

Short Communication

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Bacteria-released algal growth and morphogenesis factors regenerate axenic calli derived from the macroalga *Ulva* (Chlorophyta) and change the fatty acid profile

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Abstract: *Ulva*, a green marine macroalga, lives in a symbiotic relationship with bacteria like *Roseovarius* sp. and *Maribacter* sp., which produce algal growth- and morphogenesis-promoting factors (AGMPFs) crucial for algal development. In the absence of all AGMPFs including thallusin *Ulva* exhibit a callus-like morphotype characterised by a lack of cellular differentiation. This study introduces calli as a novel model to investigate the AGMPF action. The metabolic impacts of AGMPFs were investigated by comparing the axenic calli of *Ulva compressa* (cultivar *Ulva mutabilis*) with those exposed to AGMPFs. Adding AGMPFs regenerated the callus and promoted germling-like structures, along with changes in the metabolome. The bouquet of AGMPFs, containing (–)-thallusin, induced significant variations in fatty acid metabolism, notably an increase of palmitic acid and several polyunsaturated fatty acids, along with a change of the $\omega 6:\omega 3$ ratio (<0.7), which may directly affect growth as well as membrane and cell wall formation. The results highlight the significant implications of bacterial-derived AGMPFs instead of live bacteria and underscore the necessity of optimising AGMPF distribution in cultivation to improve the nutritional quality of *Ulva* for food and feed.

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The marine green seaweed genus *Ulva* (Chlorophyta) has drawn the attention of numerous research groups worldwide, having become a model system for studying the morphogenesis, chemical ecology and phylogeny of the Chlorophyta lineage (Blomme et al. 2023; Steinhagen et al. 2019; Wicha 2015, 2023). *Ulva* appears ubiquitously in coastal benthic regions; it copes well with stressors like changes in salinity, temperature, irradiance and polluted water (Ghaderiardakani et al. 2020). Together with its high nutrient uptake rate, these properties contribute to the appearance of *Ulva* blooms, also known as 'green tides' (Zhang et al. 2019). Therefore, many concepts for applying *Ulva* spp. were established over the past few decades for the bioremediation of wastewater polluted with heavy metals, nutrients or micropollutants (Hardegen et al. 2023; Rahhou et al. 2023; Shpigel et al. 1993). Furthermore, *Ulva* spp. can be cultivated as an alternative to land-based crops for food and animal feed (Khan and Mamat 2024). The biorefinery of, for example, the polysaccharide ulvan, protein sources, lipids and alcohols of low molecular weight open up further applications for *Ulva* in aquaculture (Bikker et al. 2016; Calheiros et al. 2021; Simon et al. 2022). Our study aimed to understand how bacteria-released algal growth and morphogenesis factors (AGMPF) promote callus regeneration and influence the metabolome and fatty acid profile of axenic calli derived from the model organism *Ulva mutabilis* Føyn, which was grown under standardised conditions in an artificial *Ulva* culture medium (UCM, Califano and Wicha 2018) (Figure 1).

Ulva mutabilis Føyn – described as conspecific with *Ulva compressa* by Steinhagen et al. (2019) – maintains a tripartite symbiotic relationship with *Roseovarius* sp. strain MS2 and *Maribacter* sp. strain MS6 in developing into a regular differentiated macroalga (Spoerner et al. 2012; Steinhagen

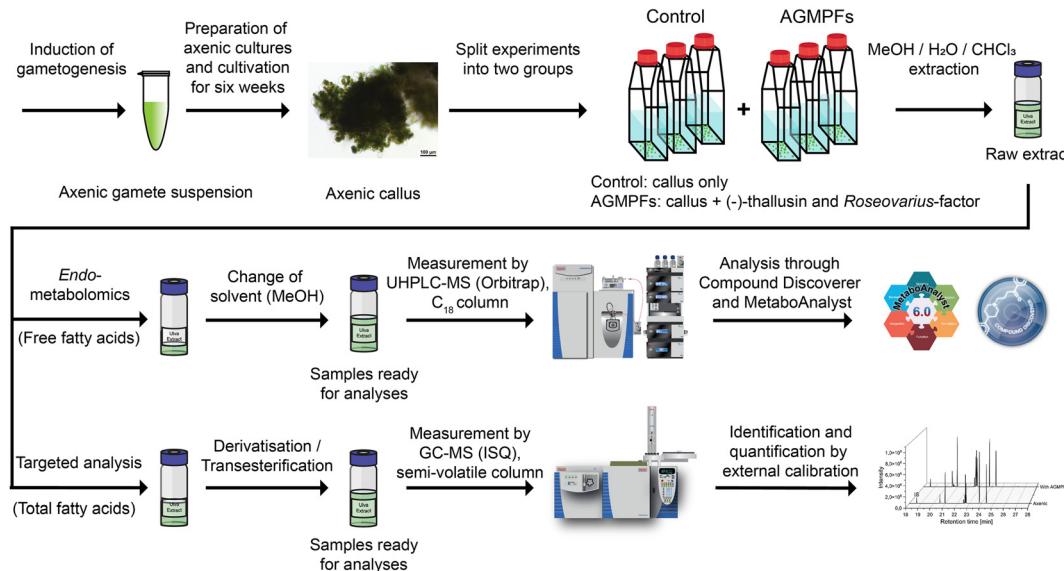


Figure 1: Experimental workflow. Gametes of *Ulva mutabilis* were gathered and separated from bacteria (Califano and Wichard 2018). These axenic gametes were cultivated for six weeks to a callus stage. Afterwards, the stock cultures of calli were unified, mixed and distributed in six culture flasks under sterile conditions. One biological triplicate served as control (in 100 % UCM), while the cultures for the AGMPF-treated biological triplicate [in sterile-filtered *Roseovarius* UCM-based supernatant (75 %) and UCM (25 %); v:v] were inoculated subsequently with (-)-thallusin (2×10^{-8} mol l^{-1} final concentration). The calli were incubated for two weeks, separated from the culture medium for solvent extraction, and flash-frozen. After further sample preparation, the ground calli were extracted using a commercial kit based on the Folch lipid extraction method (Folch et al. 1957). In the ensuing sample processing, one aliquot of each sample extract was subjected to a UHPLC-MS Orbitrap system to conduct *endo*-metabolomics. The data were analysed and evaluated using Compound Discoverer (vers. 3.3) and MetaboAnalyst (vers. 6.0). Fatty acids were derivatised to obtain methyl esters for quantification by GC-EI-MS. The analysed fatty acids were quantified based on their response factor through a comparison with an external standard mix. For detailed information, see Supplementary Material.

et al. 2019; Wichard 2023). The enhanced growth effect induced by *Roseovarius* sp. and cell differentiation mediated by *Maribacter* sp. are triggered by AGMPFs, which are released into the culture medium, including the morphogen thallusin (Alsufyani et al. 2020; Ghaderiardakani et al. 2019; Matsuo et al. 2003). Therefore, filtered bacterial supernatant can be used as a replacement for the bacterial effect (Spoerner et al. 2012).

It is not just *U. mutabilis* that benefits from the interaction with these specific bacteria; the growth and development of other *Ulva* species are also promoted within the same phycosphere (Ghaderiardakani et al. 2017). Therefore, the studied effects from bacterial interactions with *Ulva* are likely generalizable to the genus. Without the symbiotic bacteria, certain substitute species, or the corresponding AGMPFs within the chemosphere, the alga develops into a callus of unorganized cell masses (Wichard 2023). Undifferentiated callus-like morphotypes have also been investigated extensively in flowering plants, such as soybeans, and are interesting ‘seeds’ for aquaculture (Ram Kumar et al. 2021).

Given that AGMPFs shape *Ulva* significantly, a change in metabolism occurs accordingly (Califano 2022). A recent

study on the impact of individual bacterial partners on the axenic microalgae *Isochrysis galbana* reinforced these observations (Wu et al. 2023). Co-cultivation had effects on several parameters connected to primary metabolism (i.e., the growth, chlorophyll content, soluble protein content, or fatty acid profile of the microalgae) as well as in flowering plants (He et al. 2009; Ram Kumar et al. 2021).

To investigate the hypothesis regarding changes in the metabolome of *Ulva* influenced by the presence or absence of AGMPFs, (i) axenic *U. mutabilis* calli (strain FSU-UM5-1) were inoculated with AGMPFs and compared to (ii) an untreated control group of axenic calli, as well as (iii) an adult stationary *Ulva* culture containing its natural microbiome (Figure 1).

The complete development and morphogenesis occur only in the presence of all AGMPFs released by *Roseovarius* sp. and *Maribacter* sp. When only the *Roseovarius* factor or the *Maribacter* factor (i.e., thallusin) is present, morphogenesis remains incomplete, and the resulting developmental differences influence the algal metabolome. Therefore, we administered both AGMPFs together to avoid secondary effects due to different phenological stages.

A six-week-old axenic callus culture covered a colonisation area of $15 \pm 2.5 \text{ cm}^2$ on the 75 cm^2 bottom surface of the tissue culture flask in 50 ml UCM. To induce growth and morphogenesis of the calli, the sterile-filtered AGMPF cocktail was supplemented with the *Roseovarius* factor (supernatant from a *Roseovarius* sp. culture grown on glycerol in UCM) and the *Maribacter* factor, (–)-thallusin, at a concentration of $2 \times 10^{-8} \text{ mol l}^{-1}$. This concentration was 4,000 times higher than the half-effective concentration of thallusin ($\text{EC}_{50} = 5 \times 10^{-12} \text{ mol l}^{-1}$; Dhiman et al. 2022; Ulrich et al. 2022) and remained effective throughout the two-week cultivation period. The cultures underwent microscopic assessment before harvesting and extracting the *endometabolome* on day 14 after AGMPF inoculation (Figure 2 and Supplementary Material for details).

Throughout the studied period, the calli treated with AGMPFs exhibited partial recovery. They developed new tissue characterised by the absence of cell wall protrusions and a general decline in the size and number of cell protrusions (compare Figure 2A and B). Overall, AGMPFs containing (–)-thallusin in excess induced callus transformation into a growing tissue (Figure 2B). This contrasted with the

non-treated control group, where large protrusions persisted (Figure 2C and D). The well-developed branches (germling-like structures) can be separated from the callus clump and cultured individually for proper development in the presence of the AGMPFs.

To monitor changes in the metabolome upon application of the AGMPF cocktail, the calli were extracted using a mixture of chloroform, methanol and water (Folch et al. 1957). After extraction, the samples were analysed using ultra-high-pressure liquid chromatography coupled with a high-resolution electrospray ionisation mass spectrometer (UHPLC-HR-ESI-MS). The data was processed using the Compound Discoverer (vers. 3.3) and visualised using Metaboanalyst (vers. 6.0) (see Figure 1 and Supplementary Material for details).

Principal component analysis (PCA) showed the similarity between the two sample groups when PC1 was plotted against PC2 (Figure 3A). However, PC3, which accounted for 15 % of the variance, was indicated in a divergence between the control and the AGMPF-treated groups. The volcano plot (Figure 3B, Table S1) demonstrated the significant alterations in metabolite composition resulting from the addition of

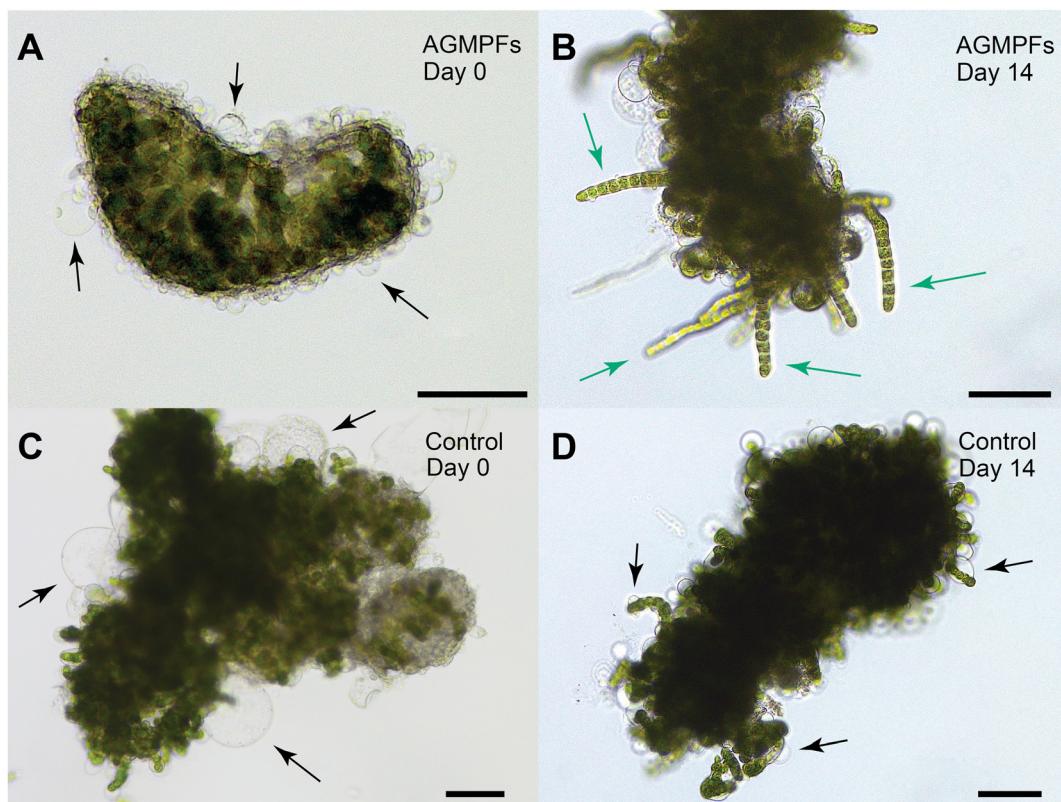
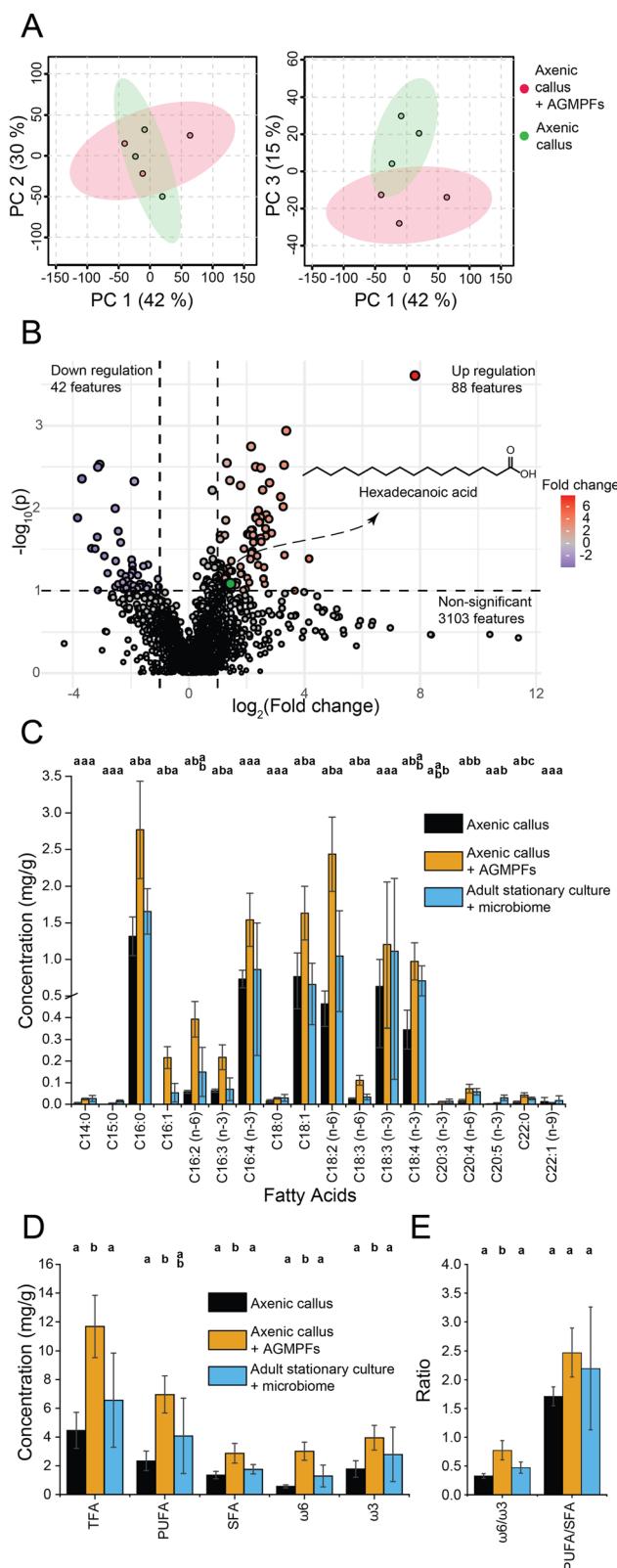


Figure 2: Bioassays with 6-week-old axenic calli of *Ulva mutabilis* and application of algal growth and morphogenesis-promoting factors (AGMPFs). Exemplary microscopic pictures of the examined treatments show (A) a callus with cell wall protrusions (arrows) on day 0 in the group with added AGMPFs and displaying (B) a callus after 14 days with AGMPF-induced germling-like structures (green arrows) and no cell wall protrusions. The control group without AGMPFs shows (C, D) the typical cell wall protrusions (arrows) on day 0 and day 14. Scale bars = 100 μm .



AGMPFs. Significantly, 88 upregulated and 42 downregulated m/z features (i.e., potential compounds) were determined in the group treated with AGMPFs, while 3,103 m/z features did not vary between the groups. The up-regulation of palmitic acid (C16:0, indicated by the green circle in Figure 3B) piqued our interest because it is the most abundant fatty acid in *Ulva* spp. (García-Poza et al. 2022; Kostetsky et al. 2018) and implied further alterations in lipid metabolism, which might affect the membrane composition and development of *Ulva* due to the application of AGMPFs. It is noteworthy that plant growth regulators can influence plant lipid composition and lipid metabolism in terms of fatty acid induction and alteration of composition (Aly et al. 2008). In contrast to these plants, *Ulva* relies on an external supply of specifically associated and necessary bacteria for enhanced algal growth and development (Wichard 2023).

For targeted total fatty analyses, the free and bound fatty acids contained in the extract were converted into fatty acid methyl esters (FAME) using methanolysis through trans-esterification (Figure 1) (Alsufyani et al. 2014). In addition to the axenic calli, stationary adult cultures of *U. mutabilis*, complete with the entire native microbiome, were surveyed for the fatty acid profile. The areas of FAME peaks analysed using gas chromatography (Figure S1) coupled with electron impact ionisation mass spectrometry (GC-EI-MS) were compared to signals from external standards to estimate the concentration per sample dry weight (Figure 3C).

In stationary adult *U. mutabilis* cultures with their associated microbiomes, high levels of C18-PUFAs were detected including linoleic acid [C18:2(n-6), 15 %], α -linolenic acid [C18:3(n-3), 15 %], stearidonic acid [C18:4(n-3), 12 %]

displays PC 2 versus PC 1, while the right plot shows PC 3 versus PC 1. (B) Volcano plot describing the 88 up- and 42 down-regulated m/z features in the AGMPF-treated samples versus the control samples (p -value threshold = 0.1, \log_2 fold change threshold = 1). The hexadecanoic acid (C16:0) is highlighted in green as the upregulated compound in samples treated with AGMPFs. (C) Bar chart illustrating the concentrations of individual fatty acids in mg per g algal dry weight and comparing the axenic calli control group (black bars) with axenic calli + AGMPFs (orange bars) and the adult algal samples (blue bars) in a stationary phase with the usual microbiome and normal tubular morphotype ('slender'). (D) Bar chart of concentrations (mg per g algal dry weight) of different groups of fatty acids in the treatment and control groups. Total fatty acids (TFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), w6-FA(n-6), and w3-FA(n-3) were compared. (E) Bar chart of w6/w3 and PUFA/SFA ratios corresponding to each treatment and control group. Error bars in (C), (D) and (E) correspond to the mean \pm standard deviation ($n = 3$). Means denoted by a different letter indicate significant differences between treatments (one-way ANOVA, Fisher or Games-Howell post hoc test, depending on homoscedasticity, $p < 0.05$) (See also Tables S1–S3 in the Supplementary Material).

Figure 3: Metabolomics and fatty acid analysis. (A) Principal component analysis (PCA) scores plot from the *endo*-metabolomic LC-MS data comparison between the axenic calli of *Ulva mutabilis* treated with the AGMPF group (red) and the axenic calli control group (green). The left plot shows PC 2 (30%) vs PC 1 (42%) and the right plot shows PC 3 (15%) vs PC 1 (42%). (B) Volcano plot of $-\log_{10}(p)$ vs $\log_2(\text{Fold change})$ for 88 up-regulated and 42 down-regulated features. (C) Bar chart of individual fatty acid concentrations (mg/g) for various fatty acids. (D) Bar chart of total fatty acids (TFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), w6-FA, and w3-FA concentrations (mg/g). (E) Bar chart of PUFA/SFA and w6/w3 ratios corresponding to each treatment and control group. Error bars in (C), (D) and (E) correspond to the mean \pm standard deviation ($n = 3$). Means denoted by a different letter indicate significant differences between treatments (one-way ANOVA, Fisher or Games-Howell post hoc test, depending on homoscedasticity, $p < 0.05$) (See also Tables S1–S3 in the Supplementary Material).

along with trace amounts of γ -linolenic acid, collectively accounting for 42 % of the total fatty acids (Table S2). C20-PUFAs such as arachidonic acid [C20:4(n-6)] and eicosapentaenoic acid [C20:5(n-3)] contributed with around 1 %, only a minor amount. Notably, the fatty acid profile of *U. mutabilis* is characterised by unsaturated C16 and C18 fatty acids (Figure S1), a typical feature of this genus, as previously reported in several studies (Alsufyani et al. 2014; McCauley et al. 2016; Monteiro et al. 2022; Pereira et al. 2012). Overall, our data aligned well with the range of concentrations previously measured in *Ulva* species; for example, those that originated from Morraceira Island (Portugal) or Brittany (France) and were cultivated under different conditions (García-Poza et al. 2022; Monteiro et al. 2022).

The fatty acid profile of the axenic callus of *U. mutabilis* was also predominantly rich in ω 3 and C18 fatty acids besides palmitic acid (C16:0), which has already been determined using the *endo*-metabolomics approach (Figure 3B, Table S1). Our analysis, excluding bacterial fatty acid contamination, found all of the typical fatty acids – such as C16:0, C16:4, C18:1, C18:2, α C18:3, C18:4 and a few C20 fatty acids – in the axenic *U. mutabilis* cultures, both with and without AGMPFs (Table S1 and S2).

However, the application of AGMPFs containing thallusin enhanced fatty acid synthesis and changed their abundance. The targeted analysis revealed that 10 out of the 18 fatty acids, including 7 PUFAs, increased significantly in cultures supplemented with AGMPFs compared to the untreated control calli. Interestingly, additional AGMPFs even increased the production of fatty acids compared to the adult stationary *Ulva* culture and its associated microbiome. The excess of AGMPFs corresponded with elevated levels of seven fatty acids – C16:0, C16:1, C16:3(n-3), C18:1, C18:2(n-6), γ C18:3(n-6) and C22:0 – whereas the concentrations of other fatty acids remained unchanged (Figure 3C, Table S3).

Distinct characteristics could be noted following the application of AGMPFs. For instance, the ratio of 18:1 to 18:2(n-6) was inverted in favour of linoleic acid, which also affected the ω 6: ω 3 ratio (Table S2). The alterations in the concentrations of polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) (Figure 3D) influenced the ratios of ω 6: ω 3 and PUFA/SFA, nearly doubling the ω 6: ω 3 ratio ($p < 0.05$) in AGMPF-treated calli (Figure 3E). With ω 6: ω 3 = 0.77, the ratio under AGMPFs-treated conditions was higher than recently published values in *Ulva* (which, for example, ranged from 0.15 to 0.37; Monteiro et al. 2022), but was still an excellent ratio (ω 6: ω 3 < 1) for human nutrition (Simopoulos 2000).

In our study, the concentration of specific fatty acids [16:4(n-3)] normalised per mg of dry mass was over ten times higher than in some previous studies (e.g., García-Poza et al.

2022) (Figure 3C, Table S3). These variations in fatty acid concentrations can result from multiple factors. Species-specific metabolic traits play a crucial role as they inherently produce and regulate fatty acids in distinct ways. Abiotic conditions such as temperature and salinity, along with variations in nutrient composition, can significantly influence lipid metabolism (Gnayem et al. 2025). Our study underscores another critical factor: the role of AGMPFs released by the associated microbiome. These bacterial-derived compounds may actively regulate fatty acid biosynthesis in *Ulva*, modulating lipid composition, and this suggests that future studies should further explore how specific AGMPFs contribute to metabolic regulation.

In any case, comparing the fatty acid composition of differentiated tissue to that of callus tissue in flowering plants (e.g., Cucurbitaceae; Halder and Gadgil 1983) yielded comparable results to those in *U. mutabilis*. The total lipid content of callus cells significantly diminished from 20–30 % to 2–4 %, and the PUFA/SFA ratio fell in undifferentiated callus cells, indicating an even more pronounced alteration than in *Ulva* calli (Figure 3D and E). Comparable to our research approach, other investigations have revealed alterations in fatty acid content by adding several growth regulators to callus cells (Pandey and Gadgil 1984).

Our results demonstrated that bacteria-derived AGMPFs triggered molecular processes that served as switches for lipid or fatty acid biosynthesis. Thus, AGMPFs function as stimulants, enhancing fatty acid production in *Ulva*, a finding that has also been suggested for bacteria-microalgae interactions (Zhang et al. 2025). Changes in fatty acid profile are often correlated with stress parameters. Certainly, the lack of, or a change in, the microbiome can harm algal morphogenesis (Marshall et al. 2006; Spoerner et al. 2012). Therefore, it is tempting to assume that the different quantities and ratios of individual fatty acids might partially explain the callus morphology and its susceptibility to stress in *U. mutabilis*, considering that those fatty acids have diverse cell functions. These functions are vital membrane components for bilayer establishment, energy storage in the form of triglycerides, and cell signalling (De Carvalho and Caramujo 2018). Membrane fluidity is, for example, higher if more unsaturated fatty acids are employed than saturated fatty acids. Many organisms alter membrane properties based on fatty acid composition, including sphingo- and phospholipids, to cope with their environment (e.g., when confronted with temperature stress). Additional chemical analysis of *Ulva* calli is necessary to evaluate the appearance of malformed cell walls and protrusion formation in relation to changes in the fatty acid profile. Using Raman spectroscopy imaging, Schultz et al. (2024) have

already demonstrated the change in chemical composition due to malformations and cell wall protrusions in axenic cultures.

Furthermore, the β -oxidation of fatty acids from triacylglycerols into fatty acyl CoA is a critical process for supporting the algae during growth. Lipids serve as storage matrices for fat-soluble vitamins, including A, D, and E. Therefore, cell functions can be impaired if essential vitamins are not available within the cell. Also, free PUFA and their derivatives, like oxylipins, often have allelopathic functions (De Carvalho and Caramujo 2018).

To sum up, an impeded lipid or fatty acid metabolism can harm the organism. Based on the change in fatty acid composition and the up- and down-regulation of many (unknown) compounds according to metabolomics, we consider that the AGMPF effect is certainly not limited to lipid metabolism only. Our results underline the importance of an optimal distribution of AGMPF during cultivation for the establishment of a healthy population of *Ulva* and the provision of high levels of unsaturated fatty acids in *Ulva* used for food and feed.

Overall, lipid and fatty acid quantities and profiles change with season, sampling location, and abiotic conditions (Gnayem et al. 2024, 2025), as well as with the availability of AGMPFs (Figure 3), which are directly related to the prevalence and functional traits of the macroalgal microbiome. Environmental changes may thus drive shifts in the microbiome or its functional traits, ultimately affecting, for example, the bioavailability of AGMPFs in the algal chemosphere and, subsequently, the fatty acid profile of *Ulva*. Therefore, measuring the quantities of AGMPFs is essential for effective monitoring of algal aquaculture. Thallusin, the AGMPF produced by *Maribacter* sp., is being monitored (Ulrich et al. 2022), whereas the AGMPF released by *Roseovarius* sp. has not yet been identified. Further research is needed to elucidate the regulatory mechanisms of these compounds and to assess their applicability to other systems, including terrestrial plants.

In summary, our study focused on the metabolic effects of AGMPFs, mainly through fatty acid profiles. Our key findings include:

- (i) Experimental design: The advantage of using bacterial-derived AGMPFs instead of live bacteria lies in their precise control and reduced risk of unintended bacteria-algae interactions, making them a powerful tool for targeted studies and applications without bacterial contaminants.
- (ii) Morphological transformation: AGMPF treatment on axenic *Ulva* calli prompts regeneration, suggesting a reversal from a callus into a differentiated form. This

transformation highlights the significance of bacterial interactions in the normal development of algal morphology and demonstrates that axenic calli can be reactivated.

- (iii) Chemical analysis: Axenic calli facilitate the identification and quantification of metabolites without background noise from microbial contaminants.
- (iv) Metabolomic shifts: Exposure to AGMPFs is associated with notable changes in fatty acid metabolism. Specifically, there is an increase in hexadecanoic acid and polyunsaturated fatty acids. These compounds play a crucial role in maintaining the structure and fluidity of the cell membrane, which are essential for cellular functionality and integrity in aquatic environments.
- (v) Implications for food and feed applications: Given that AGMPFs improve the morphological and metabolic profile of *U. mutabilis*, optimising AGMPF cultivation conditions could enhance the nutritional value of *Ulva* as a source of beneficial fatty acids.

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Data availability: The *endo*-metabolomics data are available in the public database Zenodo: <https://zenodo.org/records/14938653> (DOI 10.5281/zenodo.14938653).

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