordby, 1977). This result was the key tool in speculating the existence of chemical inhibitory substances produced by *U. mutabilis* and some released into the growth medium.

The vegetative state of *U. mutabilis* is maintained by the excretion of the regulatory factors, glycoprotein “sporulation inhibitors”, either into the cell wall or into the environment (SI-1a, SI-1b). The production of both glycoprotein factors gradually ceases during thallus maturation (Stratmann et al., 1996). When the synthesis of SI-1 breaks off and the threshold concentration i.e., below $10^{-14}$ M, is reached, gametogenesis is induced. At the same time, a second low molecular weight sporulation inhibitor (SI-2), located in the inner space of the two blade cell layers, has to be inactivated. Damaged thalli release SI-2 and hence gametogenesis can often be observed in damaged algal tissues. After onset of gametogenesis, when the sporulation inhibitors are not present anymore, an additional factor, the “swarming inhibitor” (SWI), is produced. SWI synchronizes the final gamete release and increases the mating probability. However, this metabolite might additionally function as synchronization factor by acting as a cell-cycle regulator (Wichard and Oertel, 2010), and might trigger the spontaneous gametogenesis of *Ulva* specimen in the vicinity.

The potential induction of gametogenesis by artificial removal of all SIs (SI-1 and SI-2) through washing and chopping the thallus was tested in order to follow the inducibility of gametogenesis in *U. mutabilis*.

### 3.3.5. Change of life cycle of *U. mutabilis*: induction of gametogenesis

The induction of gametogenesis of *U. mutabilis* thalli was tested only in the tripartite community following established protocols by (Stratmann et al., 1996; Wichard and Oertel, 2010). Weekly and upon artificial removal of the sporulation inhibitors, the chopped and rinsed
thalli were cultured in fresh UCM for three days. If the specimen were mature, gametogenesis was induced and gametes were formed. On day-14 and day-21, the gametogenesis of certain gametophytes was induced artificially (Fig. 22b,c). This maturity already indicated that the algae can undergo gametogenesis spontaneously. The spontaneous gametogenesis was observed on day-28 by direct and microscopic visualization (Fig. 22d). Discharged gametes could be observed on day-35 (Fig. 22e,f). By day-42, about 10-15% of the whole population in each bioreactor underwent the spontaneous gametogenesis induction and the gametes were discharged from gametangia. On day-49, new small germlings resulting from the spontaneous gametogenesis could be observed under the microscope (Fig. 22g). Based on the measurements of the regulating factors along with the observations of cell differentiation three statuses were defined describing the gametogenesis inducibility in *U. mutabilis* in the tripartite community.

“**Non-inducible status**”: Gametogenesis/sporulation is not inducible.

“**Inducible status**”: Gametogenesis/sporulation can be induced.

“**Spontaneously inducible status**”: Gametogenesis/sporulation starts spontaneously.

(The bold phrase are used throughout the whole thesis)

The change in gametogenesis inducibility can be regulated on different levels either by the concentration of the inhibitors or by their perception through the algae of those inhibitors. The activity of these inhibitors is hence at least regulated in four fashions:

1. Sporulation inhibitor (SI-1) might be decomposed by the action of bacteria. Stratmann et al. (1996) for example found certain active putative breakdown products of the SI-1 in UCM. Indeed, this led to the assumption that symbiotic bacteria are able to consume the glycoprotein inhibitors released to the medium.

2. Algal cells become no longer or less susceptible to SI-1 and/or SI-2 (Stratmann et al., 1996).


4. The older the algae the less SI-1 are released into the medium (Stratmann et al. 1996).
Results and Discussion

**Figure 22:** Microscopy pictures show the transformation of gametophytes into gametangia followed by gametes release through the life cycle of *U. mutabilis* (sl) over the sampling time in the tripartite community. Cultures started with (a) 7 days old germings on day-zero. **Circle:** an accumulation of Roseobacter sp. around the base of the thallus attached to the substratum. (b) Thallus fragments on day-14, when the artificial gametogenesis induction started. (c) Thallus fragments represent the artificial induction on day-21. (d) Thallus margin on day-28, when the spontaneous gametogenesis and gametes release started. (e,f) Thallus margin represents the spontaneous discharge of gametes from gametophyte (e) into the medium (f) on day-35. (g) Thallus margin after discharging the gametes on day-42. (h,i) New germings resulting from the spontaneous induction on day-49, and day-56, respectively.
Results and Discussion

The early “Non-inducible status” of *U. mutabilis* (sl) (Fig. 22a) lasted one week after inoculation when none of SIs nor SWI was detected in UCM (Fig. 21). The second “Inducible status” started on day-14 and lasted one week further (= till day-21), when *U. mutabilis* (sl) became fertile and ready for artificial gametogenesis induction through rinsing and chopping (Fig. 22b,c). Subsequently, the third “Spontaneously inducible status” started one week later on day-28 and the gametangia formation was observed, when the color of gametophytes changed step-wisely from light green to olive-green and finally to brownish green due to the accumulation of $\gamma$-carotene (Lee, 2008). Subsequently, gametangia were gradually discharged and the gametes were released (Fig. 22d-i) although the SWI activity was determined simultaneously approaching the highest concentration on day-28 and day-35. This might be explained by the activity of the SI-1, which was not detected in the medium due to its potential digestion by the bacteria. Thus, the inhibition of gametogenesis might be just controlled by the SI-1 linked to the cell wall and even more important by the SI-2 located in the interior of the algal bilayer. In those cases, specimens were not susceptible to the interior SI-2 anymore and consequently the gametogenesis was not inhibited by the SIs, so that gametogenesis was induced spontaneously and the SWI was released subsequently. This also means that the algal population partly lost its synchrony, which might be due to the high biomass density in the bioreactor cultures. Gametangia become insensitive to the SWI six hours after sensing the SWI (Wichard and Oertel 2010), gametes, as a result, were observed in UCM with high concentrations of the SWI on day-35 (Figs. 21, 22e,f). It is important to highlight that the sporulation inhibitor (SI-1) was extracted from the UCM of axenic cultures as reported by Stratmann et al. (1996). The highest concentration was measured when thalli were at the age of 10 days, and started to decline gradually after one week till a threshold concentration of $10^{-14}$ M was reached (Stratmann et al 1996). Although the SI-2 has a key function in maintenance the vegetative state of the alga, the present study is focusing on exo-metabolites, therefore, neither compounds in the algal tissue, nor their potential biological activity were measured. Nonetheless, the concentration of SI-2 was probably constant throughout the life cycle according to previous studies by (Stratmann et al., 1996). Stratmann and his co-workers
showed that the SI-2 was already detectable in young germlings up to the age when gametogenesis happened spontaneously.

Overall, the time regime of the developmental and generative cycles, which was observed in this study, was in agreement with previous results of several laboratories (Føyn, 1959; Løvlie and Braten, 1968; Fjeld, 1972; Wichard and Oertel, 2010). They have reported that gametophytes of *U. mutabilis* (sl) grow in a vegetative way for three weeks before they become fertile. Stratmann et al. (1996) investigated the generative cycle of *U. mutabilis* (sl), and reported that at an age between 18 and 24 days, *U. mutabilis* becomes fertile and after 4 weeks the formation of gametangia started spontaneously. Besides the short generation time, the simple structure consisting almost entirely of blade cells, suggest that *U. mutabilis* (sl) is a suitable candidate as model system in macroalgae (Stratmann et al., 1996; Spoerner et al., 2012). One the other hand, the quick change in life cycle jeopardizes the maintenance of algal aquacultures. Therefore, the understanding of the production and release of SIs along with unknown factors will be essential to predict these changes in life cycle. Further unknown factors which can be used as biomarkers and predictors of changes in the media linked to life cycle were hence investigated in the subsequent sections.

3.3.6. **Conclusion: A comprehensive view of the biological metadata profile of the tripartite community**

The success of explorative “omics” approaches relies on the comparison with other laboratories for crossvalidation. As very different biological systems, culture conditions and even field-near setups are under investigation in chemical ecology; this study aims to provide a variety of biological information to ensure the reproducibility of the experiments. In order to improve the reliability of omics data sets, as many parameters of the biological system as possible were recorded. In particular, the following parameters were collected to describe the various stages of *Ulva*-cultures in bioreactors (i.e., relative growth rate, longitudinal growth, nutrient uptake, and the inducibility of gametogenesis in *U. mutabilis* as well as monitoring of the bacteria community) under controlled conditions. *Ulva* specific data and standard parameters of macronutrients including SWI and SI concentrations are summarized below (Tab. 5).
Results and Discussion

It is notable to mention that the utilization rate (UR) and the relative growth rate (RGR) in this table were recalculated based on the statuses of gametogenesis inducibility.

Table 5: Summary of all biological data collected over the growth phases corresponding to the gametogenesis inducibility in *U. mutabilis* in the tripartite community in bioreactors under standardized laboratory conditions.

<table>
<thead>
<tr>
<th>Status of gametogenesis inducibility</th>
<th>Growth phases</th>
<th>Sampling time points*</th>
<th>RGR (% FW) **</th>
<th>UR for NO₃⁻ (%)***</th>
<th>UR for PO₄³⁻ (%)***</th>
<th>Thallus length (cm)</th>
<th>Bacterial community ****</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Non-inducible”</td>
<td>First: lag phase</td>
<td>0-7 n.d.</td>
<td>34</td>
<td>0</td>
<td>≈ 3</td>
<td>(Ro): strong</td>
<td>(Cy): strong</td>
</tr>
<tr>
<td>“Inducible”</td>
<td>Second: early exponential phase</td>
<td>14-21 8.5</td>
<td>67</td>
<td>10</td>
<td>≈ 9-12</td>
<td>(Ro): strong</td>
<td>(Cy): strong</td>
</tr>
<tr>
<td>“Spontaneous Induction” of gametogenesis</td>
<td>Third: late exponential and stationary phases</td>
<td>28-56 14.5</td>
<td>100</td>
<td>84</td>
<td>≈ 16-25</td>
<td>(Ro): strong</td>
<td>(Cy): faint</td>
</tr>
</tbody>
</table>

*The culture was inoculated on day-zero with 7 days old germlings. **RGR: relative growth rate based on weight. n.d. not determined. ***UR Utilization rate was calculated using equation (2), where Cₐ represents the concentration which was available at the end of each time of inducibility status. ****Ro: Roseobacter sp., Cy: Cytophaga sp.

To get an overall view of the metabolite profiling of *U. mutabilis* with its surrounding environment under the laboratory conditions used in the present study, all biotic and abiotic analyses were plotted comprehensively as a function of time (Fig. 23). Based on the results about the life cycle of *U. mutabilis*, the growth was divided into three phases. Each growth phase is characterized by its distinctive metadata.

Briefly, *U. mutabilis* started its life cycle with unicellular gametes to become multicellular germlings, representing the First Growth Phase, which lasted two weeks, when neither spontaneous nor induced sporulation (= gametogenesis) was possible. The average of thallus length was recorded within this phase was about 3 cm. Germlings consumed about 33% of available nitrate in UCM, in contrast to phosphate, which was not utilized (Tab. 5). *Cytophaga* and *Roseobacter* species were present in this growth phase but did not play an essential role in the life cycle of *U. mutabilis*. Afterwards, *U. mutabilis* started the Second Growth Phase, when the RGR was about 8.5% FW and the thallii length reached 12 cm in average (Tab. 5). The presence and the absence of associated bacteria during this phase had significant impact on the

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² The definition of the statuses of inducibility is found in page 66.
growth, development, and morphology of *U. mutabilis* thalli as could be observed by comparisons with axenic cultures. Gametogenesis could be induced through thalli-fragmentation, washing, and transferring in fresh UCM (Fig. 23). The utilization rate (UR) for nitrate in this phase was quite high (67%) due to the growth and the capability of macronutrient-storage by *Ulva*. Only about 10% of phosphate was utilized during this phase. The first detection of SWI in the UCM was recorded at the end of this growth phase on day 21, assuming that the SWI was released three days before (Fig. 23). Finally, the Third Growth Phase of *U. mutabilis* started on day 28 and lasted three weeks. Here, the RGR was about 14.5% FW and the length ranged between 16 and 25 cm. Nitrate was depleted completely and 83% of phosphate was consumed (Tab. 5). The bacterial community, in particular *Cytophaga* sp. seemed to be affected by e.g., nutrient depletion or unknown effects. Thus, *Cytophaga* sp. slowed down its growth in order to adapt to such nutrient-limited environment. The rate of spontaneous gametogenesis increased progressively during this last phase. Furthermore, gametes were released into the medium on day 35, although the concentration of SWI was significantly higher, which indicates that some gametangia were no longer sensitive to SWI. Ultimately, as a result an *a posteriori* hypothesis was generated from these data and will be tested by metabolomic approach.
Results and Discussion

Figure 23: Diagram summarizing all biotic and abiotic analyses, which have been performed form the onset of the algal culture (day-zero) till the mature specimen of *U. mutabilis* ready for spontaneous gametogenesis and culture termination. **N. G.**: new generation started spontaneously within the third growth phase 3. Mean values ± SD (n = 3).

**The *a posteriori* hypothesis**

"The chemosphere of the tripartite community changes throughout the growth phases of the macroalgae and biomarker of this exometabolome can be used to predict changes in the status of gametogenesis inducibility during the life cycle”

In order to investigate the dynamics of waterborne metabolites in the chemosphere of the tripartite community corresponding to the status of gametogenesis inducibility, non-targeted analyses using liquid and gas chromatography coupled with mass spectrometry were conducted, followed by chemometric analysis.
3.4. *Metabolite profiling in the chemosphere of the tripartite community in laboratory bioreactors*
Results and Discussion

Kim et al. (2011) pointed out an important strategy for metabolomic analysis. A true metabolomic analysis should systematically identify the effect of various known biological factors on the metabolome, and should give a platform from which it would be possible to study various biological questions. This serves as an observation-based systems biology approach in which (1) all kinds of observations are made (i.e. metadata), and (2) multivariate data analysis methods are used to find any correlations between these observations. This strategy was applied in the present study.

The chemometric analyses play a key role to describe, and simplify the complexity of metabolomic profiling. Unconstrained ordination chemometric analyses (e.g., principle component analysis “PCA”) are extremely useful for visualizing broad patterns across the entire data cloud whereas constrained ordination analyses (e.g., canonical discriminant analysis of principal coordinates “CAP”) enable deep insight into the correlation between the variables and collected metadata (Seger and Sturm, 2007) and figure out the differences among the treatments (Anderson and Willis, 2003). Both chemometric analyses, PCA and CAP analyses, were used in the present study.

Metabolic profiles were explored following the stepwise processes (Fig. 24). In order to cover a broad range of metabolites, two mass spectrometry techniques were used:

1. Liquid Chromatography–Mass Spectrometry analysis (LC-MS)
2. Gas Chromatography–Mass Spectrometry analysis (GC-MS)
3.4.1. Experimental design of metabolomic analyses

The experimental regime was carried out based on Vidoudez and Pohnert (2008) and Barofsky et al. (2009), with the following adaptations due to the specific requirements of macroalgal cultures:

1. The sampling of growth media was performed weekly instead of daily sampling in the present study.
2. A constant volume of 1000 mL from cultures medium was collected weekly.
3. The mass of *U. mutabilis* was collected under sterile condition in contrast to diatoms cells, which can be sampled directly during the sampling of growth media.
4. The metabolic experiments in the present study were carried out for seven weeks instead of three weeks as it was performed with diatoms due to the long lifecycle of *Ulva* compared to diatoms lifecycles.

Three biological replicates per treatment were performed in parallel with two experimental controls (UCM without any inoculation), which were treated with the same protocol as the cultures. The eluate that resulting from solid phase extraction (SPE) using Easy® cartridge was analyzed with liquid chromatography (LC) directly, and with gas chromatography (GC) after derivatization (Fig. 24). Both chromatography techniques were coupled to mass spectrometry (MS). The non-targeted analysis by LC-MS will be addressed first, followed by GC-MS.

3.4.2. Metabolite profiling in the chemosphere of the tripartite community using LC-MS analysis

LC-MS analysis produces large amounts of data with complex chemical information. The SPE samples were directly injected in the UPLC-MS using positive ion mode. The important task was to arrange data, so that relevant information can be extracted. The complexity of LC-MS raw data brings out the concept of data handling (Fig. 24), which can be roughly summarized up in three basic steps: (1) data preprocessing, (2) pretreatment, and (3) data analysis. Data preprocessing covers the methods to go from complex raw data to clean data. Raw data is comprised of mass to charge ratio and retention time (m/z, RT pair) of thousands of chemical compounds. Two software tools (commercial or freely available) were used for preprocessing of LC-MS data in this study: MarkerLynx™, and Progenesis CoMet.
3.4.3. **Determination of the range in chromatograms for metabolites collection**

Progenesis CoMet resulted in ion intensity maps, which are comparable to 2D-gel maps (Fig. 25), by which issues in sample batches were detected ([http://blog.nonlinear.com/2011/02/14/ion-intensity-maps/](http://blog.nonlinear.com/2011/02/14/ion-intensity-maps/)). For example, by examining the pattern of ion intensities in the ion map, it was possible to identify issues in the chromatography that can adversely affect the analysis, e.g., the instability in the ion spray can be seen in the ion maps, which is represented as gaps in the retention time axis of individual ions signals. Moreover, ion map provides a considerable visualization in order to collect the data from the darker areas representing a high abundance of ions in the MS signal. Based on the criteria of ion map, metabolites were collected by MarkerLynx™ in the range $m/z$ 100 - 800 amu and distributed between 0.5 – 7.0 min. 2276 metabolites resulting from the process (Fig. 25) were considered in further chemometric analyses.

![Figure 25: Ion gel-like map provided by Progenesis CoMet of the tripartite community on day-21. Determination of the retention time and the $m/z$ values for metabolites collection. The bolder the area, the higher the abundance of the MS signals. Red dots area is the selected area, black dots area is the rejected area.](image)

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3.4.4. Effect of unconstrained analysis on biomarkers obtained by LC-MS analysis

Data pretreatment methods play a role in correcting aspects that hinder the biological interpretation of metabolomics datasets by emphasizing the biological information in the dataset and thus improving their biological interpretability (van den Berg et al., 2006). Many steps are included under the said pretreatment and vary as well regarding the chemometric analyses. Pretreatment (i.e., centering, autoscaling, pareto scaling, range scaling, vast scaling, log transformation, and power transformation) depends on the data analysis method chosen since different data analysis methods focus on different aspects of the data. For instance, the unconstrained ordination analyses, including PCA, are methods of data reduction to explain as much variation as possible with as few components as possible. The constrained ordination analyses e.g., CAP finds the (dis)similarities between the samples in order to discriminate between the groups, or to determine the relation between sample and the environmental data.

In this study, PCA was conducted involving specific pretreatment after collecting 2276 metabolites (pairs of m/z and retention time) by MarkerLynx™ (Fig. 26).

The PCA scores plot (Fig. 26) indicates that 45% of the variability could be explained by the first two dimensions: component 1 accounted for 24% of the variance, while component 2 explained 21% of the variation. The scores plot resulting from the LC-MS data depicted two distinct clusters without any overlap between groups. **Group 1:** represents growth medium of control (on day-zero, and day-7), axenic and the tripartite community before inoculation and **Group 2:** represents axenic and the tripartite community from day-7 till the end (Fig. 26). The two groups were separated via component 1, and the cluster appeared to be tighter in the group 1 as compared to the group 2. In addition, the PCA analysis indicates also that no compounds were released on day-0 due to the different handling and inoculation. However, slight differences between the data within group 2 can be noticed via component 2. Thus, the differences among the data in group 2 might be covered by the unconstrained analysis.
Results and Discussion

Figure 26: PCA scores plot of samples based on metabolites (n = 2276) obtained by LC-MS analysis (positive mode) and detected in growth media of experimental control (○), axenic culture (□), and the chemosphere of the tripartite community (△) throughout the sampling time points on day: -zero (black), -7 (dark green), -14 (light blue), -21 (red), -28 (dark blue), -35 (magenta), -42 (light green), and -49 (maroon) under specific pretreatment (Pareto scale, intensities are expressed as height, and normalized to the sum of the peak intensities (TIC) in each chromatogram). Ellipses are added graphically for illustration of the two groups G1 and G2.

Clarke and Ainsworth (1993) pronounced the data analysis philosophy “let’s the data speak for themselves” through PCA clusters and encourage an exploratory attitude toward data. These quick data analyses sorted out that group 1 contains only the ingredients of medium and was not affected by the inoculation which has happened just before the sample collection. Indeed, although Roseobacter sp. and Cytophaga sp. were present since day-zero in the tripartite community which had been proven by in situ PCR (Fig. 19b) and DGGE (Fig. 19c), the PCA plot shows that these organisms did not excrete significant or detectable metabolites till
Results and Discussion

this time of sampling point or that the concentration of the released compounds was below the limit of detection (LOD) (Fig. 26). However, it is well known that morphogenetic compounds are already released during the first week upon inoculation. Those molecules, however, are highly biological active (Matsuo et al., 2005; Spoerner et al., 2012) and might be below the LOD.

With PCA, no correlation could be predicted between the samples because the Euclidean distance is the only ecological distance which can be investigated, which, in turn, depends greatly on the abundances (concentrations) of the metabolites regardless of the biomarkers that are shared. Thus, Euclidean distance is not a suitable ecological measure if it is used on raw datasets (Legendre and Legendre, 1998).

Therefore, to uncover these differences in group 2 as formed by the PCA (Fig. 26), a constrained analysis (CAP) was performed in addition. Here, besides the time factor, the influence of experimental treatments (i.e., axenic and the tripartite community) could be clearly observed by applying the suitable Bray-Curtis distance measurement as it was highly recommended by Anderson and Willis (2003) for discovering the differences between the groups even in large ecological datasets.

Owing to (1) the PCR results which proved that control samples were free of contaminants on day-zero and day-7 (Fig. 19b) and (2) the analysis of the PCA plot (Fig. 26), all biomarkers derived from the growth media of the experimental control (on day-zero, and day-7) along with the axenic culture and the tripartite community (on day-zero) were used as blank values and subtracted from all other datasets. This data preprocessing ensures that the resulted metabolites represent only the waterborne metabolites, which were excreted (and subsequently) metabolized by only the model organisms used in the present study (including U. mutabilis, Roseobacter sp., and Cytophaga sp.).

3.4.5. Effect of constrained analysis on metabolites obtained by LC-MS analysis

The constrained ordination techniques usually explain differences in species composition (biomarkers) between sites (groups) by differences in environmental variables e.g. time, treatments. Canonical analysis of principle coordinates (CAP) was applied with Bray-Curtis distance after standardization and transformation to overcome the calculation strategy used in
Bray-Curtis distance, where the final distance will be influenced more by metabolites of the largest differences in abundance (Legendre and Legendre, 1998).

Owing the metadata outcome (cf. section 3.3.6), the a priori groups were defined based on the time spent in each status of gametogenesis inducibility in U. mutabilis\(^3\). In addition, axenic culture over entire experimental time (= 49 days) was combined with early growth phase when the gametogenesis was not inducible (i.e. day-7) of the tripartite community. Thus, a priori groups were as follows:

**Group 1**: The tripartite community (day-7) combined with all sampling events of axenic cultures (day-7 till day-49).

**Group 2**: The tripartite community (sampling events from day-14 till day-21).

**Group 3**: The tripartite community (sampling events from day-28 till the day-49).

After preprocessing the data, which was collected by Markerlynx™ (= 2276 metabolites), only 1409 (62% of the total metabolites) metabolites were applied to CAP analysis, and 197 metabolites (9%) were chosen as biomarkers based on their significant contribution to CAP axes (Fig. 27b).

CAP plot (Fig. 27) resulted in low misclassification error of 5%, with squared canonical correlations of \(\delta^2_1 = 0.91\) and \(\delta^2_2 = 0.50\) \((P < 0.001, with 999 permutations)\) (Tab. 6). Thus, the classification was significant and the patterns would not mislead further interpretations. The score plot displays three groups (Fig. 27a), which were separated along both axes. The vector plot indicates the biomarkers, which contributed to this separation (Fig. 27b). CAP plot reveals that group 1 was separated from group 2 via axis 2 and from group 3 via axis 1 (Fig. 27a). Group 2 and group 3 were separated on both axes. Each group was characterized by particular biomarkers, which were represented by the vectors that contributed to CAP axes significantly (Fig. 27b). Certain biomarkers contributed equally in characterizing different groups. For example biomarkers #454, and #937 characterized group 2 and 3, and #766 characterized group 1 and 3 (Figs. 27, 28). It is notable that the characteristic biomarkers are more numerous than the common (shared) biomarkers (Fig. 27). A subset of selected biomarkers (52 biomarkers out of 197) was plotted in heatmap (Fig. 28) in order to visualize the abundance of these

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\(^3\) The definition of the statuses of inducibility is found in page 66.
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biomarkers in the growth present medium throughout the sampling time points. The selection of these 52 biomarkers was performed based on their significant contribution in characterizing the groups. The longer the vectors, the more efficient the biomarkers in characterizing the groups, e.g., biomarkers #504, #590 and #658 for group 1, biomarkers #675 and #1118 for group 2, and biomarkers #89, #249, #418 and #484 for group 3 (Figs. 27, 28).

It is important to highlight that the mechanisms of characterizing the \textit{a priori} groups that was used in this classification were:

1. The existence and the absence of a biomarker among the \textit{a priori} groups. For example, biomarker #165 characterizes group 1 the best, and it disappeared in group 2 and group 3.

2. The abundance of a certain biomarker varies significantly throughout the groups e.g., biomarker #618, which is characteristically found in high abundance in group 2, compared to its low abundance in groups 1 and 2 (Figs. 27, 28).

The combination of the CAP analysis and heatmap visualization resulted in an interesting explanation of the dynamic of the biomarkers obtained by LC-MS analysis among the \textit{a priori} groups and throughout time as well. In the first place, it became obvious which biomarkers contributed significantly to the CAP axes (Pearson correlation coefficient $|r| \geq 0.3$) (Fig. 27b), secondly in which treatment these biomarkers were detected (Fig. 28), and finally which \textit{a priori} group was characterized by these biomarkers (Fig. 27a). Taking all this information into account, one can categorize the “ups and downs” of biomarkers for a better understanding of the dynamics within the chemosphere (categories 1-11: Tab. 7). These eleven categories were produced based on: (1) the treatment where the biomarker was detected (i.e., axenic culture and the tripartite community), and (2) the trend of the abundance of the biomarker in the growth media (i.e., increasing, decreasing, or constant over time). These two criteria facilitated the prediction of the producer being either \textit{U. mutabilis} or the associated bacteria and consequently the processes that might take place in the growth media and how it could affect the trends of biomarkers (Tab. 7). Interestingly, these eleven categories can be generalized and applied to all characteristic as well as common biomarkers. Some of these biomarkers are displayed in (Tab. 7) as an example.
Results and Discussion

Figure 27: Groups correspond to the three key gametogenesis-states in *Ulva* during its growth. Separation was achieved by metabolites (n = 1409, 62% of the total metabolites) obtained by LC-MS analysis (positive mode) and found in growth media of axenic culture and the chemosphere of the tripartite community using Bray-Curtis dissimilarity distance. (a) CAP separation of the samples with the growth corresponding to the statuses of gametogenesis inducibility as groups. (b) Correlations of 197 biomarkers (9%) with the two CAP axes, with absolute Pearson coefficient correlation $|r| \geq 0.3$.

Table 6: Eigenvalues, canonical square correlation, and diagnostics statistics of the CAP analysis of the metabolites obtained by LC-MS and found in growth media of axenic culture and the chemosphere of the tripartite community throughout sampling time points.

<table>
<thead>
<tr>
<th>Constrained canonical axes</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st axis</td>
<td>2nd axis</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>($\delta^2$) Correlation</td>
</tr>
<tr>
<td>0.96</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Results and Discussion

Figure 28: The heatmap represents the abundance of the biological mean of selected biomarkers (n = 52) resulting from CAP over 49 days in axenic culture (Ax) and the chemosphere of the tripartite community (TC). The abundance of biomarkers is expressed by size and brightness of color. d: day, bio: biomarker. Red, light green and magenta arrows: characteristic biomarkers for a priori group 1, 2 and 3, respectively. Black arrows: common (shared) biomarker. Blue arrows: biomarkers representative for the mechanisms used to characterize the groups, e.g., bio #165 for presence/absence mechanism, and bio #618 for the abundance variation throughout groups mechanism.
# Results and Discussion

Table 7: The eleven categories of some selected biomarkers obtained by LC-MS analysis (positive mode) contributing significantly to CAP axes in figure 27. All biomarkers were displayed in the heat map. 52 biomarkers are shown below.

<table>
<thead>
<tr>
<th>Category</th>
<th>Detected in:</th>
<th>Trend of the abundance over time</th>
<th>#Biomarker ((m/z, RT)/\text{that characterizes the \textit{a priori group}})***</th>
<th>Produced by:</th>
<th>Predicted processes that might happen in the growth media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 1</td>
<td>Ax: ((\uparrow)) or ((\rightarrow))</td>
<td></td>
<td>#18 (130.17, 0.59)/(\uparrow), #19 (127.06, 0.63)/(\uparrow), #125 (186.12, 0.60)/(\uparrow), #165 (195.14, 5.97)/(\uparrow), #326 (230.26, 2.92)/(\uparrow), #386 (247.14, 0.63)/(\uparrow), #658 (305.19, 0.61)/(\uparrow), #1125 (447.35, 6.89)/(\uparrow), #1141 (452.38, 6.87)/(\uparrow), #1294 (535.41, 6.87)/(\uparrow), #1313 (545.42, 6.67)/(\uparrow), #1125 (447.35, 6.89)/(\uparrow), #1141 (452.38, 6.87)/(\uparrow), #1294 (535.41, 6.87)/(\uparrow), #1313 (545.42, 6.67)/(\uparrow).</td>
<td>U. mutabilis</td>
<td>(1) Inhibited by bacteria. (2) Degraded biotically. (3) Utilized by either organism in the tripartite community.</td>
</tr>
<tr>
<td>Category 2</td>
<td>Ax</td>
<td>((\downarrow))</td>
<td>#287 (217.14, 2.79)/(\downarrow), #391 (249.14, 0.61)/(\downarrow).</td>
<td>U. mutabilis</td>
<td>(1) Inhibited by bacteria. (2) Degraded (a)biotically. (3) Utilized by either organism in the tripartite community.</td>
</tr>
<tr>
<td>Category 3</td>
<td>TC</td>
<td>((\uparrow))</td>
<td>#89 (181.12, 3.37)/(\uparrow), #249 (209.19, 2.60)/(\uparrow), #379 (245.20, 3.23)/(\uparrow), #420 (257.18, 2.05)/(\uparrow), #508 (287.27, 3.09)/(\uparrow), #950 (382.96, 6.11)/(\uparrow), #962 (386.95, 6.11)/(\uparrow).</td>
<td>U. mutabilis or bacteria</td>
<td>Activated by either organism in the tripartite community.</td>
</tr>
<tr>
<td>Category 4</td>
<td>TC</td>
<td>((\downarrow))</td>
<td>#484 (271.19, 4.33)/(\downarrow), #500 (273.25, 3.70)/(\downarrow), #711 (317.24, 2.41)/(\downarrow), #766 (332.24, 5.11)/(\downarrow), #937 (378.1, 3.38)/(\downarrow.)</td>
<td>U. mutabilis or bacteria</td>
<td>(1) Inhibited by either organism in the tripartite community. (2) Degraded biotically. (3) Utilized by either organism in the tripartite community.</td>
</tr>
<tr>
<td>Category 5</td>
<td>TC</td>
<td>((\rightarrow))</td>
<td>#180 (197.16, 2.08)/(\rightarrow), #418 (257.12, 2.52)/(\rightarrow), #425 (258.12, 2.44)/(\rightarrow).</td>
<td>U. mutabilis or bacteria</td>
<td>Catabolic product.</td>
</tr>
<tr>
<td>Category 6</td>
<td>Ax + TC</td>
<td>Ax: ((\uparrow))</td>
<td>#452 (265.11, 0.62)/(\uparrow), #454 (265.16, 0.80)/(\uparrow.)</td>
<td>U. mutabilis</td>
<td>After 35 days the biomarkers</td>
</tr>
</tbody>
</table>
## Results and Discussion

<table>
<thead>
<tr>
<th>Category</th>
<th>Ax + TC</th>
<th>TC: (↑), then (↓) after specific days</th>
<th>is either:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 7</td>
<td>Ax + TC</td>
<td>(→)</td>
<td>(1) Degraded biotically. (2) Utilized by bacteria.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#1118 (443.38, 4.43) 2, #1036 (413.27, 4.35) 3</td>
<td>U. mutabilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>Category 8</td>
<td>Ax + TC</td>
<td>(↓)</td>
<td>U. mutabilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#182 (198.17, 1.63)/ 1, #310 (226.21, 2.98)/ 1, #377 (244.30, 3.02)/ 1, 2, 316 (256.29, 6.32)/ 1</td>
<td></td>
</tr>
<tr>
<td>Category 9</td>
<td>Ax + TC</td>
<td>Ax: (↓) TC: (↑) or (→)</td>
<td>U. mutabilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#70 (173.15, 5.78) 1, #446 (262.29, 2.24)/ 1, #621 (297.25, 3.92)/ 1, #750 (327.29, 4.43)/ 1</td>
<td>Catabolic product.</td>
</tr>
<tr>
<td>Category 10</td>
<td>Ax + TC</td>
<td>(1) Ax: (→) TC: (↓) (2) Ax+ TC: (→) but in general the abundance was less in TC</td>
<td>U. mutabilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#293 (218.24, 1.88)/ 1, #350 (239.17, 6.05)/ 1, #371 (243.12, 2.14)/ 1, #485 (271.22, 3.39)/ 1, 504 (274.30, 2.92)/ 1, #653 (303.26, 6.36)/ 1</td>
<td>(1) Inhibited by bacteria. (2) Degraded biotically. (3) Utilized by either organism in the tripartite community.</td>
</tr>
<tr>
<td>Category 11</td>
<td>Ax + TC</td>
<td>TC: (↓) Ax: (↑) or (→)</td>
<td>U. mutabilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>#383 (246.25, 2.45)/ 1, #539 (281.14, 0.60)/ 1, #590 (290.29, 2.97)/ 1, #618 (297.11, 0.62)/ 1, #675 (308.14, 0.61)/ 1, #944 (380.40, 5.99)/ 1</td>
<td>(1) Inhibited by bacteria (2) Degraded biotically. (3) Utilized by either organism in the tripartite community.</td>
</tr>
</tbody>
</table>

*Ax: Axenic culture, TC: The tripartite community. ** (↓): decreased, (↑): increased, (→): remained constant. *** Underlined numbers between parentheses indicate appurtenance to the same biomarker characterized two a priori groups.*
Result and Discussion

From table 7, some clear trends of characteristic biomarkers can be observed. For instance, most of the biomarkers, which characterize a priori group 1 (Fig. 27) were listed in categories 1, and 2 and some were distributed between categories 6, 8, 10 and 11. Only one biomarker was listed in category 9. Based on these categories, the common feature between all these biomarkers was the assumption that \textit{U. mutabilis} is the producer and more likely the role of the production and excretion of these biomarkers was e.g., to attract the associated bacteria in the tripartite community (Tab. 7). The biomarkers characterizing a priori group 2 were distributed between categories 9-11 (Tab. 7). In contrast to the biomarkers of groups 1 and 3, no category was specified for the biomarkers of group 2 (Tab. 7). On the other hand, the characteristic biomarkers of a priori group 3 were listed in categories 3-7, and some in categories 8 and 9. With regard to categories 3, 4, and 5, associated bacteria i.e. \textit{Roseobacter} sp. and \textit{Cytophaga} sp. were also predicted as producers in addition to \textit{U. mutabilis}. Based on these categories, the two mechanisms of characterizing the a priori groups mentioned above could be clearly noticed.

Axenic cultures over eight weeks and the tripartite community at first week of growth were not distinguishable based on LC-MS data, suggesting that the released biomarkers by \textit{U. mutabilis} on the first week of the growth were accumulated in axenic cultures due to the absence of the consumers such as bacteria. Most of these biomarkers belong mainly to categories 1 and 2, and seemed to be hydrophobic except some biomarkers (i.e., #165, #1125, #1141, #1294, and #1313). The biomarkers of categories 1 and 2 were detected only in axenic cultures, and therefore, it is supposed that these metabolites were released by \textit{U. mutabilis} and utilized by the associated bacteria or decomposed enzymatically. Thus, these metabolites were not detected in the tripartite community. Two weeks after inoculation, lipophilicity increased slightly, when biomarkers were detected in the chemosphere of the tripartite community, suggesting different classes of metabolites released into the chemosphere either by bacteria i.e., categories 3-4 or by \textit{U. mutabilis} i.e., categories 5-11.

In addition, the discriminant analysis (Fig. 27) has been proven the \textit{a posteriori} hypothesis generated from metadata (cf. section 3.3.6):

“Waterborne metabolites obtained by UHPLC-MS can be classified based on the growth phases corresponding to the statuses of gametogenesis inducibility of \textit{U. mutabilis} in the tripartite community”
Therefore, specific biomarkers obtained by LC-MS analysis might be utilized to predict the growth changes of *U. mutabilis* based on the chemosphere structure, providing a possibility to use the same biomarkers to predict the inducibility status as well.

### 3.4.6. Determination of the correlation between the biomarkers obtained by LC-MS analysis

CAP plots opened the possibility to predict: (1) the relation between the biomarkers and the *a priori* groups as discussed above (cf. section 3.4.4) with regard to the positive or negative correlation to CAP axes (Fig. 27), and (2) between the biomarkers themselves (Fig. 29). Consequently, the cosine value of canonical right angle ($\theta$) between arrows (= vectors) of each biomarkers pair (Fig. 29) provides an approximation of their pairwise correlation, e.g., arrows that point to the same direction indicate to positive correlated biomarkers, perpendicular arrows indicate to the lack of correlation, and arrows pointing to the opposite direction indicate to the negative correlated species. Those pairs might be valuable for a better understanding of the chemistry of the chemosphere.

$$r = \cos \theta$$

The biplot shows some representative biomarkers (24 out of 197) characterizing the *a priori* groups in order to clarify the canonical angle correlation between some biomarker pairs. These angles are used for specific dependencies of both variables (Figs. 27b, 29). Biomarker pairs which have a canonical angle of 180° were particularly investigated, because they indicate a reverse pairwise relationship between biomarkers. The more pronounced group of biomarkers with canonical angle less than 90° was not listed in (Tab. 8) although vast majority of biomarkers possess such correlation (Fig. 29). Notably, the canonical angel less than 90° would not give robust or extra information in large dataset because the trend of biomarkers pair will be in the same direction so that their abundance will increase or decrease at same time. In addition, the biomarker pairs of 90° canonical angle were not listed either (Tab. 8), since no correlation exists between them (Fig. 29).
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Figure 29: CAP-ordination diagram in covariance biplot scaling of figure 27 with selected biomarkers (n = 24) represented by vectors. The scale of axes 1 and 2 applies to groups; the scale of correlation with those axes applies to biomarkers.
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Table 8: The canonical angle relationship between selected biomarkers obtained by LC-MC analysis (positive mode). All biomarkers below were represented in figure 29. Essential biological metadata are given.

| Canonical angle (θ) between the vectors | Biomarker pairs | m/z | cos (θ) = r | Correlation between biomarkers pair | **U. mutabilis** | Utilization rate of NO$_3^-$ (%) | Utilization rate of PO$_4^{3-}$ (%) | **Bacteria profile**
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>180°</td>
<td>(668, 454)</td>
<td>(305.19, 265.16)</td>
<td>cos (180°) = -1</td>
<td>Negative correlation</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 2$^{nd}$/ 3$^{rd}$)</td>
<td>(34, 67-100)</td>
<td>(0, 10/84)</td>
</tr>
<tr>
<td></td>
<td>(675, 766)</td>
<td>(308.14, 332.24)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 3$^{rd}$)</td>
<td>(34, 100)</td>
<td>(0, 84)</td>
<td>Ro (S, S) Cy (S, F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(377, 249)</td>
<td>(244.30, 209.19)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 3$^{rd}$)</td>
<td>(34, 100)</td>
<td>(0, 84)</td>
<td>Ro (S, S) Cy (S, F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(504, 452)</td>
<td>(274.30, 265.11)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 3$^{rd}$)</td>
<td>(34, 100)</td>
<td>(0, 84)</td>
<td>Ro (S, S) Cy (S, F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(464, 937)</td>
<td>(267.16, 378.12)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 2$^{nd}$/ 3$^{rd}$)</td>
<td>(34, 67-100)</td>
<td>(0, 10/84)</td>
<td>Ro (S, S/S) Cy (S, S/F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(668, 454)</td>
<td>(305.19, 265.16)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 2$^{nd}$/ 3$^{rd}$)</td>
<td>(34, 67-100)</td>
<td>(0, 10/84)</td>
<td>Ro (S, S/S) Cy (S, S/F)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(416, 722)</td>
<td>(256.29, 319.23)</td>
<td>(non, induc.)</td>
<td>(1$^{st}$, 3$^{rd}$)</td>
<td>(34, 100)</td>
<td>(0, 84)</td>
<td>Ro (S, S) Cy (S, F)</td>
<td></td>
</tr>
</tbody>
</table>

*Non: non inducible, induc: inducible gametogenesis status. **Numbers separated by (/) indicate that a biomarker was found in both growth phases. *** Ro: Roseobacter sp., Cy: Cytophaga sp., S: strong, F: faint.*
Many ecological studies have discussed the pairwise correlation between the vectors using canonical angle in biplot (Campbell and Mahon, 1974; Watson, 1981; Terbraak, 1986; Terbraak, 1990). Despite some exceptions, it is clear that all groups, which are located in the opposite direction relative to the origin, i.e., group 1 and group 3, have biomarkers with obtuse canonical angle ($\theta$) equals or is close to 180°, e.g., biomarker pairs (#452, #504), (#249, #377), (#675, #766), (#454, #658). These biomarker pairs are negatively correlated, meaning:

1. The increase of either biomarker reflected the decrease of the other biomarker in the same a priori group.
2. The increase of one biomarker abundance in one group reflects the increase of the second biomarker abundance but in the counter group.

Considering that group 1 and 3 also represent the time taken for the “non-inducible status”\(^4\) and “spontaneously inducible status” (Tab. 8), it is supposed that the biomarkers which were released during the non-inducible status are negatively correlated with their derivatives that were detected during the “spontaneously inducible status”. These negatively correlated biomarkers might result from many processes which took place in the growth media such as:

1. metabolism by either organism in the tripartite community,
2. biotic degradation, e.g., enzyme mediated degradation,
3. abiotic degradation, e.g., photolysis, hydrolysis,
4. enzymatic autoxidation.

Moreover, the ($m/z$, retention time) pairs reduced the options of the prediction. Using 180° pairwise correlation, particular metabolites could be released by *U. mutabilis* during the time taken for “non-inducible status”, e.g., polysaccharides, monosaccharides and amino acids, and hence their catabolic products could be predicted during the “spontaneously inducible status”. However, given the method used in the current study, such primary metabolites were not covered by the UPLC-MS analysis, thus such molecules will not be considered.

The potential information which could be abstracted from table 8, regarding the $m/z$ of the biomarkers pair and the reproductive/growth statuses, is an estimate of the dynamic of the processes which might happen in the chemosphere. For instance, (#464, #937), and (#416, #722) are biomarker pairs, in which the first coordinate was always detected during the “non-

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\(^4\) The definition of the inducibility statuses is found in page 66.
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inducible status” with $m/z$ that was lower than the second coordinate which was detected during the time of “spontaneously inducible status”, suggesting that the second coordinate might be a catalyzed or decomposed product released at the beginning of growth. For details, the $m/z$ of the above mentioned biomarker pairs are respectively (267.16, 378.12), and (256.29, 319.23). By calculating the difference between the $m/z$ of each previous pair, the estimation was six oxygen atoms with methyl group between (267.16, 378.12), and three oxygen with methyl group between (256.29, 319.23). Thus, the relevant signals can be extracted and used for further structure elucidation.

To complete the metabolic profiling of the chemosphere in the tripartite community, GC-MS analysis was conducted and discussed in the subsequent sections.

3.4.7. Metabolite profiling in the chemosphere of the tripartite community using GC-MS analysis

Compounds screened by GC-MS represent a large proportion of the primary metabolism, and facilitate therefore comparative studies between model organisms in different systems (Fiehn, 2008). GC-MS analysis covers a wide range of compound classes, e.g., organic and amino acids, sugars, sugar alcohols, and lipophilic compounds upon derivatization. As it is shown in figure 24, the solid phase extractions of the growth media of the tripartite community and of axenic cultures were analyzed by GC-MS after derivatization.

Data processing was performed based on the methodology of Hiller et al. (2009) and Vidoudez (2010), following a four step standardized procedure: (1) correction of background noise, (2) conversion of the chromatograms to netCDF, (3) extraction of spectra by the Automated Mass spectral Deconvolution and Identification System (AMDIS) mainly for deconvolution and identification of chemical compounds, generating an input list of ion model/retention time pairs to be applied to (4) METabolomics-Ion-based Data Extraction Algorithm (MET-IDEA), which is a powerful software for extraction of individual single ion chromatographic peak areas and the subsequent determination of relative metabolite abundances. However, peaks corresponding to the retention index standards, internal standard (ribitol), UCM ingredients and any compound with $m/z$ less than 100 amu were removed from the data set. Afterwards, the means of the biomarkers resulting from experimental control in day-zero and day-7 along
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with axenic and the tripartite community cultures in day-zero were utilized as blank values and subtracted from all the remaining data sets. This yielded 399 biomarkers. 76 out of 399 metabolites (19% of the total metabolites) were identified by AMDIS. Based on the MET-IDEA outcome, the waterborne metabolites were displayed in a heatmap (Tab. 9) in order to have an insight into the conservation of identified metabolites identified by GC-MS across the species forming the model organisms; i.e., *U. mutabilis*, *Roseobacter* sp. and *Cytophaga* sp. The heatmap (Tab. 9) was divided into two parts for comparison based on the two main treatments: axenic and the tripartite community; and then each treatment was subdivided based on the sampling time. Due to the results of biological data and the detection of the time it takes in each status of gametogenesis inducibility (*cf*. section 3.3.6), metabolites could be now directly allocated to specific time of each status of gametogenesis inducibility in *U. mutabilis*. 
Table 9: The heatmap displays the abundance of identified metabolites (n = 76, 19% of the total metabolites) over 49 days in growth medium of axenic culture (no growth) and the chemosphere of the tripartite community (three statuses of gametogenesis inducibility). Metabolites are sorted according to the main substance classes.

<table>
<thead>
<tr>
<th>ID</th>
<th>Waterborne metabolites</th>
<th>Ax*</th>
<th>TC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classes</td>
<td>d-7</td>
<td>d-14</td>
</tr>
<tr>
<td>23</td>
<td>Unidentified PUFA C18 ??</td>
<td>No growth/no reproduction</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>9-Hexadecenoic acid</td>
<td>No growth/no reproduction</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Hexadecanoic acid</td>
<td>No growth/no reproduction</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Unidentified SFA C17:0 ??</td>
<td>No growth/no reproduction</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Heptadecanoic acid</td>
<td>No growth/no reproduction</td>
<td></td>
</tr>
<tr>
<td>69</td>
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<td>318</td>
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<td>331</td>
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<td>343</td>
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<td>---</td>
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</tr>
<tr>
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<tr>
<td>124</td>
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<td>Acetyl-serine ?</td>
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<td>282</td>
<td>Glutamine ?</td>
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<tr>
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<td>Glutamic acid ??</td>
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<td></td>
</tr>
<tr>
<td>65</td>
<td>Unidentified Sugar ?</td>
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<td></td>
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<tr>
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<td>Erythritol ?</td>
<td></td>
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<tr>
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<td></td>
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<td>78</td>
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<td></td>
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</tr>
<tr>
<td>130</td>
<td>Glycerol</td>
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<td></td>
</tr>
<tr>
<td>202</td>
<td>1-Heptanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>236</td>
<td>Unidentified alcohol ??</td>
<td></td>
<td></td>
</tr>
<tr>
<td>296</td>
<td>Unidentified alcohol?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>358</td>
<td>1-Octadecanol ??</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>Unidentified organic acid ??</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>4-Hydroxybutanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>Unidentified hydroxy acid C:4 ?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>Hydroxycarboxylic acid ??</td>
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Sugars (8%)

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>66</td>
<td>Erythritol</td>
</tr>
<tr>
<td>67</td>
<td>Unidentified Sugar ??</td>
</tr>
<tr>
<td>78</td>
<td>Fucose</td>
</tr>
<tr>
<td>81</td>
<td>Unidentified Sugar ?</td>
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Alcohols (5%)

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<td>1-Heptanol</td>
</tr>
<tr>
<td>236</td>
<td>Unidentified alcohol ??</td>
</tr>
<tr>
<td>296</td>
<td>Unidentified alcohol?</td>
</tr>
<tr>
<td>358</td>
<td>1-Octadecanol ??</td>
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O. m. (39%)

<p>| | |</p>
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<tbody>
<tr>
<td>59</td>
<td>Unidentified organic acid ??</td>
</tr>
<tr>
<td>61</td>
<td>4-Hydroxybutanoic acid</td>
</tr>
<tr>
<td>82</td>
<td>Unidentified hydroxy acid C:4 ?</td>
</tr>
<tr>
<td>83</td>
<td>Hydroxycarboxylic acid ??</td>
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Results and Discussion
## Results and Discussion

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<thead>
<tr>
<th></th>
<th>Metabolite Description</th>
</tr>
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<tbody>
<tr>
<td>85</td>
<td>3-Hydroxybutanoic acid or 4-Hydroxybutanoic acid</td>
</tr>
<tr>
<td>107</td>
<td>Succinic acid</td>
</tr>
<tr>
<td>108</td>
<td>Maleic acid</td>
</tr>
<tr>
<td>138</td>
<td>Mevalonic acid-1,5-lactone</td>
</tr>
<tr>
<td>142</td>
<td>Hydroxyxcarboxylic acid</td>
</tr>
<tr>
<td>149</td>
<td>2-Methyl-propanoic acid</td>
</tr>
<tr>
<td>173</td>
<td>Prephenic acid</td>
</tr>
<tr>
<td>178</td>
<td>Unidentified dicarboxylic acid (C4)</td>
</tr>
<tr>
<td>183</td>
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</tr>
<tr>
<td>185</td>
<td>Organic acid (C5)</td>
</tr>
<tr>
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<tr>
<td>246</td>
<td>Glutaric acid</td>
</tr>
<tr>
<td>256</td>
<td>Calystegine B2</td>
</tr>
<tr>
<td>271</td>
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</tr>
<tr>
<td>275</td>
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</tr>
<tr>
<td>278</td>
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<td>310</td>
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</tr>
<tr>
<td>311</td>
<td>Anthranilic acid</td>
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</tr>
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<td>Unidentified sterol</td>
</tr>
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<td>360</td>
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</tr>
<tr>
<td>377</td>
<td>2,4,6-Tribromophenol</td>
</tr>
<tr>
<td>388</td>
<td>Monomethylphosphate</td>
</tr>
</tbody>
</table>

Color shades are representative of the metabolite abundance throughout the treatments and sampling time points (row). The darker, the higher the abundance of the metabolite. *Ax: axenic, TC: Tripartite community, d (day), statuses of gametogenesis inducibility: ** "non-inducible" (non), "inducible ", and "spontaneously inducible" gametogenesis. If the reverse match score of the library identification (i.e., NIST, MP, Golm and Metabo) was lower than 800 and no standard was available, the metabolite identification is tagged with a "?", and "??" if the score was lower than 700.
Results and Discussion

In general, the identified waterborne metabolites (n = 76) could be classified into five classes: Fatty acids (24% of total identified waterborne metabolites), amino acids (24%), sugars (8%), alcohols (5%), and other metabolites (39%). In general, waterborne metabolites caused different patterns in the growth media of axenic cultures and of the tripartite community, and changed over time as follows:

**Fatty acids**

Some biomarkers e.g., biomarkers (#23, #47, #69, #257, #343, and #368) represent the fatty acids that were excreted into the medium of the axenic culture at the beginning of the sampling time until day-28, and disappeared or decreased afterward under axenic conditions. However, the same fatty acids were found in low abundance in the chemosphere of the tripartite community. A small portion of released fatty acids e.g., biomarkers #294, #314, and #318 exhibited a different pattern as they accumulated in the growth medium of the tripartite community during the time of spontaneously gametogenesis in *U. mutabilis* (Tab. 9).

---

5 Definition of the term model ion: The model ion, the ion signal showing the most rapid rise and fall, is always shown; it is assumed to be the most characteristic ion signal for a particular component.
Results and Discussion

Amino acids

Metabolite (#290): Glutamic acid (3TMS); model ion = 246, RT = 20.26 min.

The first pattern of amino acids in the chemosphere is represented by biomarkers i.e., #43, #68, #121, #124, #135, #182, #184, #190 that were detected in the medium after day-14 in axenic culture with the highest abundance then decreased gradually. The same amino acids however were detected in the chemosphere of the tripartite community during the time of “inducible gametogenesis” of *U. mutabilis*. Their abundance then either remained constant i.e., biomarkers #43, #68, #121, and #184 or decreased i.e., biomarkers #124, #135, #182, and #190.

The second pattern is shown by i.e., biomarkers #42, #205, #208, and #282, which were released into axenic culture on day-7 and depleted after that. None of these amino acids were detected in the chemosphere of the tripartite community except biomarkers (#205) (Tab. 9). On the other hand, amino acid; putatively O-acetyl-L-serine (#225) was not found in axenic culture, and tyrosine (#206) was detected in low abundance only in the last two sampling points, i.e., day-42 and day-49.
Results and Discussion

Sugars

Metabolite (#66): Erythritol (4TMS); model ion = 117, RT = 12.85 min.

Sugars accumulated in general at the end of the spontaneous gametogenesis status of *U. mutabilis* with high abundance. Erythritol (#66) was the only exception, as it was not detected in the tripartite community (Tab. 9).

Alcohols

Metabolite (#202): 1-heptanol (1 TMS); model ion = 173, RT = 9.12 min
Results and Discussion

In general, alcohols exhibited an accumulation in the chemosphere by the end of the “spontaneous status” of the gametogenesis induction of the tripartite community. Alcohols such as biomarkers #236, #296, and #358 were released into axenic culture as well as the chemosphere of the tripartite community and depleted immediately in both cultures. 1-heptanol (#202) was detected only in the chemosphere of the tripartite community on day-21 and increased afterward over time (Tab. 9).

Other metabolites

The waterborne metabolites might be released into the chemosphere as signals to aid the communication between the model organisms in the symbiotic tripartite community, as it was reported with many other macroalgae and their surrounding organisms (Kittredge et al., 1974; Fink, 2007; Hay, 2009; Goecke et al., 2010). Moreover, it is well documented that the goal of the relationship between our model system organisms is somehow to promote the growth, and the morphological completion of each other (Stratmann et al., 1996; Wichard and Oertel, 2010; Spoerner et al., 2012). Many symbiotic interactions can be assumed within the tripartite community.

Firstly from the associated bacterial side; promoting the growth and the morphology of U. mutabilis, i.e., the development of the regular U. mutabilis thallus, requires the presence of bacteria in their environment or substances provided by bacteria or other accompanying organisms (Stratmann et al., 1996; Spoerner et al., 2012). For instance, thallusin is a regulation factor which was isolated and identified by (Matsuo et al., 2005). In fact, the detection of this metabolite (C_{25}H_{31}NO_{7}) by GC-MS is not possible due to the high molecular weight of 457 amu (Fig. 3), and consequently the non volatility. Based on the working model suggested by Spoerner et al. (2012), it is predicted that associated bacteria release metabolites to communicate with each other, Roseobacter could be essential to the survival of Cytophaga.

Secondly from U. mutabilis side; U. mutabilis seems to release attracting metabolites into its chemosphere during the ”non-inducible” and ”inducible gametogenesis” status (Tab. 9) in order to attract its associated bacteria so as to meet its physical and physiological needs. On the other hand, in the late exponential phase and stationary, when gametogenesis can be induced spontaneously and the algal tissue will be disrupted.
Results and Discussion

Fatty acids

Fatty acids were detected both in medium of axenic culture and the chemosphere of the tripartite community, suggesting the production was assumed by *U. mutabilis*. The excreted fatty acids, in particular unsaturated fatty acids, were reported to serve as defense molecules (Alamsjah et al., 2009; Jüttner, 2001). Conversely, under sterile condition within the symbiotic tripartite community or axenic cultures, the defense mechanism was not in need. Moreover, the majority of released fatty acids were saturated (67% of total detected fatty acids in the growth medium) (Tab. 9). Thus, excreting fatty acids into the chemosphere might have another biological function.

Amino acids and sugars

The small molecular weight primary products i.e., sugars and amino acids are assumed to be provided by *U. mutabilis* as it was the only autotrophic macroalga in the tripartite community. Most primary metabolites such as carbohydrates, amino acids, peptides, and proteins are inducers of microbial colonization (Steinberg and de Nys, 2002). Hence, the surface of macroalgae provides a niche appropriate for colonization and reproduction of microorganisms (Mahmud et al., 2007; Englebert et al., 2008). Sugars and amino acids act as attracting substances in the chemosphere of the tripartite community for associated bacteria. This interaction was well documented between many macroalgae and surrounding bacteria (Wilson et al., 1990; Bulleri et al., 2002). The low percentage of detected sugars (8%) in the growth medium compared to fatty acids (24%) (Tab. 9) might be a result of the rapid utilization by bacteria, preventing these metabolites (sugars) from accumulating (Wiebe and Smith, 1977; Bell and Sakshaug 1980; Coveney 1982). This applies to amino acids as well, but the high percentage of amino acids (24%) could be due to the accumulation of some amino acids resulting from the decomposition and disruption of tissues.
Results and Discussion

Alcohols

As *U. mutabilis* is neither an aldehyde nor an alcohol producer (*cf.* chapter 3.1), alcohols detected in the medium are supposed to be bacterial metabolites or a result of abiotic degradation and decomposition of some metabolites taking place in the chemosphere.

Other metabolites

Many paramount biological functions can be predicted from the metabolites included under this group. Dicarboxylic acids i.e., biomarkers (#107, #108, #178, #240, #246, #289, #291, #303) were metabolites pronouncedly accumulated and detected during the time of spontaneous gametogenesis. In fact, it was reported that dicarboxylic acids with small molecular weight have a potential affect on nitrate uptake, plant growth hormone-like activity (Šmídová, 1960; Piccolo et al., 1992; Piccolo et al., 2003), as well as on reproduction and respiration of the costal dianoflagellate *Prorocentrum minimum* (Heil, 2005). Interestingly, calystegine B2 (#256) is an alkaloid detected in both axenic and the tripartite community. Calystegine B2 was administrated to influence rhizosphere ecology as nutritional sources for soil microorganisms and as glycosidase inhibitors in plants (Goldmann et al., 1996). Thus, calystegine B2 might have an influence on the chemosphere of the tripartite community.

The remaining ca. 300 waterborne metabolites are still unknown and represented 81% of the total metabolites, showing significant contribution of these unknown in the chemosphere of the tripartite community. The discriminant analysis hence is the way to investigate the efficiency of these pronounced unknowns in mediating the interaction between the model organisms.

3.4.8. **Determined biomarkers in the chemosphere of the tripartite community: the known unknowns**

The metabolites found in the chemosphere of the tripartite community along with the medium of axenic culture (*n* = 399) were analyzed by CAP based on Bray-Curtis distance after standardization and transformation (Fig. 30). Similarly to the CAP analysis of the biomarkers obtained by LC-MS analysis (*cf.* section 3.4.5), the *a priori* groups in CAP analysis were generated accordingly to the time taken for each status of the gametogenesis inducibility in *U.*
Results and Discussion

*mutabilis* in the tripartite community. Since no growth was observed in axenic cultures over whole sampling time points, biomarkers of axenic culture were combined with the young culture in the tripartite community when the gametogenesis was “not inducible”. The *a priori* groups were therefore:

**Group 1**: The tripartite community (day-7) combined with all sampling events of axenic cultures (day-7 till day-49).

**Group 2**: The tripartite community (sampling events from day-14 till day-21).

**Group 3**: The tripartite community (sampling events from day-28 till the day-49).

Out of ca. 400 biomarkers, 141 biomarkers contributed significantly to the CAP axes (Fig. 30b).

The CAP plot (Fig. 30a) resulted in a significant separation with a misclassification error of 2%; the two axes were very efficient in separating the groups (high eigenvalues), and these axes were highly related to the differences between the groups (high squared canonical correlations: $\delta_1^2 = 0.98$ and $\delta_2^2 = 0.85$) (Tab. 10). Furthermore, the permutation test confirmed that the groups had significant different location in the multivariate space ($P = 0.001$, with 999 permutations). Finally, the correlation coefficient of the first CAP axis was significant; meaning the difference explained by this axis was statistically significant (Tab. 10).

The three groups in the CAP plot were very distinct. Group 1 was separated from group 2 on both axes, and from group 3 on axis 1. In contrast, group 2 and 3 were separated on axis 2. The separation was mediated mainly by the differences in sampling time points rather than the difference between the treatments (i.e., axenic and the tripartite community). The reason behind this could be explained in twofold. Firstly, axenic culture did not exhibit any distinguishable biomarkers based on the sampling time series, that is, axenic cultures over entire sampling points (49 days) represented only one group in combination with the tripartite community in day-7. Secondly, in contrast to axenic culture, the separation of the three groups was fully dependant on the sampling time of the tripartite community. In order to decipher which metabolites were important for the separation, the absolute correlation of each metabolite with the first two canonical axes was considered, and if significant, a vector was displayed, representing the contribution of this particular metabolite to the ordination. This
Results and Discussion

graphical representation allows the determination of the metabolites important for the separation of each group formed by the CAP analysis.

Clearly, it could be observed that the biomarkers characterizing group 1 were fewer compared to the ones characterizing groups 2 and 3. Moreover, the common (shared) biomarkers were found in group 2 and 3, e.g., biomarker #262, #377, and #384. Each *a priori* group was depicted by specific biomarkers.

The same strategies that were previously adopted in characterizing the groups in CAP by the biomarkers resulting from LC-MS (cf. section 3.4.5) were used in CAP analysis of the biomarkers generated by GC-MS. These strategies are (1) presence/absence of the biomarker among the *a priori* groups, and/or (2) significant differences in the abundance of a biomarker between the *a priori* groups. In order to display these strategies, a representative biomarker in each status of gametogenesis inducibility was selected and its abundance was recorded over growth phases and gametogenesis inducibility statuses\(^6\), respectively (Fig. 31). Biomarker #246 represents glutaric acid with a \(m/z = 221\), \(RT = 9.26\) pair. Glutaric acid characterized the young culture when gametogenesis was not inducible in tripartite community in addition to axenic culture (Fig. 30), and exhibited a variation in abundance throughout the growth corresponding to the three status of gametogenesis inducibility of *U. mutabilis*. It was recorded at high abundance in the young culture, and then its abundance decreased dramatically during growth, and it was not found in aging culture when spontaneous gametogenesis was feasible (Fig. 31a). Thus, both strategies (presence/absence and concentration differences between groups) are applied on biomarker #246.

\(^6\) The definition of the inducibility statuses is found in page 66.
Results and Discussion

Figure 30: Groups correspond to the three key gametogenesis-inducibility-states in *Ulua* during its growth. Separation was achieved by the metabolites (ca. 400) obtained by GC-MS analysis and found in growth media of axenic culture and the chemosphere of the tripartite community using Bray-Curtis dissimilarity distance. (a) CAP separation of the samples with the growth corresponding to the gametogenesis statuses as groups. (b) Correlations of 141 biomarkers (35% of the total biomarkers) with the two CAP axes, with absolute Pearson coefficient correlation $|r| \geq 0.3$.

Table 10: Eigenvalues, squared canonical correlation, and diagnostics statistics of the CAP analysis of the biomarkers (n = 141) resulting from GC-MS and found in growth media of axenic culture and the chemosphere of the tripartite community throughout sampling time points.

<table>
<thead>
<tr>
<th>Constrained canonical axes</th>
<th>1st axis</th>
<th>2nd axis</th>
<th>Crossvalidation</th>
<th>Permutatest</th>
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<tr>
<td>Eigenvalue</td>
<td>$\delta^2$ Correlation</td>
<td>Eigenvalue</td>
<td>$\delta^2$ Correlation</td>
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</tr>
<tr>
<td>0.99</td>
<td>0.98</td>
<td>0.92</td>
<td>0.85</td>
<td>2%</td>
</tr>
</tbody>
</table>

Group 1: Axenic (day-7 till day-49) + Tripartite community (day-7)
Group 2: Tripartite community (day-14 till day-21)
Group 3: Tripartite community (day-28 till day-49)
Biomarker #83 characterized the growth time when gametogenesis was inducible (Fig. 30), and was identified as hydroxyl carboxylic acid ($m/z = 117$, RT = 7.66). Moreover, it was a representative of the presence/absence mechanism as it was present only in the growth time when gametogenesis was inducible and absent in the young and aging culture (Fig. 31b). During the growth when the gametogenesis spontaneously was induced, biomarker #313, identified as 4-hydroxybenzoic acid and/or 3-hydroxybenzoic acid ($m/z = 276$, RT = 12.77), was detected in high abundance (Fig. 31c), while its abundance was low during the growth before reaching spontaneous induction (Fig. 31c), meaning that biomarker #313 represents both strategies in characterizing the growth phase of spontaneous gametogenesis.
Results and Discussion

Figure 31: GC-MS chromatograms of three selected and identified representative biomarkers characterizing the *a priori* groups shown in figure 30. (a) Glutaric acid characterizing the axenic cultures as well as the growth phase when gametogenesis was not inducible. (b) Hydroxy carboxylic acid-C4 characterizing the growth phase when gametogenesis was inducible. (c) 4-Hydroxybenzoic acid characterizing the phase when the spontaneous gametogenesis started.
Results and Discussion

The longer the vector, more efficient the biomarker in characterizing the group. Some of these characteristic biomarkers are listed in table 11 to display their model ion and retention time as well. The selection of the listed biomarkers was performed based on the significant contribution for characterizing each group separately.

Table 11: Model ion and retention time of biomarkers found in axenic culture medium and the chemosphere of the tripartite community over 49 days. Shown here are some selected biomarkers (n = 85) which contributed significantly to the CAP axes ($|r| \geq 0.3$).

<table>
<thead>
<tr>
<th>ID</th>
<th>Biomarkers</th>
<th>Model ion</th>
<th>RT (min)</th>
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Model ion: The model ion, the ion signal showing the most rapid rise and fall, is always shown; it is assumed to be the most characteristic ion signal for a particular component.
### Results and Discussion

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A priori group 3 (n = 48)

"Spontaneously status" of gametogenesis
Results and Discussion

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If the reverse match score of the library identification (i.e., NIST, MPI, Golm, or Metabo databases) was lower than 800 and no standard was available, the metabolite identification is tagged with a “?”, and “??” if the score was lower than 700.

Based on table 11, the vectors (characteristic biomarkers) were classified into four chemical classes over all a priori groups in descending order, i.e., other metabolites (15%), fatty acids (8%), amino acids (7%), and alcohols (3%). 67% of the characteristic biomarkers remained unknown and contributed significantly in the characterization of the growth phases corresponding to the status of gametogenesis inducibility. However, the time taken for each inducibility status was characterized by specific chemical classes making the chemosphere in each status chemically distinguishable (Fig. 32).

For instance, fatty acids, notably saturated fatty acids (Tab. 11) and amino acids were the remarkable biomarkers during the growth phase when the gametogenesis was not inducible (Fig. 32), whereas other metabolites could be found (25%) during the “inducible status” in the second growth phase (Fig. 32). It is notable, that the only growth phase that was characterized by substances of all chemical classes i.e., fatty acids, amino acids, alcohols and other metabolites the most abundant class was the phase when gametogenesis spontaneously induced (Fig. 32).

In addition, the CAP plots (Fig. 30) reveals no negative correlation between the characteristic biomarkers, and consequently no groups were located in the counter direction of each other (Fig. 30a) in contrast to what was observed with characteristic biomarkers resulting from LC-MS analysis (Fig. 27a, 29).
Results and Discussion

Figure 32: Pie charts display the abundance (%) of the various substance classes of identified biomarkers characterizing the chemosphere of the tripartite community over the time taken for the statuses of gametogenesis inducibility in *U. mutabilis*.
Results and Discussion

It can be argued that the absence of the 180° pairwise correlations between the characteristic biomarkers might be attributed:

(1) to the nature of the metabolites analyzed by GC-MS as they are mainly primary metabolites (Fiehn, 2008). Consequently, the demand and the utilization rate of these metabolites by the organisms involved in the tripartite community were high.

(2) to the strategy of subtraction blank values required eliminating unspecific peaks, thus a part of the extracellular compounds was not represented in the data analysis.

(3) to the effect of the cartridges capacity to retain only specific waterborne metabolites. However, based on the comprehensive comparison between solid phase extractions (SPE) cartridges performed by (Masque et al., 1998), the PS-DVB Easy® cartridge used in the current study for extraction is the recommended cartridge to recover most polar compounds from the water body because of the hydrophilic surface containing relatively high aromatic sites which allow $\pi - \pi$ interaction with unsaturated analytes.

Fiehn (2008) addressed two major challenges remaining in GC-MS-based metabolomics: (1) rapid annotation of unknown peaks; and (2) integration of biological background knowledge aiding data interpretation. The first challenge still remains in the present study, as 67% of the characteristic biomarkers are still unknown. However, the second challenge was managed in the present study by gathering the metadata and generating the a posteriori hypothesis (cf. section 3.3.6), which facilitated the multivariate analyses by generating the a priori groups based on the growth corresponding to the status of gametogenesis inducibility of *U. mutabilis*.

During the time of “non-inducible status” of the alga, all the detected substances are likely produced by the alga as the chemometric data analyses reveals that axenic cultures and the young tripartite community grouped together based on metabolic profiling. Interestingly, some studies attempted to measure the bacterial uptake of dissolved organic carbon (DOC) produced and released by algae in particular phytoplankton. These metabolites seemed to be turned over rapidly by bacteria (Wright and Shah, 1975; Wiebe and Smith, 1977). Kinetic analysis indicated that the rate of DCO production by algae is equal to the rate of the uptake by microbes. Based on these analyses, it was supposed that the concentration of DCO in axenic algal cultures increases over time or remains constant (Nalewajko et al., 1976) in contrast to non-axenic
cultures, where bacterial uptake can keep pace with the rate of release (Chrost and Faust, 1983). This might explain why axenic culture of *U. mutabilis* over entire experimental time (49 days) grouped to the young tripartite community (on day-7) (Fig. 30).

Those algal substances, released into the chemosphere during the time of “non-inducible status”, belong mainly to the amino acids, saturated fatty acids, and other metabolites (Tab. 11, Fig. 32). Amino acids are known for their attracting power, making the releasing alga a favorable source of primary metabolites for associated bacteria. For instance, Kirchman (2002) found out that bacteria belonging to the *Cytophaga-Flavobacteria* cluster are capable of utilizing proteins and chitin. In addition, the results of Schweitzer et al. (2001) predicted that *Cytophaga-Flavobacteria* are capable of using amino acids. Miller et al. (2004) demonstrated that *α-proteobacteria* phylogenetically related to the *Roseobacter* clade are strongly attracted to amino acids and DMSP metabolites, while being only mildly responsive to sugars and the tricarboxylic acid cycle intermediates. Interestingly, a study by Cottrell and Kirchman (2000) tested the ability of both bacteria clusters *α-proteobacteria* and *Cytophaga-Flavobacter* to utilize amino acids. *Cytophaga-Flavobacter* was found to consume chitin, N-acetylglucosamine, and proteins but was generally underrepresented in consuming amino acids. The amino acid-consuming assemblage was usually dominated by the *α-proteobacteria*.

During the time spent in the “inducible gametogenesis status”, another substance class, carboxylic acids came into the fore. Those molecules are most likely produced by the bacteria, because these pathways are well known investigated in *α-proteobacteria*, e.g., glucose catabolism (Furch et al., 2009). These bacterial compounds might be taken up by macroalga and contribute hence to the chemically mediated interaction of algae and bacteria. As a consequence, the spontaneous gametogenesis induction starts. Evidently, it was demonstrated also in diatoms that many stimuli secreted by associated bacteria such as vitamin 12 increased algal growth (Haines and Guillard, 1974; Cole, 1982; Croft et al., 2005).

When gametogenesis was spontaneously induced within the late exponential and stationary growth phase, there were a lot of similar patterns in terms of substance classes and the dynamics observed as found in diatom cultures (Paul et al., 2013; Vidoudez and Pohnert, 2012). These algal and bacterial substances are classified mainly into amino acids, dicarboxylic acids,
saturated fatty acids, and aromatic compounds. Thus, these metabolites might be detected in the chemosphere in this specific time due to the catabolism and the decomposition of tissues. Interestingly, *U. mutabilis* released 3-(or 4)-hydroxybenzoic acid (#313), a potential biomarker was found in the third growth phase during the “spontaneously inducible status” (Fig. 30b), when *U. mutabilis* grew healthy and reproduced spontaneously (Fig. 23). The same phenomenon was observed by Vidoudez (2010), who detected 3(or 4)-hydroxybenzoic acid in the culture medium, where *Skeletonema marinoi* was actively growing. Accordingly, Vidoudez hypothesized that 3(or 4)-hydroxybenzoic acid might be released to gather iron (Vidoudez, 2010). Thus, biomarker #313 might play the same function in the chemosphere of the tripartite community. In addition, *Roseobacter* lineage is one of the few dominant marine clades that exhibit a unique opportunity to use aromatic compounds including phenolics such as 4-hydroxybenzoate as primary growth substrates, and consequently catabolize such phenolic products via β-ketoadipate pathway (Buchan et al., 2000; Buchan et al., 2004). More interesting, it was suggested that 4-hydroxybenzoic (#313) is a likely precursor of 2,4,6-tribromophenol (#377) in *U. lactuca* (Flodin and Whitfield, 1999). Even more interesting yet, the precursor and the product were detected as characteristic biomarkers during the same inducibility status (Tab. 11). The decrease over time of 4-hydroxybenzoic acid was associated with the increase of 2,4,6-tribromophenol.

3.4.9. The relationship between the waterborne metabolites: Tyrosine, 4-hydroxy-benzoic acid, and 2,4,6-tribromphenol

Based on the biosynthesis suggested by Flodin and Whitfield (1999), 2,4,6-tribromophenol (biomarker #377) is supposed to be synthesized from tyrosine (metabolite #206) via 4-hydroxybenzoic acid (biomarker #313) (Fig. 33). Thus, the abundance of these metabolites in the chemosphere of the tripartite community and axenic culture medium at each sampling time point were compared and shown in the heatmap (Tab. 12). From this heatmap, it can be clearly observed the following:

- The high and constant abundance of tyrosine over sampling time points in axenic culture was associated by the absence of 2,4,6-tribromophenol in general, and by low abundance in
particular sampling points, i.e., on day-35 and day-42. This pattern continued as well in the young tripartite community till day-14 (Tab. 12).

- In contrast, the depletion of tyrosine in the tripartite community after day-14 resulted in an increase of 2,4,6-tribromophenol abundance (Tab. 12).

- No specific pattern of 4-hydroxybenzoic acid could be generalized (Tab. 12). However, it was found in low, high abundance, or was depleted completely in the growth medium. For instance, (1) 4-hydroxybenzoic acid was detected in low abundance, either when the abundance of tyrosine was high and 2,4,6-tribromophenol was not detected i.e., in axenic culture on days 7, 14, 28, and 49 and in the tripartite community on day-7, or when tyrosine was depleted and 2,4,6-tribromophenol increased, i.e., in the tripartite community on days 28 and 35 (Tab. 12). (2) 4-hydroxybenzoic acid was completely depleted when the abundance of tyrosine was high, and that 2,4,6-tribromophenol was low i.e., axenic culture on days 35 and 42, and the tripartite community on day-14 (Tab. 12). (3) 4-hydroxybenzoic acid was detected at a high abundance both when tyrosine depleted completely and the abundance of 2,4,6-tribromophenol was low i.e., in the tripartite community on days 21, 42, and 49, or when 2,4,6-tribromophenol was not detected and the abundance of tyrosine was low i.e., axenic culture on day-7 (Tab. 12).

This fluctuation of 4-hydroxybenzoic acid abundance in growth media might be a result of its multifunctionality in the chemosphere of the tripartite community as it was addressed previously (cf. section 3.4.8).

The inverse relationship between the abundance of tyrosine and 2,4,6-tribromophenol in the growth media suggests that *U. mutabilis* might synthesize 2,4,6-tribromophenol using tyrosine via 4-hydroxybenzoic acid. Moreover, this biosynthesis is mediated by bromoperoxidase (BrPO) (Hewson and Hager, 1980), which was found in green algae e.g., *Ulvella lenza* (Ohshiro et al., 1999). Based on Flodin and Whitfield (1999) study, the biosynthesis of 2,4,6-tribromophenol is facilitated by the alga. At the same time, Li et al. (2009) showed that 2,4,6-tribromophenol is released by the brown algae *Eisenia bicyclis* and *Ecklonia kurome* and subsequently inhibits the settlement and survival of the Japanese abalone *Haliotis discus hannai*. For these reasons, 2,4,6-tribromophenol might be synthesized in vivo by *U. mutabilis* from tyrosine via 4-
Results and Discussion

Hydroxybenzoic acid in the presence of BrPO and released into the growth medium. Bromophenols are common volatile compounds among red (Etahiri et al., 2007; Xu et al., 2009) and brown (Chung et al., 2003; Shibata et al., 2014) algae. Flodin and his co-workers have addressed the existence of tribromophenol in green alga *U. lactuca* (Flodin et al., 1999; Flodin and Whitfield, 1999). This extracellular compound was reported as antibacterial (Flodin and Whitfield, 1999; Etahiri et al., 2007; Xu et al., 2003; Liu et al., 2011) and as a deterrent substance against marine herbivores (Liu, 2011; Shibata et al., 2014). These allelopathic affect however cannot be predicted within the symbiotic relationship of the tripartite community under sterile condition, assuming another biological activity of 2,4,6-tribromophenol. Further studies are needed to investigate the biosynthesis of 2,4,6-tribromobenzoic acid in *U. mutabilis*.

Table 12: The heatmap shows the changes in the abundance of tyrosine, 4-hydroxybenzoic acid, and 2,4,6-tribromophenol in the growth medium of axenic and the tripartite community in each sampling time over 49 days.

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</tbody>
</table>

Color shades are representative of the abundance at each time sampling throughout the biomarkers (column). The darker the color, the more pronounced the substance. *d: day. Data was standardized by sum-standardization to all areas of peaks.*
Results and Discussion

Figure 33: Proposed biosynthesis pathway for the formation of 2,4,6-tribromophenol in U. lactuca. The pathway from tyrosine to 4-hydroxybenzoic acid is based on the study by Landymore et al. (1978), and from 4-hydroxybenzoic acid to 2,4,6-tribromophenol is based on the study by Flodin and Whit (1999). (1): Tyrosine. (2): 4-Hydroxyphenylpyruvic acid. (3): 4-Hydroxyphenyllactic acid. (4): 4-Hydroxyphenylacetic acid. (5): 4-Hydroxymandelic acid. (6): 4-Hydroxybenzaldehyde. (7): 4-Hydroxybenzoic acid. (8): 3,5-Dibromo-4-hydroxybenzoic acid. (9): 2,4,6-Tribromobenzoic acid. The compound marked with (*) were detected in axenic growth media and the chemosphere of the tripartite community and the inserts (a, b, and c) show the mass spectra of TMS-derivatives of these compounds, respectively.
3.4.10. Conclusion

Ultimately, metabolomic analysis using two different analytical platforms proves the clarity of the hypothesis generated from the metadata, in which the time taken for each gametogenesis inducibility status in *U. mutabilis* influenced the chemosphere of the tripartite community. For this reason, waterborne metabolites were divided in response to the statuses of gametogenesis inducibility in *U. mutabilis* (Figs. 27, 30). Despite the fact that the chemical properties of the metabolites obtained by GC-MS and LC-MS analyses were different, both LC- and GC-based metabolomics can be used to monitor the growth and which also allows conclusion about the status of the gametogenesis inducibility in *U. mutabilis* under the condition used in bioreactors cultures.

In general, *U. mutabilis* tends to release the potential biomarkers by the end of the “inducible status” time till the mid of the time spent in “spontaneously inducible status” during the second and third growth phases. The high percentage of unknown biomarkers were shown to contribute significantly in characterizing the *a priori* groups, opening the doors for further analytical work in order to investigate the chemical structures of these pronounced compounds and their functions in structuring the chemosphere of *U. mutabilis* and its associated bacteria.

Based on the promising outcome of metabolomic profiling using bioreactor cultures, I aimed in the future studies:

1. to apply the same model system used in laboratory (the tripartite community) in land based aquaculture to possibly collect elevated amounts of identified biomarkers for structure elucidation.
2. to predict the changes of gametogenesis inducibility in aquaculture in order to maintain subsequently the tripartite community in its vegetative state.
3. to compare the waterborne metabolites collected under controlled conditions and in aquaculture.

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8 The definition of the inducibility statuses is found in page 66.
3.5. Exo-Metabolomics of algal Aquacultures

Aquacultures at the Ramalhete Marine Station of the Algarve Marine Sciences Centre (CCMAR) in Faro, Portugal, conducted in 2011 from the 10th March to the 5th May.
Results and Discussion

In general, the global challenge in performing seaweed aquaculture is the sustainability of seaweed farming for long term. Many applications can be considered with seaweed. Recently, production of alternative fuels from non-starch biomass has also attracted the attention towards utilization of marine algae, including seaweed, as sources of fuels production (Varela et al., 2006). Seaweed has also been proposed as a mass source for production of functional food ingredients, pharmaceuticals, and cosmetics (Bixler 1996; Bobin-Dubigeon et al. 1997; Cumashi et al. 2007). Ulva species are particularly rich in rare cell-wall polysaccharides (Ulvan) in addition of e.g., ionic polysaccharides containing sulphate groups, uronic acids, and essentially rhamnose and xylose, or xylose, galactose and arabinose, and have been proposed as being an important source of dietary fibers, especially soluble fibers (Lahaye 1991; Lahaye and Axelos 1993). In addition, Pereira et al. (2012) concluded that macroalgae can be considered as a potential source for large-scale production of essential PUFAs with wide applications in the pharmacological industry. Ulva spp. were documented to possess high amounts of C18 fatty acids, in particular αALA (Pereira, 2012). Ulva has simple growing requirements and can produce lipids and proteins in large amounts over short periods of time in simple land based aquacultures. However, wound activated transformation of lipids into oxylipins could also result in a depletion of valuable unsaturated fatty acids and might hence jeopardize the value of Ulva as a resource for e.g., PUFAs in aquacultures (Wichard et al., 2007). In this context, the chemotaxonomic significance of PUAs was proven by comparison with the morphogenetic and phylogenetic properties of about 100 collected Ulva isolates (cf. chapter 3.1). The tube-like morphotype did not produce any C10-PUAs, while the sea lettuce-like morphotype produced these compounds. For instance, U. lactuca is characterized by its sheet-like structures that are two cells thick and classified in the category of sea-lettuce Ulva species that produces PUAs (cf. chapter 3.1). With regard to FAs depletion due to PUAs and oxylipins production, this finding might be conflicted with the studies of Magnusson et al. (1996); SandJensen (1988); and Naldi and Viaroli (2002), who reported that U. lactuca and U. rigida respectively have a considerable importance in marine ecology due to the high photosynthetic rates and high C- and N- nutrient uptake capacity, which resulted in rapid growth, making it a suitable species for aquaculture. On the other hand, based on the present results, U. mutabilis does not produce PUAS and it can
be thus taken into account as a resource for e.g., PUFAs in aquacultures (cf. chapter 3.1). However, further investigations are needed to confirm that oxylipins production rather than PUAAs has no effect on PUFAs depletion.

Martinez-Porchas and Martinez-Cordova (2012) provided a comprehensive review on algal aquaculture. They suggested many strategies in order to advance in the sustainability of aquaculture from two aspects: site selection, and species selection. Regarding these suggestions, (1) *U. mutabilis* aquaculture in the present study was performed at the Ramalhete Marine Station in Faro, Portugal, which is a native habitat of *U. mutabilis* (Føyn, 1958) to avoid introducing of exotic species causing many and diverse environmental problems, and to also avoid environmental effects as much as possible (e.g., temperature, light/dark cycle). (2) From the species selection point of view, the bioreactors results helped to gather the as much knowledge as possible about the biology and ecology of the model organisms (e.g., life cycle, nutritional requirements, gametogenesis inducibility status, and some relative ecological information). In the present study, many abiotic parameters in aquacultures were monitored prior to metabolomic analysis and metadata collection, offering more controlled material to assess treatment effects conclusively, and helping eliminate other potentially negatively interfering environmental factors.

### 3.5.1. Experimental design

The big challenge in transferring the approach used in batch or bioreactor cultures in the laboratory into land based aquaculture was to start the aquacultures in volume of 200L of Instant Ocean/ tank with purified axenic germlings aged 7 day. In this way, the metabolomic analysis in aquaculture will be comparable with bioreactor cultures.

Two treatments were conducted in parallel: **The defined bacterial community**, where axenic *U. mutabilis* germlings were inoculated with *Roseobacter* and *Cytophaga* species and the so-called **“inappropriate community”**, which was inoculated only with axenic *U. mutabilis* germlings.
3.5.2. Monitoring of environmental (abiotic) parameters

Temperature

Since temperature plays an important role in *U. mutabilis* growth (Nordby, 1977), it was essential to record the temperature hourly inside each tank over experimental time (Fig. 34a). Over the cultivation time (= 49 days), the temperature ranged from 13 to 27 °C during the day, and from 13 to 24 °C at night. The night temperature was lower than the day temperature during the first 35 days. After that, both night and day temperatures were almost equal and ranged from 18 to 25 °C (Fig. 34a).

According to *U. mutabilis* literature, the recommended temperature is 21-22 °C (Nordby, 1977). Taylor et al. (2001) found the optimal temperature prompting the growth of *U. curvata*, *U. rigida*, *U. compressa*, and *U. linza* are 20, 15, 10 and 15 °C, respectively. In light of these data, no extreme change in temperature was recorded during the entire experimental time of aquaculture. Thus, the influence of temperatures was minimal.

Although an attempt was made to minimize the influence of evaporation by covering the tanks, temperature surely had an effect on the evaporation and subsequently on salinity.

Salinity

Salinity was recorded weekly along with the sampling of the culture media for chemical analyses. During the long-term experiment, salinity fluctuation was not important, and limited between 27.3 and 28.8 psu (Fig. 34b). This is due to the aquaculture experimental design and its application (Fig. 24). To reduce the evaporation, the tanks were covered with a tygon film. Salinity increased slightly anyway. Regarding the high salt tolerance of *Ulva*, the increase is only minor and did not affect its overall growth.

“Green tide” algae are generally reported to have tolerance for broad ranges of salinity (Taylor et al., 2001). Choi et al., (2010) observed that the maximum and the minimum growth rate of *U. pertusa* were recorded at 20 and 40 psu under salinity regimes from 5 to 40 psu, respectively. Similarly, the highest growth rates recorded for *U. rigida* and *U. linza* were obtained at 23.8 psu, and for *U. curvata* at 27.2 psu (Taylor et al., 2001). Accordingly, the range of salinity in *U. mutabilis* aquacultures was within the recorded range for optimal growth rate.
of other *Ulva* species. In addition to salinity, *Ulva* also is well known for its tolerance to a variety of pH values up to 10.

### pH

The effect of pH on the growth and on reproduction of macroalgae is well known (Middelboe and Hansen, 2007a, Middelboe and Hansen, 2007b). Whereas *Ulva* does not grow below pH 6.5 (Menéndez et al., 2001), it is highly adapted to alkali environments and grows even at pH 10 (Maberly, 1990).

In aquacultures, there was no significant change recorded in pH values during the six weeks after inoculation (*P* > 0.05) although pH value increased slightly within the range of 8.3 – 9.2 from day-7 to day-28 (Fig. 27). However, pH value dropped gradually after 28 days (*P* > 0.05), and significantly (*P* < 0.05) at the end of the culturing time (day-49) (Fig. 27).

The slight increase in pH value (alkalinity) over cultivation time might be attributed to the decrease of CO$_2$ (aqueous) due to the photosynthetic uptake related to the increase in biomass and growth of algae (Goldman, 1973). This suggests the necessity of the measurement of CO$_2$ to correlate its pattern with the biomass and pH in order to meet the need of industrial applications. The pH value of the suspension medium has also been shown to affect the sporulation processes in two *Ulva* species. For instance, the optimum pH for inducing sporulation in *U. mutabilis* is assumed to be 8.0 - 8.5 (Nordby, 1977), and the optimum pH for spore release in *U. pertusa* was reported to be between 7 and 9 (Han et al. 2008).
Results and Discussion

Figure 34: Weekly changes in environmental factors of _U. mutabilis_ aquacultures maintained in Instant Ocean. Values are expressed as mean ± SD, n = 3. Different letters indicate significant difference in the measurements over time (Tukey-posthoc test; *P* < 0.05). (a) Temperature of the day and night in aquaculture. (b) Salinity. (c) pH values.
3.5.3. **Change of U. mutabilis life cycle in well-defined bacterial community and “inappropriate community”**

As a feedstock of well-defined germlings inoculated with the two bacterial strains (*Roseobacter* and *Cytophaga* species) was used (Fig. 35a), reproducible conditions in aquacultures were achieved for the first time, comparable to freshly collected *Ulva* species from the coast. One week after inoculation, small alga with a pronounced well shaped thallus could be observed in the defined bacterial community (Fig. 35b), in contrast to the "inappropriate community", where the competitive microorganisms started growing rapidly due to the lack of the right microbiome along with the lack of the bacterial morphogenetic compounds. Thus, even dead thalli and germlings were observed (Fig. 35e). On day-14 and 28, the thalli in the defined bacterial community continued to grow normally (Figs. 35c, d), and in the "inappropriate community", malformed cell walls and contaminants overlying the thalli could be observed (Figs. 35g,h). On day-42, thalli were torn into two parts in the defined bacterial community, whereas in "inappropriate community" alga was coated intensively by diatoms and other microorganisms (data not shown).

The results of control tanks (= no inoculation at all) along with the “inappropriate community” tanks were discarded due to the high contamination level observed in those tanks. Thus, within the scope of this project, only the results of the defined bacterial community (*U. mutabilis* + *Roseobacter* sp. and *Cytophaga* sp.) was addressed.

Under the microscope (Fig. 35), the influence of the tripartite community can be clearly seen in aquacultures (the defined bacterial community), which resulted in the development of normal *U. mutabilis* slender like thalli with its typical morphology (Figs. 35a-d) as it was observed in the tripartite community cultured in bioreactors (Figs. 1a, 2a). Although contaminants can be observed in the tripartite community, the young feedstock can overcome these adverse negative effects as it benefits from the mutualistic interactions with the symbiotic bacteria (Spoerner et al., 2012). Regarding the suggestion of (Martinez-Porchas and Martinez-Cordova, 2012), algae selection is one of the main reasons behind the sustainability of aquacultures. Evidently, the present observation supported this suggestion and added in addition the selection of appropriate microbiome as a considerable reason of aquaculture sustainability.
Results and Discussion

Figure 35: Microscopy pictures show changes in phenotype of *U. mutabilis* during the life cycle in well-defined bacterial community\(^9\) inoculated with *U. mutabilis*, *Roseobacter* sp. and *Cytophaga* sp. (a - d) and in “inappropriate community” inoculating only with *U. mutabilis* (e - h). (a) Germling with rhizoid surrounded by *Roseobacter* sp. (black arrow) on day-zero (onset of the aquaculture). (b) Longitudinal growth of *U. mutabilis* thallus on day-7. (c) Thallus representing the rapid growth on day-14. (d) Thallus with unknown aggravation (black arrows) on day-28. (e) Malformed cell wall with bubble like structures (red arrows) on day-zero in "inappropriate community". (f) Dead germling (yellow circle) on day-7. (g) Thallus with cell wall covered with bubble like structure (yellow circles) surrounded by contaminants (red arrows) on day-14. (h) Thallus overlying with contaminants (yellow arrows) on day-28.

\(^9\) The definition of the communities is found in page 120.
Results and Discussion

3.5.4. **Monitoring of the growth of U. mutabilis in the defined bacterial community**

*U. mutabilis* met all the biotic and abiotic factors that promoted its growth and development. The growth hence was estimated by measuring the length of the thalli (Fig. 36a). Accordingly, remarkable longitudinal growth patterns were observed during the life cycle of *U. mutabilis*. From day-zero till day-7, the growth was relatively slow approaching an average of thallus length 2.6 cm ± 1 (SD), and then the growth increased rapidly with an average of thallus length 9.8 cm ± 0.6 (SD) on day-14. From day-14, the thallus length increased gradually reaching the maximum length over experimental time on day-35 (15.6 cm ± 0.8). After that, the longitudinal growth drooped to 10 cm ± 1.2 on day-42. By the same token, the growth continued till day-49. The mass obtained by the end of aquacultures ranged between 600 to 700 g fresh weight in 200L after removal of the water.

It is important to note that the decrease of the longitudinal growth which was recorded in the defined bacterial community from day-35 to day-42, was not observed in the tripartite community in the bioreactor. In aquacultures, the thalli were torn into two parts during this particular time which was not the case in the tripartite community of the bioreactor where thalli grew continuously approaching an average of length 25 cm ± 1.2 (SD) (Fig. 18).

3.5.5. **Estimation of bacteria growth by qPCR**

qPCR was used to quantify DNA concentration of *Roseobacter* clade in aquaculture (Fig. 36b), reflecting its abundance in the defined bacterial community over time. In the final analysis, there was a general trend of increasing *Roseobacter* clade abundance over experimental time (49 days). The increase in DNA followed the pattern of bacterial growth. After the lag phase lasting for two weeks, the growth started exponentially (Fig. 36b), indicating that *Ulva* provides enough carbon sources for bacterial growth. Moreover, the typical rapid increase of bacterial growth was controlled by *Ulva* since no significant increase was recorded after 21 days ($P < 0.05$). *Rosoebacter* clade specific primers of the 16S DNA gene were used for estimation of the growth of *Roseobacter* species. The qPCR data was in agreement with the result of DGGE analysis of *Roseobacter* pattern in the tripartite community (Fig. 19). Herewith, one could somehow predict that *Cytophaga* sp. followed the same growth pattern as was seen in DGGE of
Results and Discussion

bioreactor culture (Fig. 19). Certainly, Cytophaga sp. provided the morphogenetic compounds in aquacultures as normal Ulva thallus development and cell wall formation could be observed. The prospective unknown factor released by Cytophaga sp. was quantified in the growth media of the aquacultures through an accompanying study (Grüneberg and Wichard, unpublished results). Indeed, the morphogenetic factor accumulated in the medium. As this factor has not been found so far in any other marine bacteria strains (Spoerner et al., 2012), this chemical analysis was a good proxy for the presence of Cytophaga throughout the whole cultivation. It is notable that bacterial and algal culture achieved a stationary phase.

Figure 36: Estimation of culture growth in the defined bacterial community. (a) U. mutabilis growth as a function of time in aquacultures by measuring length of thallus. Values are expressed as mean ± SD, n = 3. (b) Roseobacter clade including Roseobacter sp. by qPCR. DNA was extracted from filters (0.2 µm pores size) upon filtration of Instant Ocean. Products of 365 bp length were quantified. Data on day-28 is not available. Values are expressed as mean ± SD, n = 3. Different letters indicate significant difference between concentrations over time (Tukey-posthoc test; P < 0.05).

3.5.6. Nutrients depletion and excretion in growth media of the defined bacterial community

Nitrate, nitrite, and phosphate were measured weekly over time course of the experiment in the growth media of the defined bacterial community. The concentration on day-zero represents the concentration of the growth medium before inoculation of bacteria was performed (Fig. 37).
Results and Discussion

**Nitrate**

Nitrate concentration remained constant during a week after the inoculation, i.e. on day-7 (Fig. 37a). Indeed, Ulva did not grow much either during this lag phase. Then, on day-14, nitrate was depleted significantly ($P < 0.05$) (utilization rate of 56.6%). A week later, nitrate decreased slightly until day-28 ($P > 0.05$). At this point, 95% of the total initial amount of nitrate was already depleted; however Ulva continued growing till day-35, when nitrate was not detectable overall in the growth medium (Fig. 37a). In comparison, nitrate was depleted completely after 28 days in bioreactor cultures (Fig. 20a). According to the recommended value (85 mg/L) by Stratmann et al. (1996), the initial concentration of nitrate in both bioreactors and aquacultures, was respectively at the same value (85 mg/L) (Fig. 20), or at the optimum value (100 mg/L) (Fig. 37a). Surprisingly, no extreme difference was observed between nitrate depletion in aquacultures and bioreactors (cf. section 3.3.3) although in aquacultures other microorganisms in addition of the model organisms might utilize nitrate. This finding reflects the strength of the model system in controlling the community and the competition with other organisms. As nitrate uptake would result in nitrite release, external nitrite was measured.

**Nitrite**

Nitrite was released by U. mutabilis during the first weeks of culture reaching a significantly higher concentration in the medium on day-14 ($P < 0.05$) (Fig. 37b), before being taken up again on day-21. It was depleted completely by day-28. The increase of external nitrite concentration was associated to the decrease of nitrate concentration (Fig. 37a). Regarding the result of Bona (2006) in U. rigida, the reduction of NO$_3^-$ to NO$_2^-$ mediated by nitrate reductase is dependent mainly on photosynthetic activity. A high net photosynthesis rate increases intracellular levels of NADH, which is the physiological electron donor for NO$_3^-$ reduction in green algae (Azuara and Aparicio, 1985; Bona, 2006). For this reason, it is supposed that the high concentration of nitrite on day-14 and day-21 was due to the high activity of photosynthesis.
Results and Discussion

**Phosphate**

With similar initial concentration as added into UCM in bioreactors (6.69 mg/L) (Fig. 20), the initial concentration of phosphate in aquaculture was (7.00 mg/L) (Fig. 37c). Phosphate concentration decreased gradually over time. The significant decrease was remarked on day-14 ($P < 0.05$) by an utilization rate of 49.5% compared to 45% in bioreactors on day-28 (Fig. 20a). After that, fluctuation in concentration was observed but still globally decreasing reaching the utilization rate of 87% by the end on day-49 (Fig. 37c). The early consumption of phosphate in aquacultures might be due to the coexisting of other organisms in addition to the inoculated organisms in aquacultures that certainly shared the phosphate consumption (e.g., diatoms).

The differences in the utilization rates (UR) of phosphate and nitrate confirmed that nitrate was still the limiting growth factor as it was proven for the bioreactor cultures (*cf.* section 3.3.3). It can be seen from the above analysis, that nitrate approached an utilization rate of 56% on day-14 and it was completely consumed after day-28. Conversely, only 50% of phosphate was utilized on day-14 and no complete depletion of phosphate was recorded over time.

3.5.7. Regulation of life cycle: regulation of gametogenesis by sporulation and swarming inhibitors

The vegetative state (from day-14 to day-35) of *U. mutabilis* lasted three weeks in the defined bacterial community, hence the activity of sporulation (SI-1a and 1b), and swarming (SWI) inhibitors were measured in the growth media (Fig. 38). The activity of SIs was not detected overall in the growth media, whereas SWI activity was detected on day-21 (1.8 unit/mL) and remained constant ($P > 0.05$) during the next two weeks (1.9 and 2.0 units/mL, respectively). After 35 days, no activity of SWI was detected in the growth media (Fig. 38).

As SWI-activity was recorded on day-21 for the first time, it indicates that the SWI was released at the earliest three days before when certain individuals went through the onset of gametogenesis spontaneously after 18 days (Wichard and Oertel, 2010). Similarly, the activity of SIs was detected in bioreactors neither in axenic nor in tripartite communities (Fig. 21). Nevertheless, the activity of SWI was detected on day-21 as in the defined bacterial community.
Results and Discussion

Figure 37: Weekly changes in nutrient concentration in aquaculture of *U. mutabilis* maintained in Instant Ocean. (a) Nitrate. (b) Nitrite. (c) Phosphate. Values are expressed as mean ± SD, n = 3. Different letters indicate significant differences between concentrations over time (*Tukey-posthoc* test, *P* < 0.05).

Figure 38: Weekly changes in sporulation (sum SI-1a+1b) and swarming (SWI) inhibitors in growth media of the defined bacterial community. Error bars based on mean values ± SD (n = 3). Different letters above the bars indicate significant differences of SWI activity throughout cultivation (*Tukey-posthoc* test, *P* < 0.05).
The clear differences between SWI in bioreactors and aquacultures were as follows; (1) The highest activity of SWI in aquacultures growth media (2 units/mL) was 2 times lower than the highest activity in bioreactors (approaching 4 units/mL). (2) After 35 days, the activity of SWI was not detected anymore in growth media of aquaculture and decreased to 50% in bioreactors and continued at the same rhythm till the end. It is suggested thus that SI-1 was not detectable in the defined bacterial community due to the same reason which was addressed with the tripartite community (cf. sections 3.3.4), which was in general attributed to the bacterial utilization and the rapid turnover of SI-1 (Wichard and Oertel, 2010). Together with gametogenesis induction assay, the status of gametogenesis inducibility of *U. mutabilis* in the defined bacterial community could be described as follows: The ‘’Non-inducible status’’ lasted one week after inoculation (= day-7). The ‘’inducible status’’ started from day-14 till day-21. Afterward, the ‘’spontaneously inducible status’’ was observed on day-28 till the end.

In comparison with the gametogenesis status of *U. mutabilis* in the tripartite community, it can be clearly noticed that the statuses of gametogenesis inducibility in the tripartite community (cf. sections 3.3.5 and 3.3.6) was successfully achieved in the defined bacterial community.

**3.5.8. A comprehensive view of the biological metadata profile of the defined bacterial community in aquaculture and of the tripartite community in bioreactors**

The comparison of the metadata of the defined (aquacultures) and tripartite (bioreactors) communities was an approach demonstrating that the scaling-up resulted in predictable and comparable data using a defined feedstock for inoculation (Tab.13).

**Growth phases:** In short, the longitudinal growth in the tripartite community was increasing over time, whereas in the defined bacterial community the growth decreased significantly after 35 days because the thalli were torn to two parts. For the reason, the declining phase was observed in the defined bacterial community after day-35 in contrast to the tripartite community, where the growth did not approach this phase within the experimental time.

**Gametogenesis inducibility status:** importantly, no difference was noticed in time spent for each status of gametogenesis inducibility in *U. mutabilis* in the defined bacterial and the tripartite communities. Each status of gametogenesis inducibility started at the same time in both communities.
Results and Discussion

**Nutrients depletion**: consumption in general was higher in the defined bacterial community than in the tripartite community due to the presence of others organisms in aquacultures (i.e., the defined bacterial community). Nitrate was evidently the limiting growth factor in both communities.

**Bacterial community**: the growth trend of *Roseobacter* sp. in both defined and the tripartite community seemed to be similar and toward an increase. Thus, the growth trend of *Cytophaga* in the defined bacterial community might be somehow as same as in the tripartite community, assuming that *Cytophaga* sp. tended to grow slowly by day-42 as it was observed in the tripartite community (cf. section 3.3.2, DGGE). In summary, the metadata of the defined bacterial community had a very good reproducibility, robustness, and recovery when compared with metadata of the tripartite community.

Table 13: Comparison between the metadata collected during the metabolomic analysis of the defined bacterial community in aquacultures and of the tripartite community in bioreactors.

<table>
<thead>
<tr>
<th>Time spent in each status of gametogenesis</th>
<th>Inducibility (days)</th>
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<tr>
<td>&quot;Non-inducible&quot; gametogenesis</td>
<td>&quot;Inducible gametogenesis&quot;</td>
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</tr>
<tr>
<td>Bioreactors</td>
<td>0-7</td>
</tr>
</tbody>
</table>

Thallus length (cm)/ Inducibility status

<table>
<thead>
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<td>Bioreactors</td>
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<td>9-12</td>
<td>16-25</td>
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</table>

Nutrient utilization rate (%)/ Inducibility status*

<table>
<thead>
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<tr>
<td>Bioreactors</td>
<td>NO$_3$</td>
<td>34</td>
<td>67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>PO$_4$$^{3-}$</td>
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<td>10</td>
<td>84</td>
</tr>
</tbody>
</table>

Bacterial community/ Inducibility status**

<table>
<thead>
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<th>strong</th>
<th>strong</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cy</td>
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<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bioreactors</td>
<td>Ro</td>
<td>strong</td>
<td>strong</td>
<td>strong</td>
</tr>
<tr>
<td></td>
<td>Cy</td>
<td>strong</td>
<td>strong</td>
<td>Faint</td>
</tr>
</tbody>
</table>

* Utilization rate was calculated using equation (2). ** Ro: *Roseobacter* sp., Cy: *Cytophaga* sp. n.d.: not determined.
3.6. The chemosphere of the defined bacterial community

3.6.1. Metabolite profiling in the chemosphere of the defined bacterial community using LC-MS analysis

As same as the downstream process shown in figure 24, the waterborne metabolites were collected by MarkerLynx™ software tool with the same parameters used for collecting the metabolites from the LC-MS chromatograms (positive ion mode) of the growth media in tripartite community (cf. sections 3.4.2 and 3.4.3). In addition, samples that were collected from tide-pool dominated by *Ulva* and tide pool not dominated by different macroalgae. Both tide pools were located in the Ria Formosa nearby the marine station Ramalhete (cf. section 5.2.3) and sample were used in order to compare the waterborne metabolites found in natural sources (tide pools) with those determined in aquacultures consisting of Instant Ocean inoculated with the model organisms. This resulted in 1429 metabolites, which were analyzed directly by unconstrained chemometric (i.e., PCA) and by constrained chemometric (i.e., CAP analysis) after preprocessing the dataset.

3.6.2. Effect of unconstrained analysis on metabolites obtained by LC-MS analysis

The PCA scores plot shown in figure 39 shows the samples collected from the defined bacterial community in aquacultures over experimental time (49 days) and the samples collected from the tide pools. Moreover, PCA scores plot shows that 37% of the variability could be explained by component 1, compared to 22% of the variability represented by component 2. The separation can be observed mainly via component 1 between the defined bacterial community on day-zero and the other sampling time points. Similarly, the defined bacterial community on day-7 was separated via component 1 from the other sampling time points. Via component 2, only the defined bacterial community on day-14 and day-21 were separated from the other sampling points. An accumulation between the rest of sampling points (i.e., from day-35 till day-49) along with pools samples can be clearly noticed.
Figure 39: PCA scores plot of samples based on metabolites obtained by LC-MS analysis (positive mode) \((n = 1429)\) and detected in the chemosphere of the defined bacterial community in day-zero \(\bigcirc\) (before inoculation), day-7 \(\bigcirc\), day-14 \(\bigcirc\), day-21 \(\bigcirc\), day-35 \(\bigcirc\), day-42 \(\bigcirc\), and day-49 \(\bigcirc\), in addition to the samples of tide pools \(\square\) under specific pretreatment (Pareto scale, intensities are expressed as height, and normalized to the sum of the peak intensities \(\text{TIC}\) in each chromatogram).
In total, there were four clear groups distributed via component 1 and 2 (Fig. 39).

**Group 1**: the defined bacterial community on day-zero (before inoculation),

**Group 2**: the defined bacterial community on day-7,

**Group 3**: the defined bacterial community on day-14 and day-21,

**Group 4**: the defined bacterial community on day-35 till day-49 in addition to the samples collected from the tide pools. From this separation, three considerable notes can be concluded:

1. Growth medium on day-zero can be considered as a blank sample and can be hence subtracted from all measured chromatograms in order to eliminate any signals due to impurities in aquaculture tanks, medium and due to the added vitamins.

2. The preliminary groups separation resulting from PCA matches the inducibility status\(^{10}\) of the gametogenesis i.e., ”non-inducible status”, “inducible “and “spontaneously inducible” gametogenesis (Tab. 13).

3. Regarding the fundamental idea of unconstrained analysis (PCA), the differences between groups can be masked (Anderson and Wills, 2003). Thus, discriminant analyses (e.g., CAP) was needed to uncover such masked differences.

3.6.3. **Effect of constrained analysis on metabolites obtained by LC-MS analysis**

After preprocessing the dataset, 330 metabolites (23% of the total metabolites = 1429) were analyzed by the CAP. The \textit{a priori} groups were generated based on (1) the statuses of gametogenesis inducibility of \textit{U. mutabilis} in the defined bacterial community and (2) the result of PCA plot score (Fig. 39). As a consequence, the \textit{a priori} groups were:

**Group 1**: the defined bacterial community on day-7,

**Group 2**: the defined bacterial community (sampling events from day-14 till day-21),

**Group 3**: the defined bacterial community (sampling events from day-28 till day-49) with tidal pools samples.

The CAP analysis was run based on the Bray-Curtis distance after transformation and standardization (Fig. 40). The significant contribution to the CAP axes shows that 110 biomarkers out of 330 metabolites (i.e., 8% of the total metabolites) were found to be of high

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\(^{10}\) The definition of the inducibility statuses is found in page 66.
Results and Discussion

relevance for separation the a priori groups (= misclassification error of 6%). The two axes are very efficient in separating the groups (high eigenvalues) and these axes are highly related to differences between the groups (high correlation $\delta_1^2 = 0.99$ and $\delta_2^2 = 0.98$) (Tab. 14).

From the CAP plots (Fig. 40) and the heatmap (Tab. 15), the distribution of some common biomarkers can be clearly observed between the first and second a priori groups e.g., biomarkers #21, #315, and #319, meaning these biomarkers characterized these two groups equally. Furthermore, few biomarkers were found to characterize both a priori groups 2 and 3 e.g., biomarkers #241, #263, and #316. At the same time, a unique aggregation can be noticed for the biomarkers characterizing a priori group 3 and natural pools (Fig. 40b). As the a priori groups also represented the statuses of gametogenesis inducibility, the inducibility statuses were hence used in the subsequent description and discussion instead of a priori groups. Interestingly, none of the biomarkers found during either “non-inducible” or “inducible status” were detected in pools samples (Tab. 15). However, the mechanism of presence/absence used in characterizing the groups can be clearly noticed during the growth when the “inducible gametogenesis status” began e.g., biomarkers (#147, #167, #171) and when the “spontaneously inducible status” was feasible e.g., biomarkers (#47, #106, #130, and #137). Nevertheless, the significant changes mechanism of the biomarker abundance throughout the samples can be represented by some biomarkers found during the last inducibility status e.g., #104, and #305. The special aggregation of biomarkers which was observed when the gametogenesis was induced spontaneously along with tidal pools samples, suggesting that some metabolites which released during this particular inducibility status (e.g., biomarkers: #224, #248, and #250) were not specifically related to the status of “spontaneously inducible status” and might be a result of decomposition the tissues.
Results and Discussion

Figure 40: Groups correspond to the three key gametogenesis-states\textsuperscript{11} in \textit{U. mutabilis} during its growth. Separation was based on the metabolites (\(n = 330\), 23\% of the total metabolites) obtained by LC-MS analysis (positive mode) and found in the chemosphere of the defined bacterial community and of the samples of tide pools using Bray-Curtis dissimilarity distance. (a) CAP separation of the samples with growth corresponding to gametogenesis statuses as groups. (b) Correlations of the 110 (8\%) biomarkers with the two CAP axes, with absolute Pearson coefficient correlation \(|r| \geq 0.5\).

Table 24: Eigenvalues, canonical square correlation, and diagnostics statistics of the CAP analysis of the biomarkers obtained by LC-MS analysis (positive mode) and found in the chemosphere of the defined bacterial community throughout sampling time points and of the samples of tide pools.

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<th>Constrained canonical axes</th>
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<td>0.97</td>
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</table>

\textsuperscript{11} The definition of the inducibility statuses is found in page 66.
Table 15: The heatmap represents the abundance of the biological mean of biomarkers shown to be of high relevance by CAP analysis (n = 110) over 49 days in the chemosphere of the defined bacterial community in aquaculture, in addition to the samples of tide pools.

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<td>“Inducible status”</td>
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<td>“Spontaneously inducible status”</td>
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12 The definition of the inducibility statuses is found in page 66.
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</table>

*Results and Discussion*

G. *priori* group 2 (n = 21)

G. *priori* group 3 (n = 3)

G. *priori* group 3 (n = 50)
Results and Discussion

Color shades are representative of the biomarker abundance throughout the sampling time (row). * G: group. The darker the color, higher the abundance. The shaded area in yellow represents the common biomarkers found in two a priori groups.
Results and Discussion

Unlike the tripartite community in bioreactor cultures, both constrained (PCA) and unconstrained (CAP) ordination chemometric analyses revealed the same separation, which can be correlated to the inducibility status. Thus, a biomarker obtained by LC-MS analysis could be a considerable indicator to predict the time of the various statuses of gametogenesis inducibility of *U. mutabilis* in aquacultures. In addition, these excreted biomarkers might indicate (1) the defense and competition mechanisms against other organisms including diatoms and protists, which were common contaminants in non-sterile aquacultures (Figs. 35a-d), or (2) the symbiotic interaction between *U. mutabilis* and its associated bacteria. Therefore, the waterborne metabolites found in the chemosphere of both the defined and tripartite communities can be used as sensitive and valuable proxies to determine the status of the culture including the inducibility status of gametogenesis in *U. mutabilis*.

3.6.4. *Comparison between biomarkers obtained by LC-MS analysis in the chemosphere of the defined bacterial community in aquacultures and of the tripartite community in laboratory bioreactors*

A search for the common biomarkers released into the chemosphere of (1) the tripartite community in bioreactors, and (2) the defined bacterial community in aquacultures was performed. Changes in the inducibility statuses of gametogenesis in *U. mutabilis*, during its growth, were very similar in both communities. Thus, the comparison was being based on the common biomarkers that were excreted into the chemosphere during the growth of *U. mutabilis* corresponding to the statuses of gametogenesis inducibility (Tab. 16).

**Table 16:** Common biomarkers obtained by LC-MS analysis (positive mode) and found in the chemosphere of defined and tripartite communities in aquaculture and bioreactor, respectively. Here are shown only the biomarkers which contributed significantly to the CAP axes (Pearson correlation coefficient $|r| \geq 0.3$).

<table>
<thead>
<tr>
<th>m/z</th>
<th>RT</th>
<th>ID in the defined bacterial community</th>
<th>ID in the tripartite community</th>
<th>Status of gametogenesis inducibility of <em>U. mutabilis</em> in</th>
<th>The defined bacterial community (aquaculture)</th>
<th>The tripartite community (laboratory bioreactors)</th>
</tr>
</thead>
<tbody>
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<td>376</td>
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<td>induc.</td>
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</table>
Results and Discussion

Interestingly, the common biomarkers between the two communities were detected in the chemosphere at the same gametogenesis inducibility status of *U. mutabilis*, suggesting these biomarkers could be used as a sensitive proxy to predict the respective growth phase in the presence of its associated bacteria despite of variations in culture condition (i.e., sterile and non-sterile conditions). These pronounced biomarkers that were found in the chemosphere of both communities indicate that *Ulva* has really conditioned its chemosphere much more than other microorganisms in the defined bacterial community. Further investigation is needed to elucidate the structures of these biomarkers. The additional biomarkers that were found in the chemosphere of the defined bacterial community but not in the tripartite community might be a result of the non-sterile condition in aquacultures, where *U. mutabilis* needs to compete with other microorganisms for e.g., nutrition and to prevent biofouling on algal surface (Figs. 35a-d). Conversely, the biomarkers which were only detected in the chemosphere of the tripartite community might be utilized or decomposed by the other organisms found in the non-sterile defined bacterial community e.g., diatoms, and protists.
Results and Discussion

3.6.5. *Metabolite profiling in the chemosphere of the defined bacterial community using GC-MS analysis*

349 waterborne metabolites were obtained by GC-MS analysis and quantified by Met-IDEA. 98 metabolites out of 349 (28%) were identified by AMDIS, and the remaining 251 (72%) metabolites are still unknown. The identified metabolites are listed in table 17 to show their conservation over sampling points and in the natural pools as well. Mainly, the dominant *Ulva*-pools were pools (1) and (2). However, pools (3) and (5) were included to see the difference, if any existed, between the dominant and non dominant *Ulva*-pools. More details about the environmental factors, i.e., temperature, pH, salinity, size and depth of these pools are mentioned in material and method. The heatmap (Tab. 17) was divided based on the sampling time points and subdivided further based on the status of gametogenesis inducibility of *U. mutabilis* in the defined bacterial community (Tab. 13). The inducibility statuses were used in order to facilitate the comparison purpose between the chemosphere of the defined bacterial community and tripartite community as the gametogenesis inducibility of *U. mutabilis* in both communities were very similar.
Table 17: The heatmap represents the abundance of identified metabolites ($n = 98$, 28% of the total metabolites) over 49 days in the chemosphere of the defined bacterial community (three statuses of gametogenesis inducibility) and in natural samples (four pools). Metabolites are grouped according to the main substance classes.

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### Results and Discussion

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Amino acids (8%)

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Sugars (13%)

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145
### Results and Discussion

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## Results and Discussion

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<td>Dehydroascorbic acid dimer; L(++)-Ascorbic acid (BP)</td>
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<td>Adenosine</td>
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Color shades are representative of the abundance of each metabolite throughout the samples and sampling time points (row). The darker the higher the abundance of the metabolite. *non inducible status* (non induc.). If the reverse match score of the library identification (i.e., NIST, Golm, MPI, and Metabo) was lower than 800 and no standard was available, the metabolite identification is tagged with a “?”, and “??” if the score was lower than 700.
Results and Discussion

Throughout the 98 identified exo-metabolites, five classes can be seen in the heatmap (Tab. 17), Fatty acids (40% of the total identified metabolites), amino acids (8%), sugars (13%), sterols (5%) and all other metabolites (34%) were combined together. In general, the main difference between the chemosphere of the defined bacterial community and the natural samples is the abundance of the metabolites, which was higher in the defined bacterial community than in natural pools. For the scope of this chapter, a comparison between the chemosphere of the defined and tripartite communities in aquaculture and bioreactor, respectively, was addressed in subsequent sections.

**Fatty acids (FAs)**

(1) 40% of the total identified waterborne metabolites were FAs in the chemosphere of the defined bacterial community (Tab. 17) whereas only 19% FAs were detected in the chemosphere of the tripartite community (Tab. 9).

(2) Excreted FAs varied between saturated (#190, #230, and #265), monounsaturated (#24, and #226), and polyunsaturated (#224, and #260) fatty acids in the chemosphere of the defined bacterial community (Tab. 17) as well as in the tripartite community (Tab. 9).

(3) PUFAs were more pronounced in the chemosphere of the defined bacterial community (54% of the total detected FAs), whereas in the chemosphere of the tripartite community PUFAs represent only 16% of the total detected FAs and the dominant FAs were the SFAs approaching 69%.

(4) FAs (#258, #260, #262, and #268) were detected in the chemosphere of the defined bacterial community (Fig. 41) and represent an interesting pattern of C18 FAs (18:3 n-3, 18:2 n-6, 18:1, 18:0, respectively). Such pattern was not detected in the tripartite community.

(4) Interestingly, no complete depletion of FAs could be noticed in the chemosphere of both communities over 49 days.
Results and Discussion

Figure 41: TIC-Chromatogram of GC-MS analysis, containing TMS-derivatives of C18 fatty acids found in the chemosphere of the defined bacterial community on day-21.
Results and Discussion

**Amino acids (AAs)**

A significant difference was noticed between the total detected AAs in the chemosphere of the tripartite community (22%) (Tab. 9) and in the defined bacterial community (8%).

**Sugars**

(1) 13% of the total identified metabolites were detected as sugars in the chemosphere of the defined bacterial community while only 8% were found in the tripartite community.

(2) An accumulation of sugars was observed in the chemosphere of both communities during the time of spontaneous gametogenesis.

**Sterols**

Only one sterol whose structure is unknown was detected in the chemosphere of the tripartite community. However, 5% of the total identified metabolites in the chemosphere of the defined bacterial community were identified as sterols.

**Other metabolites**

Many different classes were combined in this group (i.e., organic acids and their derivatives, fatty acids methyl ester, organophosphorus compounds, etc.).

(1) Dicarboxylic acids were pronounced metabolites within this substances class and represented 21% in the chemosphere of the defined bacterial community, while 30% dicarboxylic acids were found in the chemosphere of the tripartite community.

(2) 2,4,6-Tribromophenol was detected in the chemosphere of the tripartite community, but not in the defined bacterial community.

**Unknown metabolites**

Besides the 98 identified metabolites, 251 (72%) of pronounced unknown metabolites were found in the chemosphere of the defined bacterial community compared to 323 unknown metabolites in the chemosphere of the tripartite community.
The high percentage of free fatty acids in the chemosphere of the defined bacterial community compared to that of the tripartite community in bioreactors might reflect the defense mechanism of such molecules taking place within the defined bacterial community as reported for diatom biofilm by Jüttner (2001). DellaGreca et al. (2010) reported that the freshwater unicellular green alga *Chlorella vulgaris* releases fatty acid into the culture medium. This result is in consistent with the result of the present study (Fig. 41) due to the fatty acids released by *C. vulgaris* so-called chlorellin are mixture of C18 fatty acids: stearic, oleic, linoleic, and linolenic acids (DellaGreca et al. 2010). Chlorellin at low concentration stimulated the growth of both algae: *C. vulgaris* and *Pseudokirchneriella subcapitata* (green microalga). But when the concentration was higher than 6.5 mg.L\(^{-1}\), chlorellin produced inhibitory effects on both species as a result of autoxic action (DellaGreca et al. 2010). Moreover, Desbois and Smith (2010) addressed a comprehensive overview of the antimicrobial potency and anti-inflammatory properties of various saturated and unsaturated free fatty acids including C18 fatty acids. They were shown to have broad spectrum of bioactivities such as antimicrobial activity (Benkendorff et al., 2005; Galbraith and Miller, 1973), anti-algal activity (Kakisawa et al., 1988; Alamsjah et al., 2009). In addition, Liaw et al. (2004) investigated the infochemical activity of fatty acids to regulate the bacterial swarming of *Proteus mirabilis*. Similar effects might hence happen to the motile marine bacteria in the defined bacterial community in addition of the potential defense mechanism.

However, due to the fact that these C18 fatty acids were not detected in the tripartite community (laboratory bioreactors), furthermore the defined bacterial community in aquaculture was not under sterile condition. One can argue that polyunsaturated fatty acids, notably C18 FAs, might be released by other organisms as well.

A further explanation is that (1) *U. mutabilis* does not need to release defense molecules under sterile condition i.e., bioreactor cultures in contrast to non-sterile condition like aquacultures, where *Ulva* requires to activate a defense mechanism to compete with and defend against other organisms. (2) The exo-metabolites reflect sometime the biosynthesis of such molecules in the organism, thus the C18 FAs which were found in the chemosphere of the defined bacterial community, reflect on the other hand the ability of *U. mutabilis* to produce
these fatty acids in vivo. This finding was proven earlier (cf. chapter 3.1) in addition it is consistent with the result found by Pereira et al. (2012), who concluded that linoleic acid (LA; C18:2 n-6) was the main PUFA among most chlorophytes, and Ulva sp. was the only exception, in which higher percentages of ALA (16%) was detected (Pereira et al., 2012). Moreover, ALA (18:3, n-3) was considered as a characteristic PUFA of the Ulvales (Khotimchenko et al., 2002; Kumari et al., 2010). (3) even more interesting, the results of DellaGreca et al. (2010); and Fergola et al. (2007) demonstrated that the concentration of charollin (a mixture of C18 fatty acids) is much higher (1.90 mg.L$^{-1}$) in the mixed culture (i.e., C. vulgaris and P. subcapitata) than in the single species culture of C. vulgaris (0.4 to 0.85 mg.L$^{-1}$). This result supported the idea that C18 fatty acids were not detected in the sterile tripartite community due to LOD. Further studies are needed to investigate the ability of U. mutabilis to release these C18 fatty acids e.g., upon stress.

In addition, the chemosphere of the defined bacterial community was distinct from the chemosphere of the tripartite community by the sterolic substances. However, sterols were found in low abundance (only 5%) in the chemosphere of the defined bacterial community compared to other chemical classes. Guschina and Harwood, (2006) reported that sterols usually undergo an intensive abiotic degradation; this might explain their low abundance in the chemosphere of defined bacterial community (aquacultures) and their absence in the chemosphere of the tripartite community (bioreactors). Ultimately, the comparison between the waterborne metabolites of the defined bacterial community in aquaculture and of the tripartite community in bioreactor revealed that:

(1) New class and compounds detected in the chemosphere of the defined bacterial community such as sterols and C18 fatty acids were not found in the tripartite community. On the other hand, some metabolites were pronounced only in the chemosphere of the tripartite community i.e., alcohols and 2,4,6-tribromophenol.

(2) The common classes, found in the chemosphere of both communities, were detected in higher abundance in the defined bacterial community than in the tripartite community e.g., FAs (40% in the defined bacterial community vs. 21% in the tripartite community) and sugars (13%
in the defined bacterial community vs. 8% in the tripartite community). Therefore, scaling up the cultures is recommended in order to obtain elevated amount of the exo-metabolites.

The large numbers of unknown metabolites (251, 72% of the total metabolites) released into chemosphere of the defined bacterial community is supposed to play a crucial role in the relationship between the model system organisms and their environment. Therefore, a discriminant analysis was needed in order to emphasize the potential contribution of such unknown as well as the known metabolites in chemosphere of *U. mutabilis* and its associated bacteria under unsterile condition.

### 3.6.6. Determined biomarkers in the chemosphere of the defined bacterial community: the known unknowns

The CAP analysis was applied on 349 waterborne metabolites. The *a priori* groups were generated based on the time spent in each status of gametogenesis inducibility of *U. mutabilis* in the defined bacterial community (Tab. 13). Thus, the *a priori* groups were:

- **Group 1**: the defined bacterial community on day-7,
- **Group 2**: the defined bacterial community between day-14 till day-21,
- **Group 3**: the defined bacterial community between day-35 till day-49,
- **Group 4**: All tidal pools samples (i.e., 1, 2, 3, and 5).

These groups were separated as shown in figure 42a with misclassification of 2% (Tab. 18), meaning that the groups were extremely distinct in the multivariate space (Fig. 42). The two axes were very efficient in separating the groups (high eigenvalues) and these axes were highly related to the differences between the groups (high square correlation $\delta_1^2 = 0.94$ and $\delta_2^2 = 0.82$) (Tab. 18). Furthermore, the permutation test confirmed that the groups had significant different locations in the multivariate space ($P = 0.001$, with 999 permutations). All biomarkers with Pearson correlation coefficient of $|r| \geq 0.5$ ($n = 100, 29%$ of the total metabolites) were plotted in figure 42b to investigate their contribution to separates the *a priori* groups based on the statuses of gametogenesis inducibility in *U. mutabilis* in the defined bacterial community.
Results and Discussion

Figure 42: Groups correspond to the gametogenesis-state in *Ulva* during its growth and to tidal pools samples. Separation was based on the metabolites \( n = 349 \), obtained by GC-MS analysis and found in the chemosphere of the defined bacterial community and tidal pools samples using Bray-Curtis dissimilarity. (a) CAP separation of the samples with statuses of gametogenesis inducibility and the samples of tide pools as groups. (b) Correlations of the 100 biomarkers (29% of the total metabolites) with the two CAP axes, with absolute Pearson coefficient correlation \( |r| \geq 0.5 \).

Table 18: Eigenvalues, canonical square correlation, and diagnostics statistics of the CAP analysis of the biomarkers obtained by GC-MS analysis and found in the chemosphere of the defined bacterial community and the samples of tide pools throughout sampling time points and tidal pools.
## Results and Discussion

<table>
<thead>
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<th>Permutatest</th>
</tr>
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<td>Eigenvalue</td>
<td>($\delta^2$) Correlation</td>
<td>Eigenvalue</td>
<td>($\delta^2$) Correlation</td>
</tr>
<tr>
<td>0.97</td>
<td>0.94</td>
<td>0.91</td>
<td>0.82</td>
</tr>
</tbody>
</table>
It can be clearly seen that the 100 biomarkers were characterizing the \textit{a priori} groups the best, resulting in two types of biomarkers: characteristic and common biomarkers.

Table 19: Model ion and retention time of biomarkers found in the chemosphere of the defined bacterial community over 49 days. Shown here all biomarkers ($n = 100$) which contributed significantly to the CAP axes ($|r| \geq 0.5$).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{A priori groups} & \textbf{Status of gametogenesis inducibility\textsuperscript{13} in \textit{U. mutabilis}} & \textbf{ID} & \textbf{Biomarkers}\textsuperscript{*} & \textbf{Model Ion} & \textbf{RT (min)} \\
\hline
\textit{A priori} group 1 (n = 4) & “Non-inducible gametogenesis status” & 116 & Unknown & 205.2 & 10.99 \\
& & 159 & Pentonic acid ? & 277.2 & 12.41 \\
& & 300 & Unknown & 149 & 17.68 \\
& & 311 & Unidentified PUFA ?? & 105.1 & 18.24 \\
\hline
\textit{A priori} group 2 (n = 24) & “Inducible gametogenesis status” & 68 & Unknown & 184 & 9.38 \\
& & 92 & Unknown & 179.1 & 10.19 \\
& & 95 & Unknown & 103.1 & 10.30 \\
& & 120 & Unknown & 199.1 & 11.14 \\
& & 132 & Unknown & 280.2 & 11.96 \\
& & 133 & Unknown & 217.1 & 11.50 \\
& & 135 & Unknown & 109.1 & 13.46 \\
& & 139 & Unknown & 123.1 & 13.77 \\
& & 148 & Glucose & 280.2 & 11.96 \\
& & 189 & Unknown & 109.1 & 13.46 \\
& & 198 & Unknown & 108.1 & 14.95 \\
& & 227 & Unknown & 309.2 & 14.72 \\
& & 228 & Unidentified MUFA ?? & 311.2 & 14.75 \\
& & 234 & Unidentified PUFA ?? & 108.1 & 14.95 \\
& & 253 & Unidentified SFA ?? & 143.1 & 15.59 \\
& & 254 & Mannitol & 319.2 & 15.60 \\
& & 271 & Unknown & 109 & 16.27 \\
& & 272 & Galactosylglycerol ? & 204 & 16.29 \\
& & 277 & Unknown & 194.1 & 16.55 \\
& & 289 & Unknown & 194.1 & 17.04 \\
& & 309 & Adenosine & 236.1 & 18.10 \\
& & 313 & Unknown & 204.1 & 18.49 \\
& & 337 & Unknown & 204.1 & 21.06 \\
& & 345 & Unknown & 129.1 & 22.77 \\
\hline
\end{tabular}
\caption{Model ion and retention time of biomarkers found in the chemosphere of the defined bacterial community over 49 days. Shown here all biomarkers ($n = 100$) which contributed significantly to the CAP axes ($|r| \geq 0.5$).}
\end{table}

\textsuperscript{13} The definition of the inducibility statuses is found in page 66.
## Results and Discussion

### A priori group 3 (n = 37)

“Spontaneously gametogenesis status”

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### Common biomarkers found in a priori groups 3 and 4 (n = 25)

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Common biomarkers found in a priori groups 3 and 4 (n = 25)
### Results and Discussion

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**A priori group 4 (= samples of tide pools: (1), (2), (3), and (4)) (n =3)**

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<tr>
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<td>149</td>
<td>15.26</td>
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</table>

**Common biomarkers found in a priori groups 1 and 4 (n = 1)**

|   | 307 Unidentified PUFA?? | 105.1 | 17.97 |

* SFA: saturated, MUFA: monounsaturated, PUFA: polyunsaturated fatty acid, AA: amino acid. If the reverse match score of the library identification (i.e., NIST, Golm, MPI, and Metabo) was lower than 800 and no standard was available, the metabolite identification is tagged with a “?”, and “??” if the score was lower than 700.

The visualization of the characteristic and common biomarkers among a priori groups resulting from the CAP analysis (Fig. 42) revealed that the biomarkers, which characterized the inducible reproductive state (4 biomarkers) were much fewer than those characterizing the growth phases when the statuses of “inducible gametogenesis” and “spontaneously inducible gametogenesis” were observed. Most biomarkers (approximately 92 out of 100) were found to characterize the growth phase when gametogenesis was “inducible” and “spontaneously inducible”, either as characteristic or as common biomarkers found during both inducibility statuses. Only three biomarkers characterized the tidal pools samples.
Results and Discussion

Surprisingly, sugars and amino acids did not contribute to the characterization of the early growth phase when gametogenesis was “non-inducible”. Unsaturated fatty acids were remarkable biomarkers during the time of “inducible status” compared to growth phases before and after the “inducibility status” (Tab. 19). However, the low molecular weight carboxylic acids were pronounced biomarkers during the growth when the spontaneously inducible status” (Tab. 19). The contribution of the 100 biomarkers to the characterization of the growth phases corresponding to the gametogenesis inducibility statuses depended either on the mechanism of (1) presence/absence or (2) the significant changes of the abundance over groups. For instance, biomarkers #116, and #234 were only detected respectively during the time of the “non-inducible” and “inducible” statuses (Figs. 43a,c), while biomarkers #228, #36, #186, and #229 exhibited significant changes in their abundance over time (Figs. 42b,d-f).

Interestingly, the unknown biomarkers provided an important contribution in characterizing the growth phases based on gametogenesis inducibility statuses as ca. 70 out of 100 biomarkers resulting from the CAP analysis are still unknown. Even more interesting, only the unknown biomarkers were found in the chemosphere of both defined bacterial and the tripartite communities (Tab. 20), providing the probability to use these common unknown biomarkers to indicate the growth phases of *U. mutabilis* and subsequently the status of gametogenesis inducibility under any condition (i.e., sterile or non-sterile condition).
Results and Discussion

Figure 43: The abundance trend of selected biomarkers as a function of time corresponding to the statuses of gametogenesis inducibility in *U. mutabilis* in the defined bacterial community. (a) #116: unknown biomarker characterizing the time taken for “non-inducible status”. (b,C) #228: unidentified MUFA, and #234: unidentified PUFA characterizing the time taken for “inducible status”. (d-f) #36: nonanoic acid, #186: trans-p-coumaric acid, and #229: tyrosine characterizing the time spent in “spontaneously inducible status”. Star indicates the status of gametogenesis inducibility characterized by the biomarker.

14 Definition of the inducibility statuses is found in page 66.
Results and Discussion

<table>
<thead>
<tr>
<th>Common biomarkers</th>
<th>Status of gametogenesis inducibility in <em>U. mutabilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT</strong></td>
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</table>

Among the *a priori* groups shown in figure 42, the biomarkers distribution reflected the duration of each inducibility status of gametogenesis in *U. mutabilis*. Within the scope of this chapter, the comparison between metabolic profiling in the tripartite community and the defined bacterial community using GC-MS analysis revealed that the growth corresponding to the status of gametogenesis inducibility in *U. mutabilis* had and influence on the chemosphere structure of both communities. Nevertheless, GC-MS is limited to volatile, thermally stable, and energetically stable compounds (Lei et al., 2011). It is less adjustable to large, highly polar metabolites due to their poor volatility. Some metabolites meet GC-MS requirement either by their native state e.g., short-chain alcohols, acid, esters and other metabolites or after derivatization e.g., primary metabolites, sterols and fatty acids. Due to the rapid turnover of primary metabolites, sugars were not shown to contribute in characterization of the growth when gametogenesis was not inducible in both communities (i.e., tripartite community and the defined bacterial community) although they play an important role in the community as carbon sources have to be provided by the macroalgae for feeding the heterotrophic bacteria. Fatty acids, notably MUFAs and PUFAs were remarkable biomarkers that discriminated between the growth phases corresponding to the inducibility statuses of gametogenesis in *U. mutabilis* under non-sterile conditions (i.e., the defined bacterial community) as they characterized significantly the phases when gametogenesis was either “non-inducible” or “inducible”. In contrast, MUFAs and PUFAs did not contribute to separate the growth phases in tripartite community. This finding might suggest the function of unsaturated fatty acids as molecular defense. 2,4,6-tribromophenol characterized the time when the spontaneous gametogenesis
Results and Discussion

was feasible in tripartite community, but it was not detected overall in the chemosphere of the defined bacterial community. As the defined bacterial community was non-sterile, it is suggested that tribromophenol underwent biodegradation process, which is in agreement with Aguayo et al. (2009), who reported the ability of bacterial communities in a lake to degrade tribromophenol.

In summary, metabolite profiling provided a preliminary understanding of the chemosphere structure during the changes of the growth and life cycle of *U. mutabilis* when grown with its associated bacteria *Roseobacter* sp. and *Cytophaga* sp.
4. Conclusion
Conclusion

The purpose of my PhD project was to investigate the chemosphere of the tripartite community consisting of *Ulva mutabilis* and its associated bacteria: *Roseobacter* sp. and *Cytophaga* sp. Therefore, I conducted the non-targeted metabolomic analysis to understand the structure of the chemosphere during the life cycle of *U. mutabilis* when grown with its associated bacteria. As polyunsaturated aldehydes (PUAs) play a crucial role in extracellular signalling between organisms and in chemical defense mechanism, I specifically performed the targeted metabolomic analysis to survey PUAs potential production in *Ulva* spp. collected at various sampling sites in the lagoon of the Riau Formosa (Portugal).

The survey of PUAs allowed me along with morphogenetic and phylogenetic analyses to reveal its chemotaxonomic significance. Only sea lettuce-like algae produced elevated amounts of PUA, whereas tube-like species did not release these compounds upon cell damage. Although PUAs were not determined in the growth medium in this study, they might affect the population dynamics of the phytoplankton, grazers as well as the microbial community during e.g., bloom events upon release by *Ulva*. Because of the general importance of PUAs in chemical ecology, the biosynthetic pathway was investigated in detail. Interestingly, *Ulva* can produce 2,4-decadienal via the octadecanoid and eicosapentaenoic pathway catalyzed via a 9-lipoxgenase/hydroperoxide-lyase and 11-lipoxgenase/hydroperoxide-lyase, respectively. *Ulva* uses hence the whole set of polyunsaturated fatty acids upon cell damage. The lipoxygenases/lyase mediated depletion of polyunsaturated fatty acids might jeopardize the commercial value of the algal biomass in land based aquacultures. Thus, it is suggested to consider this additional criterion for selecting the appropriate *Ulva* species. Here, *U. mutabilis* might be selected as feedstock to be cultivated under controlled condition to increase the concentration of a given product (e.g., PUFAs) in biomass production.

My results confirmed that *U. mutabilis*, only when grown with its associated bacteria, passes through three stages of life cycle. These stages are defined based on the inducibility status of gametogenesis: “non-inducible status”: young germling, “inducible status”: when gametogenesis is inducible in thalli upon removal the sporulation inhibitors and “spontaneously inducible status”: mature thalli. Thus, I hypothesize that:
Conclusion

“The chemosphere of the tripartite community changes throughout the growth phases of the macroalgae and biomarker of this exometabolome can be used to predict changes in the status of gametogenesis inducibility during the life cycle”

Over the growth phases, diverse metabolites and substance classes were released into the chemosphere including fatty acids, sugars, amino acids, and many other primary metabolites, in addition to the secondary metabolites. Although the axenic unialga *U. mutabilis* excreted metabolites into the growth media during the lag growth phase, but these metabolites did not undergo marked metabolic turnover and accumulated as a result over time. These results indicate that the associated bacteria influenced the excretion of algal metabolites and might reflect in turn the chemically mediated interaction between *U. mutabilis* and its symbiotic bacteria.

My research provides novel information about the chemosphere and its dynamics. The life cycle of *U. mutabilis*, defined by the inducibility status of the gametogenesis, is one of the essential factors influence the dynamics of the metabolites excretion into the chemosphere. Most of the metabolites were shown to be secreted into the chemosphere one week before the onset of spontaneous gametogenesis onwards. Whether these metabolites have an ecological relevance is not yet clear, further investigations with fractionation extracts might uncover their possible ecological role.

Scaling up the cultures in near-field experiment enabled me to detect some metabolites in the chemosphere of tripartite community such as C18:n fatty acids, which were not detected under the laboratory condition. However, conducting the metabolic survey under controlled laboratory conditions helped to conserve some metabolites, for instance 2,4,6-tribromphenol, from the decomposition *via* abiotic and biotic factors which were highly variable in field-near experiment. These identified biomarkers might play a crucial role in chemical-mediated processes within the chemosphere. I, therefore, recommend conducting algal metabolic survey under controlled laboratory conditions in addition to the field-near conditions in order to assess the metabolite profiling in a broad sense.

The combination between different instrumental analyses along with the chemometric data analysis shed light on potential biomarkers can be utilized in the future studied to predict the
Conclusion

life cycle of *U. mutabilis* under any cultivation condition, when all the morphogenetic and growth factors are available. This will help to maintain *U. mutabilis* in the vegetative stage as long as possible, particularly, in land based aquaculture. Thus, I propose that these biomarkers can be used to determine the time when the sporulation inhibitors should be added into the growth medium of the tripartite community and so the spontaneous gametogenesis in *Ulva* will be arrested.

Taking all this together, future research will be needed for

- Determination PUAs in the medium and testing their ecological relevance in structuring the microbiome.
- Structure elucidation of biomarkers, which were detected in the tripartite community under all the cultivation conditions conducted in this study, and testing them in bioassay to prove that these biomarkers can be used as a sensitive proxy to determine the change of the community, and/or the algal life cycle.
5. Materials and Methods
5.1. Chemicals and consumables

5.1.1. Analyses of Polyunsaturated aldehydes (PUAs) and polyunsaturated fatty acids (PUFAs)

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) and VWR (Darmstadt, Germany). UHPLC-grade methanol, acetonitrile, water, and formic acid were obtained from Biosolve (Valkenswaard, Netherlands). Deuterium labeled fatty acids, 5Z,8Z,11Z,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid ([²H₈]-ARA), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic-19,19,20,20,20-d₅ acid ([²H₅]-EPA), 9Z,12Z-octadecadienoic-9,10,12,13-d₄ acid ([²H₄]-LA), 5,8,11,14-eicosatetraynoic acid (ETYA) as well as the stearidonic acid (SDA, C18:4 n-3) were purchased from Cayman Chemical Company (Michigan, USA). Linolenic acid (LA, C18:2 n-3), α-linolenic acid (α-LEA, C18:3 n-3), and γ-linolenic (γLEA, C18:3 n-6) were purchased from Sigma-Aldrich. Ultra pure water (0.055 µS) obtained by a MicroPure water purification system (Thermo Scientific, Germany) was used for the preparation of aqueous solutions.

5.1.2. Nutrient analyses

Unless otherwise mentioned, all chemicals were obtained from Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany) or VWR (Dresden, Germany).

5.1.3. DNA extraction and downstream analyses

DNA extraction: QIAamp DNA Mini Kit (QIAGEN). EtOH(abs), VWR (Dresden, Germany). Polycarbonate filter (Millipore ISOPORE (TM), 0.2 µm GTTP 25 mm, Sigma Aldrich) in a polysulfone filter holder for syringes Polymerase chain reaction (PCR): ChromoSolv® water (filter-sterilized), Sigma Aldrich, 1.5 µl Bovine serum albumin (BSA) A7030, Sigma Aldrich. Forward primer (357fGC) Reverse (Biomers.net, Germany), Reverse primer (907rM), (Biomers.net, Germany). All other reagents were obtained from Fermentas. Quantitative real-time Polymerase chain reaction (qPCR): Sybr® Green (Invitrogen™, Carlsbad, CA, USA). Denaturing gradient gel electrophoresis (DGGE): Sybr® Gold (Invitrogen™, Carlsbad, CA, USA).
5.1.4. Metabolomics

The grade of the solvent used in all metabolomic analysis was: methanol (Chromasolv Plus >99.9%, Sigma-Aldrich). Acetone (HiPerSolv, VWR). Chloroform (HiPerSolv, VWR). Tetrahydrofuran (THF, HiPerSolv, VWR). Water (Chromasolv® Plus, Sigma-Aldrich). Pyridine (Chromasolv® Plus, Sigma-Aldrich). Ethanol (LiChrosolv®, Merck). Acetonitrile (ULC/MS, Biosolve, Valkenswaard, the Netherlands).

5.2. Strains

5.2.1. Ulva species collected for polyunsaturated aldehydes (PUAs) and polyunsaturated fatty acids (PUFAs) analyses

A total of 100 Ulva isolates were collected in the lagoon Ria Formosa (Portugal) in May 2010. Fully grown thalli (ca. 4 - 5 weeks old thalli) were carefully washed with filtered seawater, subsequently transferred to Ulva culture media (UCM) and cultured under standardized conditions without silicate in standing flasks (V = 200 mL) (Stratmann et al., 1996; Wichard and Oertel, 2010). The morphotype of the collected algae was determined according to taxonomic key (Brodie et al., 2007).

5.2.2. Ulva mutabilis and bacterial strains used in bioreactors and aquaculture

The laboratory strain of U. mutabilis mutant (slender-G[mt+]) is a direct descendant of the original isolates collected by B. Føyn (Føyn 1958). This strain was originally maintained in the presence of their natural microbial flora by A. Løvlie (University of Oslo). These two bacterial symbionts of U. mutabilis, Roseobacter and Cytophaga species were isolated and characterized as described in Spoerner et al. (2012).

5.2.3. Tide pools samples

Samples were collected from the beach at Ramalhete station and described in an early stage of this project by Dr. Thomas Wichard (Friedrich Schiller University of Jena).

Pool 1: T = 21°C, pH 8.0, salinity 39, floating Ulva sp., size: 2.5 m x 3.8 m, depth 9 cm
Pool 2: T = 19°C, pH 8.0, salinity 39, Ulva sp., Fucus sp., size 2.2 m x 2.7 m, depth 10 cm.
Pool 3: T = 22°C, pH 8.0, salinity 39, Blindigia sp., size 3.6 m x 2.9 m, Depth 14 cm
Pool 5: T = 21°C, pH 8.0, salinity 40, sea grass (certainly no Ulva in pool 5), size 10 m x 4.0 m, depth 8 cm.
5.3. Culturing

5.3.1 Culture Media

All lab experiments were conducted on *Ulva* culture medium (UCM), prepared as described in Stratmann et al. (1996). This medium is free from silicate with pH 8.2 prior autoclaving (121°C, 30 min, in Nalgene Polypropylene (PP) 1 L bottle).

Table 21: Ingredients of Ulva culture media (UCM).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>19.14 g.L⁻¹ NaCl; 7.28 g.L⁻¹ Na₂SO₄; 10 H₂O, 8.68 g.L⁻¹ MgCl₂; 6 H₂O, 1.24 g.L⁻¹ CaCl₂; 6 H₂O, 85 mg.L⁻¹ NaNO₃, 6.6 mg.L⁻¹ (NH₄)₂SO₄.</td>
</tr>
<tr>
<td>II</td>
<td>0.7 g.L⁻¹ NaH₂PO₄·H₂O, 8.8 g.L⁻¹ NaHCO₃, 10.0 g.L⁻¹ Tris-OH.</td>
</tr>
<tr>
<td>III</td>
<td>7.84 g.L⁻¹ KBr, 54.2 g.L⁻¹ KCl, 1.95 g.L⁻¹ SrCl₂; 6 H₂O</td>
</tr>
<tr>
<td>IV</td>
<td>668.4 mg.L⁻¹ EDTA, 1140 mg.L⁻¹ H₂BO₃, 199 mg.L⁻¹ FeSO₄; 7H₂O, 3.9 mg.L⁻¹ CuSO₄·5H₂O, 12.6 mg.L⁻¹ Na₂MoO₄·2H₂O, 36 mg.L⁻¹ MnCl₂·4H₂O, 44 mg.L⁻¹ ZnSO₄·7H₂O, 3.3 mg.L⁻¹ Co(NO₃)₂·6H₂O, 2.3 mg.L⁻¹ NH₄VO₃, 3.9 mg.L⁻¹ KJ, 263 µg.L⁻¹ Na₂SeO₃·5H₂O, 9.3 µg.L⁻¹ As₂O₃, 6.6 µg.L⁻¹ Na₂WO₄·2H₂O, 3.4 µg.L⁻¹ TeO₂, pH adjusted to 8.0 with NaOH.</td>
</tr>
<tr>
<td>V</td>
<td>0.05 mg.L⁻¹ B12, 0.2 g.L⁻¹ thiamine·HCl, 0.1 g.L⁻¹ niacin, 0.1 g.L⁻¹ Ca·panthothenate, 0.04 g.L⁻¹ pyridoxine·HCl, 0.01 g.L⁻¹ p-aminobenzoic acid, 5 mg.L⁻¹ biotin, 0.8 g.L⁻¹ thymine, 1.0 g.L⁻¹ inositol, 0.26 g.L⁻¹ erotic acid, 0.2 g.L⁻¹ folinic acid (citrovorum), 2.5 mg.L⁻¹ folic acid, 0.04 g.L⁻¹ putrescine. 2HCl, 5 mg.L⁻¹ riboflavin, 0.02 g.L⁻¹ pyridoxamine. 2HCl, 0.36 g.L⁻¹ choline·Cl.</td>
</tr>
</tbody>
</table>

For preparation UCM, 1L of solution I was supplemented with 10 mL of solutions II-IV and 2 mL of solution V. The pH was adjusted to 8.1 with HCl. Solution I was autoclaved; Solutions II-V were filter-sterilized. Ultra pure water (0.055 µS) obtained by a MicroPure water purification system (Thermo Scientific, Germany) was used for the preparation of culture media.
5.3.2. Culture conditions of *U. mutabilis*

The standard culture conditions for *U. mutabilis* were 20°C (measured in the culture dish), illumination 17 h from 04:00 to 21:00 h [middle European standard (summer time) (‘’long day’’)]. The light was provided by fluorescent tubes (Osram T8 36W 840).

5.3.3. Culture conditions of bacteria

*Roseobacter* and *Cytophaga* species were cultivated in full medium broth (Roth) at 21°C until usage.

5.3.4. Preparation of axenic *U. mutabilis* gametes

The gametogenesis induction and gametes purification were performed as described in the study of Wichard and Oertel, (2010); and Spoerner et al., (2012).

Briefly, the intact mature gametophytes of *U. mutabilis* mutant “slender” (3-4 weeks old) were induced for gametogenesis by mincing the thalli manually into 1–3 mm fragments using a herb chopper prior to washing in fine sieve with distilled water three times to remove the sporulation inhibitors (SI-1 and SI-2), the fragments were suspended in UCM growth medium in standard Petri dishes (40 mL) and exposed to standard growth conditions. In the morning of the third day after induction, the gametes were released by removal of the swarming inhibitor (SWI) by changing the medium and application of light.

For gametes purification, gametes were separated from accompanying bacteria by making use of their phototactic behavior. The gametes assembling at the brightest spot of the Petri dish were aspirated with a pipette in 2 mL Eppendorf tube. Afterward, the gametes were applied to the wide end of a 25-cm-long sterile Pasteur pipette containing sterile UCM. After about 20-30 min, the gametes assembled in the tip of pipette were collected. After repeating this procedure twice, the gametes were diluted till reaching the faint green color and used for inoculation of a sterile culture. Sterility was tested by plating gamete samples on agar plate (marine broth + 2% agar, Roth) and PCR reactions of 16S DNA. Axenic gametes (~ 6 x 10³ gametes) were incubated overnight in 250 mL sterile UCM in polycarbonate tissue culture flasks (BD Falcon, Franklin Lake, USA) in the dark, allowing settlement of the germ cells. Furthermore, incubation was conducted under standard conditions. The axenic germlings were used for further inoculations. Some
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culture flasks were supplemented with the symbiotic bacteria for one week. Then, the axenic germlings were used for further inoculations in bioreactors and aquacultures. Before inoculation, bacterial cultures were centrifuged (1 min, 5,000g) and bacterial pellets were washed three times by resuspension with sterile UCM. The Optical density (OD$_{620\text{nm}}$) of 0.0001 was used for inoculation.

5.4. Fatty acids (FAs) analyses and determination of polyunsaturated aldehydes (PUAs)

5.4.1. Direct measurement of PUAs with solid phase micro extraction (HS-SPME)

200 mg of the frozen Ulva sample, upon wounding by grinding under liquid nitrogen, was added to 250 µl Ulva culture medium (UCM) in 4 mL glass vial, spiked with 2 µl of 2-decanon (1 mmol.L$^{-1}$ in methanol) and directly a divinylbenzene/carboxen/polydimethylsiloxane-coated (50 µm) SPME fiber (Supelco, Taufkirchen, Germany) was introduced in the headspace. Extraction was performed for 15 min at room temperature at the physiological pH 8.2. Evaporation of the volatiles from the fiber was directly performed within the injection port (250 °C) of a ISQ GC-EI-MS (Single Quadrupole GC-MS Systems, Thermo Scientific, Bremen, Germany) equipped with a DB5 capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness, Agilent, Germany). The temperature program was 40 °C [3 min, splitless] ramped with 15 °C min$^{-1}$ to 300 °C. Unsaturated aldehydes were identified by comparison with reference standards (Pohnert, 2000).

5.4.2. In situ determination and quantification of polyunsaturated aldehydes

PUA quantification was performed as described previously (Wichard et al., 2005b). O-(2,3,4,5,6-pentafluorbenzyl) hydrazylamine hydrochlorine (25 mmol.L$^{-1}$, PFBHA-HCl) was added before thawing of the 100 – 200 mg (fresh weight) damaged Ulva thalli in few µl UCM. O-pentafluorobenzyl-oxime derivates were identified and quantified using a GC-EI-ToF (Time of Flight) Mass Spectrometer (GCT, Waters, Micromass, Manchester, UK) equipped with a DB5 capillary column (40 m × 0.25 mm internal diameter, 0.25 µm film thickness, Agilent) (Wichard et al., 2005b).
5.4.3. Direct measurement of oxylipins with UHPLC-ESI-ToF-MS

Under liquid nitrogen 100 - 200 mg of *Ulva* was ground and subsequently incubated in 500 µl UCM. After incubation for 15 min at room temperature, 500 µl of methanol were added to stop any further enzymatic reaction and the sample was centrifuged (14,000 rpm, 15 min). The supernatant was centrifuged again in order to remove left over cell debris and concentrated under nitrogen stream. 10 µl of the sample were immediately measured by UHPLC-ESI-ToF-MS (Acquity™ ultra performance liquid chromatography (Waters, Milford, MA, USA) coupled to a Q-ToF Micro mass spectrometer (Waters, Micromass, Manchester, UK). For determination of the 10-hydroxy-5,8-decadienoic acid on a 50 mm Acquity UPLC BEH C<sub>18</sub> column (2.1 mm, 1.7 µm), a solvent system of 0.1 % acetic acid in (mobile phase A) water and acetonitrile (mobile phase B) was used: 0 - 0.2 min: 0 % B, 4.2 min 100 % B, 5 min 0 % B with a flow rate of 0.6 mL min<sup>-1</sup>. For improved separation of 6-hydroxy-7-octenoic acid and 8-hydroxy-6Z-octenoic acid, the same solvent system was used but the gradient was adjusted on a 50 mm Phenomenex C<sub>18</sub> column (2.1 mm, 1.7 µm) to: 4 min 0-20 % B, 5 min 25 % B, 6 min 100 % B, 6.5 min 0% B with a flow rate of 0.45 mL min<sup>-1</sup>. For identification, the analytes were compared with synthetic standards (Barofsky and Pohnert, 2007).

5.4.4. Fatty acid analysis

100 – 200 mg (fresh weight) of algal samples were ground under liquid nitrogen and analyzed according to (Wichard et al., 2007; Pohnert et al., 2004): [<sup>2</sup>H<sub>27</sub>]-myristic acid was added as an internal standard (2 µL of a 10 mg mL<sup>-1</sup> solution in methanol) and the sample was treated with the methylation mixture (0.5 mL methanol/acetyl chloride, freshly prepared 20:1, v/v) and hexane (0.6 mL) in 1.5 mL glass vial. The mixture was heated for 10 min at 100 °C in pressure-resistant glass vials. After cooling in an ice bath, distilled water (0.5 mL) was added and the sample was vortexed for 1 min. For fast phase separation, the sample was centrifuged. The removed hexane layer was dried over sodium sulfate and directly analyzed on the ISQ GC-EI-MS system equipped with a DB5 capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness, Waldbronn, Germany). The temperature program was 60°C (1 min), 30 °C min<sup>-1</sup> to 120 °C, 5 °C min<sup>-1</sup> to 250 °C and 20 °C min<sup>-1</sup> to 300 °C (2 min). The fatty acid methyl esters were
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identified by comparison with commercially available standards (Sigma-Aldrich, Taufkirchen, Germany) and by evaluation of their mass spectra.

5.4.5. **Elucidation of the biosynthetic pathway of PUAs in Ulva with isotope labeled**

Precursors 100 µg of commercial available deuterium-labeled polyunsaturated fatty acids (1 mg mL⁻¹ in ethanol) were added into a 1.5 mL glass vial. The solvent was evaporated under a nitrogen stream, before 10-100 mg frozen fragments of Ulva were added and thawed in the presence of 200 µl UCM. Samples were worked up as described above and analyzed by GC-EI-MS and/or by UHPLC-ESI-ToF-MS. For inhibition of potential lipoxygenases, Ulva fragments were pre-incubated with 100 µmol.L⁻¹ ETYA at room temperature for 15 min before applying additional labeled PUFAs.

5.5. **Experimental set ups of bioreactors and quacultures**

Metabolomic analysis was conducted by the model system organisms *Ulva mutabilis* (slender-G[m+t]) and its associated bacteria *Roseobacter* sp. and *Cytophaga* sp.

5.5.1. **Bioreactor cultures (10 L and 25 L)**

Unless otherwise stated, all supplies were purchased from Roth (Karlsruhe, Germany).

**Bioreactor design**

Large volume cultures were grown in 25 L polycarbonate (PC) bottles (Nalgene, VWR, Dresden, Germany). The culturing vessel is shown in (Fig. 44). The bottles had one inlet (blue arrow), and two outlets (green and magenta arrows). The air inlet was connected inside the bottle to a glass tube with the exit at ~ 1 cm from the bottom of the bottle (Fig. 44a). Air was pumped by an aquarium air pump through a glass wool pre-filter and a sterile HEPA-Vent (Ø 50 mm, Whatman) filter for sterilization ((Fig. 44b-c). The outlet of the filter was connected to the inlet of the bottle, allowing bubbling of the cultures via the glass tube. For sampling, one end of a Teflon tube (inner Ø: 1 mm) was fitted through the outlet (1) of the bottle and the other end was lying the bottom of the bottle (Fig. 44d). Outlet (1) was connected via silicon tubing to a dripping chamber (Fig. 44e). This chamber, built by inserting a 1 mL PC syringe into a 2.5 mL PC syringe, preventing contact between the sterile liquid of the bottle and the liquid at the
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sampling outlet, then the chamber was connected to sterile polypropylene (PP) Luer tubing connecter (C65.1, female, Roth) via silicon tubing (Fig. 44f). This Luer tubing connecter was replaced after each sampling by a new sterile one. The outlet (2) of the bottle served as an air outlet and was connected to silicon tubing. This tubing was fitted to sterile HEPA-Vent (Ø 50 mm, Whatman) filter (Fig. 44g) and connected to opened stopcock Luer Lock (A) (S7396, Sigma Aldrich) (Fig. 44h) via silicon tube. A hose clamp was attached to the connecting tube (Fig. 44i) in order to control and create pressure to ease the sampling flow without using the stopcock.

Illumination was provided by a rack of 2 fluorescent tubes placed above the bottle with light period [04:00-21:00h]; 7-h and dark period [21:00-04:00h]. The distance between the bottle and the light rack was adjusted to have a PAR of 60-120 µmol photons m\(^{-2}\) s\(^{-1}\) at the middle of the bottle.

**Culture media preparation for bioreactors**

Before conducting the experiment, the bottles were bleached by liquid detergent overnight and acid washed (10% HCl) several hours. The bottles were then thoroughly rinsed with deionized water and left overnight filled with deionized water. The next day, the bottles were again rinsed with deionized water before attaching the transfer cap and tubing. The bottles were filled with *Ulva* culture medium (UCM) and autoclaved (121°C, 30 min). The bottles were first secured tightly with lids without holes. After all tubing, air filters, and other stuff were autoclaved (Fig. 44), they were connected to new lids with one inlet and two outlets and autoclaved (121°C, 20 min). Under sterile condition, the inoculation in sterile tissue culture flasks 250 mL were added by opening the lids and the bottles were immediately secured tightly by new sterile lids connected with all tubing, air filter and other stuff. The airflows were adjusted to ensure similar bubbling in all bottles (visual estimation). The inoculation cultures were then pumped by creating a slight vacuum in the large bottle. All bioreactors cultures were transferred to the algal chamber and incubated under standard conditions at 20 °C.
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Figure 44: Design of 25 L bioreactor culture in polycarbonate bottle. (a) Bubbling tube (Duran glass, Ø 4 mm). (b) Glass wool pre-filter. (c) Sterile HEPA-Vent (Ø 50 mm, Whatman) filter. (d) Sampling tube (Teflon, Ø 1 mm). (e) Dripping chamber, made of a 1 mL syringe inserted into a 2.5 mL syringe (PC). (f) Luer tubing connector (PP) (C65.1, female, Roth). (g) Sterile HEPA-Vent (Ø 50 mm, Whatman) filter. (h) Stopcock Luer Lock (A) (S7396-10EA, Sigma Aldrich). (i) Hose clamp to control the sampling flow. Inlet: air inlet. Outlet (1): sampling outlet. Outlet (2): air outlet.

Algal and bacterial culture preparation

Two treatments were studied: cultures of axenic algae and of the tripartite community. Both treatments were inoculated under sterile condition with axenic germlings prepared one week earlier in tissue culture flasks 250 mL as described in section (5.3.4) to reach the density of (ca. $5 \times 10^5$ germlings/ 25 L). In case of the tripartite community cultures, the bottles were inoculated first with associated bacteria ($\text{OD}_{620 \text{ nm}} = 0.0001$, reached after diluting the original measured $\text{OD}_{620 \text{ nm}}$), and then with axenic germlings. Three replicates of each axenic ($3 \times 25$ L)
and the tripartite community (3 × 25 L) were conducted. In parallel, two replicates of control (2 × 10 L) containing only UCM were performed.

**Sampling processing**

Sampling of the cultures was achieved as follows: the bottle was shaken gently by hands to ensure a homogenous culture. The air outlet was closed using hose clamp (Fig. 44i), resulting in the building of slight overpressure in the bottle. The sampling outlet (2) was then opened using Luer tubing connecter (Fig. 44f) and the culture was pushed out by the overpressure. The first 20 mL were discarded and then the culture was collected in 1 L glass bottles. The sampling outlet was finally closed by a new sterile Luer tubing connecter before re-opening the air outlet.

### 5.5.2. Aquacultures (200 L)

Aquacultures were conducted at Ramalhete Station, Faro, Portugal, in 2010 from 10th March until 5th May.

**Aquacultures set-up**

Nine of 200 L cylindrical and conical tanks, made of polyester resin reinforced with fiberglass (Fig. 45a) were used. The tanks before being used were washed with 10% HCl, fresh water, and then bleached with commercially available bleach liquid. The bleach liquid was removed; and fresh water was added and neutralized with an excess of sodium hyposulfate (Na$_2$S$_2$O$_3$) to prevent the presence of residual bleach, and washed with fresh water. After that, the tanks were filled with 10 μm filtered artificial seawater (33.2 g L$^{-1}$ of Instant Ocean, Aquarium Systems, Sarrebourg, France) in the rate of 1.7 L h$^{-1}$. The tanks were continuously aerated to avoid alga accumulation at the bottom which would affect significantly algal growth. The air line was filtered from moisture and bacteria through HEPA-Vent (Ø 50 mm, Whatman) filters (Fig. 45e), and injected to the tanks from the bottom via 6 mm diameter polyethylene hose ended in a glass Pasteur pipette (Fig. 45f). The tanks were covered with a Tygon film (Fig. 45b).
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Figure 45: Aquaculture set-up of 200 L in (a) cylindrical and conical tanks. (b) Tygon film. (c) Pump. (d) Silicon tubing (Ø int. 2mm × Ø ext. 4mm 228-0703 VWR). (e) HEPA-Vent (Ø 50 mm, Whatman) filters. (f) Inlet of air and outlet of samples. (g) The right direction where the disconnection should be made to get the samples.

Algal and bacterial culture preparation

Two different cultures were performed in aquacultures. “Inappropriate community cultures” (3 × tanks, cf. section 5.5.2) inoculated only with axenic germlings, defined bacterial community cultures (3 × tanks) inoculated with axenic germlings in addition to Roseobacter and Cytophaga species with same optical cell densities (final OD₆₂₀ nm = 0.0001) used in bioreactors. In parallel, three tanks were treated with only Instant Ocean as control. Axenic germlings were prepared in the laboratory at Jena University and transferred to Ramalhete Station. It is noteworthy, that the first sampling process (blank sample) took place before the inoculation.
5.6. **Collection of metadata for metabolomic analysis**

5.6.1. **Sampling of growth media**

*Bioreactors cultures*

Based on sampling process described in (4.3.3), one liter from each culture in addition of control was collected in 1L sterile Erlenmeyer flask. This process was repeated weekly on day (zero, 7, 14, 21, 28, 35, 42, and 49), and the extraction performed immediately to avoid any metabolomic alteration as possible. It’s worth mentioning that the first 1L on day-zero was collected from the tripartite community after the bacterial inoculation and before *U. mutabilis* inoculation in both axenic and the tripartite community.

*Aquacultures*

Three liters from each tank were collected weekly in foldable water carrier 5L (Fig. 45). Additional 200 mL was collected weekly for further analyses such as nutrient analyses, DNA extraction and swarming and sporulation inhibitors bioassay as described in sections (5.6.2, 5.6.3, and 5.6.5, respectively). Samples were filtered under sterile condition and kept at -80 °C until the end of the experiment. Once all samples were collected, they were extracted and analyzed.

5.6.2. **Nutrient analyses**

Phosphate and nitrate concentrations were determined in the growth media by photo spectroscopy according to (Parsons et al., 1984; Zhang and Fischer, 2006). The volume was adapted for measuring in 1 cm half micro cuvettes (Roth). Each sample was measured three times on a Specord M82 photo spectrometer (Carl-Zeiss, Jena, Germany). Nitrite was determined in addition in growth media of aquaculture.

Utilization rate was calculated according to (Miyamura, 2010):

\[
\text{Utilization Rate (100\%) } = \frac{C_i - C_a}{C_i} \times 100
\]

Where \(C_a\) is the concentration of the nutrient after specific week, and \(C_i\) is the initial concentration of the nutrient in the culture medium.
5.6.3. DNA extraction and downstream analyses

**DNA extraction, sequencing and phylogenetic analysis**

Total DNA was extracted from 20 mg of cultured algal material using a modified Cetyl trimethylammonium bromide (CTAB) based method for sequencing of the chloroplast located RuBisCo gen (rbcL). All rbcL sequences of all collected samples were obtained from genomic DNA using the following primer pairs: rbcL START - atg gct cca aaa act gaa ac (Shimada et al., 2003) and 1385r - aat tca aat tta att tct ttc c (Calie and Manhart, 1994). Sequences were blasted at GenBankTM for homologies and are available at NCBI (National Center for Biotechnology Information) with the accession no. from KJ417440 to KJ417458. Sequences were aligned with reference sequences obtained from GenBankTM (see Tab. 3 for GenBankTM accession numbers of reference sequences) using Geneious software (Version 4.8.2). The resulting data set consists of 29 taxa (including ten reference taxa) with 1299 sites of the rbcL gene sequence. The alignment was analyzed using Maximum Likelihood by the software PhyML implemented in Geneious software using default settings. Bootstrap analysis was performed in PhyML with 1000 replicates.

**DNA extraction from the growth medium**

Weekly and immediately after sampling under sterile condition, 10 mL of culture medium was filtered using a polycarbonate filter (Millipore ISOPORE (TM), 0.2 µm GTTP 25 mm, Sigma Aldrich) in a polysulfone filter holder for syringes, and stored in -80 °C till extraction using QIAamp DNA Mini Kit (QIAGEN). Based on the protocol of QIAmp DNA Mini and Blood Mini Handbook (2nd Edition, Nov 2007) with some modification, DNA was extracted from PC filter. Briefly, 600 µL Lysis Buffer (prepared by: 20 mg mL⁻¹ Lysozyme dissolved in TETeX (20 mmol.L⁻¹ Tris-HCl (pH 8.0) + 2 mmol.L⁻¹ EDTA + 1.2% Triton)) was added to the PC filter, and incubated at 37°C for at least 30 min. Then, 600 µL of Buffer AL, and 20 µL of Proteinase K were added respectively, and vortexed immediately and thoroughly for 15 sec. The samples were incubated at 56 °C for at least 30 min, followed by incubation at 95 °C for not longer than 15 min. 600 µL EtOH(abs) was added to the samples, and they were vortexed and centrifuged (1 min to remove drops), preparing solution (1). 700 µL of solution (1) was applied to QIAmp Mini spin columns without wetting the rim connected with collection columns, centrifuged at 8000 rpm for 1min,
and the filtrate was discarded. Then, the spin columns were placed in new collection columns. Further 700 µL of solution (1) was applied to QIAmp Mini spin columns, centrifuged at 8000 rpm for 1 min, and discarded the filtrate. After that, 500 µL of Buffer AW1 (guadinine hydrochloride) was added to the spin column, centrifuged at 8000 rpm for 1 min, and the filtrate was discarded. 500 µL of Buffer AW2 (70% EtOH) was added to the spin columns, centrifuged at 14,000 rpm for 3 min, placed in new collection columns, and discarded the filtrate. Again, spin columns were centrifuged at 14,000 rpm for 1 min. Spin columns were placed in clean 1.5 mL micro-centrifuge tubes, 50 µL of Buffer AE was added, incubated at RT for 1 min, and centrifuged at 8,000 rpm for 1 min. The filtrate was applied to the columns again, incubated at RT for 1 min, and centrifuged at 8,000 rpm for 1 min. DNA extract was frozen at -80 °C for Nanodrop, PCR and DGGE analyses.

**A quantification via Nanodrop technology**

For estimation the total DNA in growth medium of the tripartite community and axenic cultures, 1 µL of DNA extract was applied in pedestal of Thermo Scientific Nanodrop 2000 spectrophotometer. DNA was measured at the wavelength of 260 nm. The 260/280 nm ratio was calculated for each sample to assess sample purity. Once the ratio was 1.8, this indicated the purity of DNA extract. After each measurement the pedestal was cleaned by wipe. Waster was applied as a blank measurement.

**Polymerase chain reaction (PCR)**

PCR amplification was performed using 16S rDNA bacterial primers 357fGC (CGC CCG CCG CGC GCG GCC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) and 907rM (CCG TCA ATT CMT TTG AGT TT) (Muyzer et al., 1995). Primers were obtained from (Biomers.net). Amplification method of DNA extract was applied according to (Sneed and Pohnert, 2011b). Briefly, each 50 µL PCR reaction contained in addition to 1 µL of DNA extract (was added at the end after all reagents, primers, and buffers), 1.5 µL bovine serum albumin (BSA) (20 mg mL⁻¹) (Sigma Aldrich), 1 µL dNTP mix (10 mmol L⁻¹), 5 µL DreamTaq™ Buffer (10x), 2 µL of each primer (10 µmol L⁻¹), and 0.5 µL DreamTaq™ DNA Polymerase, and 37 µL of MicroPure water from
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purification system (Thermo Scientific, Germany) (water was autoclaved and additionally filter-sterilized).

Amplification was performed using a TGradient Thermocycler (Biometra, Germany) beginning with an initial denaturation step (5 min at 95 °C), followed by 10 touch-down cycles, lowering the annealing temperature by 1 °C each cycle beginning at 65 °C (1 min at 95 °C, 1 min at 65 °C, 2.5 min at 72 °C) and then 25 cycles with an annealing temperature of 55 °C (1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C). A final extension step at 72 °C for 3 min completed the amplification. PCR products were quantified by comparison with the GeneRuler™ Express DNA Ladder run on a 1% agarose gel (Sigma Aldrich, Germany) (Sneed and Pohnert, 2011b).

**Quantitative real-time Polymerase chain reaction (qPCR)**

DNA extracts, based on extraction method described in DNA extraction from the growth medium, were applied to qPCR analysis (with minimum concentration of total DNA 10 ng µL⁻¹) to quantify *Roseobacter* species in the defined bacterial community. In addition a pure DNA extract from *Roseobacter* species was provided as a positive control of serial dilution as follows: 1 ng, 100 pg, 10 pg, and 1 pg.

qPCR reagent Sybr® Gold (Invitrogen™, Carlsbad, CA, USA) was used according to the manufacturer’s instructions and 8 pmol per reaction of the following primers were added: Forward primer (ROSfw), 5’–GATTTGCATTCAGGAGGTCA–3’; and reverse primer (ROSrev), 5’–GTTAGTGTACTTGACTTGGAC–3’ (both were obtained from Biomers.net), and used to quantify *Roseobacter* clade (Soller et al., 2000) in the samples. The PCR amplification (5 min at 95 °C, and then 40 cycles of two steps consisting of 15 min at 95 °C, 58 min at 60 °C) was performed with StepOne Real-time PCR System (Applied Biosystem, USA). All samples were processed for melting curve analysis.

For calibration curve, cycle threshold (C_T) values were plotted vs. log concentrations of the positive control. Afterward, the exact abundance of *Roseobacter* clade was quantified using the calibration curve.
**Denaturing gradient gel electrophoresis (DGGE)**

Denaturing gradient gel electrophoresis (DGGE) was conducted based on (Sneed and Pohnert, 2011b) using a DCode™ Universal Mutation Detection System (Bio-Rad). Standards were prepared by combining the PCR products of the direct amplification of six known bacterial strains (*Cytophaga* sp., *Roseobacter* sp., and *Halomonas* sp., *Sulfitobacter* sp., *Dinoroseobacter shibea*, and *Celluphaga* sp.) so that each standard lane contained 200 ng of DNA from each bacterial strain. These standards were loaded at the sides and the center of the gels to monitor “band smiling” and for comparison between the samples and the standards across gels. Samples and standards were loaded onto an 8% acrylamide gel (acrylamide:bis-acrylamide ratio 37.5:1, v:v) with a denaturant gradient from 20 – 70% denaturant (100% denaturant contained 7 M urea and 40% formamide). Electrophoresis was run for 12 hours at 100 V and 60 °C. Gels were stained with Sybr® Gold (Invitrogen™, Carlsbad, CA, USA). Imaging was performed using the BioDocAnalyze (BDA) digital system (Biometra, Germany).

5.6.4. **Growth rate**

In tripartite and defined communities, the length of *U. mutabilis* was measured by ruler one week post inoculation (day-7). The length average of three thalli from each population was calculated and plotted as function of time. Fresh weight of thalli after removal water was measured on the scale from day-14. The relative growth rate (RGR) was calculated according to (Lüning, 1990; Olischlager et al., 2013):

\[
RGR \ (\text{per day}) = \frac{100 \cdot \ln(W_2 / W_1))}{(t_2 - t_1)}
\]

Where \(W_1\) = fresh weight (g) at time point 1, \(W_2\) = fresh weight (g) at time point 2, \(t_1\) and \(t_2\) = time in days. RGR is given in % per day. In axenic cultures, the growth was estimated by measuring the diameter of the callus-like colonies.

5.6.5 **Bioassays to investigate the gametogenesis inducibility in U. mutabilis**

The gametogenesis inducibility was investigated by following bioassays:

**Induction of gametogenesis in thalli**

On day-7 upon inoculation, the gametogenesis induction was tested artificially based on the procedure mentioned in section (5.6.5, *Induction of gametogenesis in thalli*). Up to five thalli
were picked up from each population over the sampling time points, chopped by forceps, rinsed three times with UCM, resuspended in new UCM in three Petri dishes (Ø = 10 mm), and incubated three days under standard growth condition. In the morning of the third day, UCM was changed and subsequently the swarming inhibitor (SWI) was removed. After exposing to the light, the process of discharging gametes from the gametangia was tested.

**Sporulation and swarming inhibitors assay**

The activity of SI-1 (sum of SI-1 and its active breakdown products) and swarming inhibitor was tested in the growth medium (n = 3) weekly according to the protocols (Stratmann et al., 1996; Wichard and Oertel, 2010).

**5.6.6. Environmental Variables**

**Temperature, Salinity and pH value** were measured only in aquacultures as they were maintained constant in bioreactors.

**Temperature, and Salinity** were measured with YSI 85 Oxygen Conductometer Salinity and Temperature (YSI, Ohio, and USA), whereas **pH value** was measured with Oxyguard pH 4/10 (WMT, USA).

**5.6.7. Statistical analysis**

For all metadata post-hoc comparisons were performed using Tukey’s HSD (honestly significant difference) test on the probability level of $\alpha = 0.05$ to determine pairwise differences between treatments (Minitab 16.2.4, USA).

**5.6.8. Microscopy**

Chosen and representative gametophytes of each population over the sampling time points were observed weekly with an inverted microscope (Leica DM IL LED, Germany). Pictures were taken with a digital firewire colour camera (DFC280, Leica, Heerbrugg, Switzerland).

**5.6.9. Filtration**

One liter collected from each bioreactor culture was filtered through a GF/C filter (glass microfiber, pores ~1.2 µm, Whatmann, VWR), by filtration under moderate vacuum (~500 mBar).
5.6.10. **Solid phase extraction (SPE)**

After filtration, 1 L filtrate was extracted using EASY® cartridges (Chromabond 3 mL, polar modified polystyrene-divinylbenzene copolymer, 200 mg, Macherey-Nagel, Düren, Germany). Cartridge was first conditioned with 5 mL methanol and 5 mL water. The filtrate in Erlenmeyer flask was passed through the EASY® cartridges, via Teflon tubing, at 1 L hour⁻¹. After washing with 5 mL deionizer water, the EASY® cartridges were air-dried, and eluted by gravity with 4 mL of methanol:THF (1:1) in 4 mL glass vial. Vials were closed with caps fitted with PTFE-butyl-PTFE septa (VWR). 1 mL was dedicated to UPLC/ESI-MS analysis (5.7.1), and 3 mL was dedicated to GC-MS analysis (5.7.2).

5.7. **Instrumentation and chromatographic conditions**

5.7.1. **UPLC/ESI-MS analysis**

A Waters Acquity Ultra Performance LC equipped with 30-mm Fortis UPLC C18 column (2.1 mm, 1.7 µm) at a column temperature of 21°C was used for separation. The injection volume was 10 µL, and each sample was injected three times. The mobile phases were A = 0.1% formic acid and 2% acetonitrile in water and B = 0.1% formic acid in acetonitrile. The linear LC gradient with a flow rate of 0.6 mL min⁻¹ was ramped within 7 min from 0 to 100% B, and then held till for 2 min at 100% B, then 9.5 min at 0% B, and 10 min at 0% B. The outlet of the diode array detector was coupled to a Q-TOF micro–mass spectrometer (Waters) operated with an ESI source in positive TIC mode with a scan rate of 1 scan s⁻¹, an interscan delay of 0.1 s, and a scan range from 100 to 1000 m/z (Barofsky et al., 2010).

5.7.2. **GC/ES-TOF-MS analysis**

Metabolomic analysis by Gas Chromatography coupled with mass spectrometry was adapted from the protocol of Hiller et al. (2009), Vidoudez and Pohnert (2012).

**Derivatization**

Five µL of internal standard (ribitol, >99%, Sigma-Aldrich, 4 mmol.L⁻¹ in water) was added to a volume of 1.5 mL of each sample. After vortexing for 5 min, the samples were sonicated for 10 min in an ultrasound bath. For each batch (20 samples), 20 µg of methoxyamine hydrochloride (Sigma-Aldrich) was dissolved in 1 mL of pyridine by sonication for 5 min in an ultrasound bath.
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Fifty microliters of this solution was added to each sample of a batch and the vials were closed with caps fitted with PTFE-butyl-PTFE septa (VWR, Germany). After vortexing for 1 min, the samples were incubated at 60°C for one hour followed by 9 hours at room temperature. Sylilation reagent was prepared by adding 40 µL of retention Index (RI) mix (decane, pentadecane, nonadecane, octacosane, dotriacontane, all at 1 µmol.L⁻¹ and hexatriacontane at 0.5 µmol.L⁻¹ in hexane, all >99%, Sigma-Aldrich) with a glass syringe into 1 mL of N-methyl-N-trifluoroacetamide (MSTFA, in 1 mL vials, Macherey-Nagel, Düren, German). An n-alkane (RI) mix was analyzed to allow reproducibility of the measurements. Fifty microlitres of this sylilation reagent was added with a glass syringe into the samples before incubating them for one hour at 40°C. The samples were then transferred into glass inserts of 1.5 mL vial and were immediately analyzed by GC-MS.

**GC-MS parameters**

A Waters GCT premier (Waters, Manchester, UK) orthogonal reflectron time-of-flight (TOF) mass spectrometer (MS) coupled to an Agilent 6890N gas chromatograph (GC) equipped with a DB-5ms 38 m column (0.25 mm internal diameter, 0.25 µm film thickness, with 10 m Duraguard pre-column, Agilent, Waldbronn, Germany) was used for GC-EI-MS measurements. The split/splitless injector was fitted with a gold plated inlet seal with dual Vespel rings (Restek, Bad Homburg, Germany). The samples were injected with a 7683B autosampler (Agilent, Waldbronn, Germany) equipped with a 10 µL tapered, fixed needle, PTFE-tipped plunger syringe (23-26s/42, Agilent, Waldbronn, Germany). Samples were run in random order. A new deactivated glass liner (4 × 6.3 × 78.5 mm inner Ø × outer Ø × length, Agilent, Waldbronn, Germany) was used for every batch of 20 samples. The used liners were shipped to be cleaned and deactivated by CS Chromatography service (Bremen, Germany). The GC parameters for the analysis were as follows. Carrier gas: Helium 5.0. Carrier gas flow: Constant flow at 1 mL min⁻¹. Injection pre dwell time: 0.1 min (hot needle injection). Oven starting temp. 60°C for 1 min. Oven ramp to 310 °C at 15 °C min⁻¹. Oven final temp. 310 °C for 9.3 min. Injector temp. 300 °C. Injection volume: 1 µL. Injector mode: Split 5. Measurements were performed over 30 min. Parameters of EI source: Electron energy: 70 eV. Trap current: 200 µA 4.3. Calibration of the MS parameters (beam steering, focusing lenses, dynamic range extension (DRE)) was performed.
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before the analysis of the samples from every experiment. The MS parameters, Source temp. 300 °C. Transfer line temp. 280 °C. Scan rate: 5 scans s⁻¹. m/z: 50-800. DRE: activated.

5.8. Data collection and preprocessing

5.8.1. Data collection from the LC-MS chromatograms

Biomarkers were collected from UPLC-MS raw data by using two software tools:

(1) Progenesis CoMet (http://www.nonlinear.com/progenesis/qi/, version 2.0) to determine the range of intensities and of retention time for biomarkers collection using an ion intensity map, a 2D gel-like representation of the ions in an LC/MS runs. It is a map of ions in the run, with the darker areas showing the higher intensities in the MS signal. Retention time increases from top to bottom, while the mass/charge ratio increases from left to right. By examining the pattern of ion intensities on these maps, it is sometimes easy to identify issues in the chromatography that can adversely affect the analysis. Thus, (1) the instability in the ion spray, (2) a completely blocked ion spray, (3) contaminants and column leaching, and (4) electronic noise in the MS signal, helped to avoid time consuming downstream analyses, trying to analyze data that should be rejected due to impurities. Using these criteria, Best range of intensities and retention time to collect the biomarkers were from m/z: 100-800 and retention time from 0.5 till 7.0 min; see figure 25.

(2) MarkerLynx™ (Waters, Version 4.1) was used, and the parameters were as shown in table 22.

Table 22: MarkerLynx™ parameters used for biomarkers collection.

<table>
<thead>
<tr>
<th>Peak Detection</th>
<th>ApexPeakTrack</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak width at 5%</td>
</tr>
<tr>
<td></td>
<td>Peak width at 5% height (seconds)= default (15)</td>
</tr>
<tr>
<td></td>
<td>Peak-to-peak baseline ratio = default (0)</td>
</tr>
<tr>
<td></td>
<td>Noise elimination = default (0.10)</td>
</tr>
<tr>
<td></td>
<td>Intensity threshold = 10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Deisotoping</th>
<th>Performed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Alignment</td>
<td>m/z window = 0.05 amu</td>
</tr>
<tr>
<td></td>
<td>Retention time window = 1 min</td>
</tr>
</tbody>
</table>
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5.8.2. Data collection from the GC-MS chromatograms

Data processing and smoothing

All chromatograms were background-noise corrected with the Component Detection Algorithm (CODA) implemented in Masslynx™ (version 4.1, Waters, UK). The MCQ index used was 0.8 and the smoothing window was 3 points. The chromatograms were then converted to netCDF files using the Masslynx™ DataBridge (Micromass, V4.1).

Extraction of spectra

Converted spectra were treated in batch jobs in the Automated Mass spectral Deconvolution and Identification System (AMDIS) (version 2.65, NIST, http://www.nist.gov/, 2006). The following parameters in table 23 were used:

<table>
<thead>
<tr>
<th>Identification</th>
<th>Minimum match factor: 30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type of analysis: Simple</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Deconvolution</td>
<td>Component width: 32</td>
</tr>
<tr>
<td></td>
<td>Omitted m/z: 147, 176, 193, 207, 219.</td>
</tr>
<tr>
<td></td>
<td>Adjacent peak subtraction: 2</td>
</tr>
<tr>
<td></td>
<td>Resolution: Low</td>
</tr>
<tr>
<td></td>
<td>Sensitivity: Medium</td>
</tr>
<tr>
<td></td>
<td>Shape requirement: Low</td>
</tr>
<tr>
<td>Library</td>
<td>Target Compounds Library: Golm</td>
</tr>
</tbody>
</table>

The CDFs files and the corresponding AMDIS files were fed into METabolomics-Ion-based Data Extraction Algorithm (MET-EDEA) (version 2.03, http://bioinfo.noble.org/download/, 2006) with the following parameters (Tab. 24).

The resulting data sets of peak areas were imported into Excel (Office 2007, Microsoft, Redmont, USA). The peaks corresponding to the retention index standards, ribitol.
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**Table 24:** MET-IDEA parameters used for identification and deconvolution mass spectra.

<table>
<thead>
<tr>
<th>Chromatography</th>
<th>Type: GC</th>
<th>Average peak width: 0.08</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMDIS transfer: 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximum peak width: 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peak start/stop slope: 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adjusted retention time accuracy 0.25, peak overload factor: 0.9</td>
</tr>
<tr>
<td>MS</td>
<td>Type: TOF</td>
<td>Mass accuracy: 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mass range: 0.3</td>
</tr>
<tr>
<td>General Type</td>
<td>Exclude ion: 73, 147, 193, 281, 341, 415</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower mass limit: 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ions per component: 1</td>
<td></td>
</tr>
</tbody>
</table>

**Identification of metabolites generated by GC-MS**

The spectrum of each peak retained for the analysis was manually examined and identification was attempted using the software MS search (version 2.0 d, NIST, http://www.nist.gov/, 2005). The following libraries were used: NIST, Golm Metabolome Database (Version: 121_VAR5_ALK_MSP, http://gmd.mpimp-golm.mpg.de/) and MPI of Molecular Plant Physiology (Version: Q_MSRI_ID2004-03-01) A structure was accepted if the reverse match was higher than 800 and if the retention index was close to the index provided in the libraries. The structure was accepted with a tag in cases where the reverse match was higher than 800, but that the retention index and visual inspection of the spectra corresponded to a structure.

**Important nomenclature:**

“?” if the reverse match is between 700 and 800.

“??” if the reverse match is between 500 and 700.

**5.9. Chemometric analyses**

**5.9.1. Unconstrained ordination analysis**

**Principle component analysis PCA**

For each measurement, the resulting two-dimensional data (component 1 vs. component 2) were Pareto-scaled. The intensities were measured as a height; and mean centered. The
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identified m/z signals were then normalized to the sum of the peak intensities (TIC) in each chromatogram and analyzed using the PCA software implemented in the MarkerLynx™ software package (Version 4.1).

5.9.2. Constrained ordination analysis

Data preprocessing

(1) The mean of (the data derived from control (only UCM, in case of bioreactors) along with the data generated from day-zero of all treatments underwent PCA analysis) was calculated.

(2) The median of the data at each sampling time point (from day-7 till day-49) was calculated.

(3) The mean of the data generated from Step 1 was subtracted from the median of the data resulting from Step 2 (Step 2 – step 1 = clean data).

(4) All negative values were converted to zero.

(5) If the sum of each biomarker throughout the samples resulted in zero, the biomarker was discarded.

Canonical analysis of principle coordinates (CAP)

(1) Normalization was performed by (a) transformation of the data to square root, and then (b) standardization of the transformed data by dividing each peak area by the sum of all peak areas within one sample.

(2) The data sets were then exported as txt files for discriminant analysis mode (CAP).

(3) CAP analysis was performed with the software CAP12 (http://www.stat.auckland.ac.nz/~mja/) (Anderson and Willis, 2003) using the following parameters: Bray-Curtis dissimilarity for the distance measure and discriminant analysis. The number of principal coordinates axes were chosen by the program. For validation of the classification 999 random permutations tests were automatically performed.

The resulting first two canonical axes and sample coordinates were then imported into SigmaPlot (version 11.0, Systat Software, USA) for graphical illustration. The biomarkers were screened for significant Pearson correlation coefficients with the CAP axes. Compounds having such a correlation coefficient with one of the CAP axes were retained as significantly characteristic compounds. The correlation coefficients were scaled to the CAP range of the
coordinates (usually between 0.3 - 0.5) to generate the vectors corresponding to each significant compound.

5.10. Heatmap

The heatmap was performed with the biological mean of the biomarkers or metabolites, meaning that the average of the biological replicates (n = 3) was used to perform the heatmap. Two software tools were used to implement heatmap: Tableau (version 8.1, USA), and Excel (Office 2007, Microsoft, Redmont, USA).


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Curriculum vitae

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Education

2010-2014
PhD in chemical ecology in the bioorganic analytic group of Prof. Dr. Pohnert, (FSU) Jena, Germany.

2006-present

2002-2006
Master in organic chemistry in natural product research group of Prof. Dr. Hassan Albar, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia

1996-2000
Bachelor in chemistry, College of science, Jeddah, Kindom of Saudi Arabia

Experience

2010-2013
Practical course supervision. Course: UV and IR spectroscopy. Friedrich Schiller University, Germany.

04.2012-09 .2012
Project student supervision, Friedrich Schiller University, Germany.

2006-2009
A member of the green chemistry research performing laboratory experiments for the Curriculum of Principles of Organic Chemistry, using the technology of green microscale chemistry, Kingdom of Saudi Arabia.

2006-2010
Lecturer in chemistry department, Taif University, Chemistry Departmant, Kingdom of Saudi Arabia.

Field experience

10. 03-05.05 2011
Aquacultures at the Ramalhete Marine Station of the Algarve Marine Sciences Centre (CCMAR) in Faro, Portugal.

Workshops

18.10. 2013
Applying Statistics – Understanding Your data with SPSS, Jena School for Microbial Community (JSMC), Jena, Germany.

6 –7.03. 2012
Structure elucidation of small molecules using NMR and MS-based methods, JSMC, Jena, Germany.

20.10-01.12.2011
Scientific Writing and Publishing for Natural Scientists, JSMC, Jena, Germany.

27.09-23.10. 2003
Cell and tissue culture, King Fahad Centre for medical research, Jeddah, Kingdom of Saudi Arabia.
Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Ich erkläre, dass die übersetzte arabische Version der Zusammenfassung mit der deutschen und englischen Version identisch ist.

Jena, 27.05.2014

Taghreed Abdurahem Alsufyani