Supplementary Material for

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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Content

Table S1: Top 20 ranked dysregulated features (*m/z*) obtained by UHPLC-MS metabolomics, including hexadecanoic acid (red)

#	Molecular formula	Measured *m/z* ([M±X], Da)	Calculated mass (M, Da)	Theoretical monoisotopic mass (M, Da)	Adduct	RT (min)	Δ (ppm)	log_2(Fold change)	raw p-value
1	C9H10N2	147.09151	146.08423	146.08439	$[M+H]^+$	2.91	-1.2	7.81	0.00025
2	C22H47N10O13P	689.30002	690.30730	690.30616	$[M-H]^-$	5.92	1.6	3.37	0.00115
3	C16H28O2	251.20126	252.20854	252.20893	$[M-H]^-$	8.00	-1.8	2.15	0.00178
4	C17H23F3N2S	345.15591	344.14863	344.15340	$[M+H]^+$	5.72	13.9	1.31	0.00283
5	C31H73N9O12P2	826.49273	825.48545	825.48539	$[M+H]^+$	6.89	0.1	-3.08	0.00295
6	C28H49NO15	638.30209	639.30937	639.31021	$[M-H]^-$	5.92	-1.3	3.29	0.00299
7	C18H30N6O4	395.24224	394.23496	394.23285	$[M+H]^+$	5.58	5.4	2.32	0.00314
8	C6H8BrN7O	271.99091	272.99819	272.99737	$[M-H]^-$	6.75	3.0	-3.14	0.00317
9	C11H16O2	181.12214	180.11486	180.11503	$[M+H]^+$	6.49	-0.9	2.49	0.00324
10	C45H57N8OP	757.45019	756.44291	756.43929	$[M+H]^+$	7.24	4.8	-3.69	0.00440
11	C26H54N10O10S	697.36505	698.37233	698.37450	$[M-H]^-$	6.45	-3.1	1.42	0.00461
12	C13H24O4	243.15978	244.16706	244.16745	$[M-H]^-$	4.71	-1.1	-1.88	0.00473
13	C35H57NO7P2	664.35303	665.36031	665.36102	$[M-H]^-$	6.96	-1.1	2.79	0.00492
14	C25H40S2	405.26305	404.25577	404.25714	$[M+H]^+$	6.15	-3.4	1.79	0.00531
15	C32H55NO8P2	642.33329	643.34057	643.34029	$[M-H]^-$	6.95	0.4	2.55	0.00602
16	C31H53N2O2P3	623.32769	578.32949	578.33198	$[M+FA-H]^-$	6.32	-4.5	3.17	0.00725
17	C28H51NO12	594.34763	593.34035	593.34112	$[M+H]^+$	5.92	-1.3	3.26	0.00958
18	C35H53N2O4P3	657.31188	658.31916	658.32181	$[M-H]^-$	5.17	-4.0	-2.54	0.01008
19	C28H31ClN8O7	314.10806	626.20156	626.20042	$[M+2H]^{2+}$	5.42	1.9	2.41	0.01064
20	C43H45N3O	620.36266	619.35538	619.35626	$[M+H]^+$	6.15	-1.4	2.68	0.01201
90	C16H32O2	257.24682	256.23954	256.24023	$[M+H]^+$	6.64	-1.9	1.25	0.07004

Table S2: Mean relative abundance of fatty acids in *Ulva*, expressed as a percentage of their total quantified amount (mean ± SD %, $n = 3$). Fatty acid composition was determined in axenic callus cultures with and without the application of algal growth and morphogenesis-promoting factors (AGMPFs) and in adult stationary cultures with their associated microbiome. (n.d. = not detected).

Fatty acids (%)	Axenic callus		Axenic callus + AGMPFs		Adult stat. culture + microbiome	
C14:0	0.160	± 0.081	0.209	± 0.012	0.419	± 0.171
C15:0	n.d.		0.050	± 0.005	0.247	± 0.078
C16:0	29.897	± 2.233	23.619	± 2.67	28.380	± 9.897
C16:1	n.d.		1.838	± 0.129	0.729	± 0.257
C16:2(n-6)	1.378	± 0.441	3.351	± 0.138	2.026	± 0.868
C16:3(n-3)	1.493	± 0.415	1.843	± 0.179	0.963	± 0.293
C16:4(n-3)	16.707	± 1.792	13.114	± 1.135	11.941	± 3.604
C18:0	0.374	± 0.194	0.239	± 0.015	0.509	± 0.272
C18:1	16.706	± 2.249	13.932	± 1.264	10.162	± 1.082
C18:2(n-6)	10.485	± 0.538	20.790	± 0.984	15.203	± 3.397
C18:3(n-6)	0.629	± 0.121	0.948	± 0.023	0.537	± 0.071
C18:3(n-3)	13.379	± 3.988	10.673	± 7.251	14.626	± 6.952
C18:4(n-3)	7.759	± 0.216	8.249	± 0.934	11.717	± 3.113
C20:3(n-3)	0.035	± 0.009	0.094	± 0.009	0.204	± 0.08
C20:4(n-6)	0.348	± 0.04	0.610	± 0.073	0.975	± 0.304
C20:5(n-3)	0.038	± 0.008	0.032	± 0.029	0.619	± 0.568
C22:0	0.266	± 0.017	0.368	± 0.035	0.468	± 0.184
C22:1(n-9)	0.345	± 0.541	0.042	± 0.028	0.275	± 0.352

Table S3: Fatty acid quantification (derived from Figure 3D, E) in *Ulva*. Data is presented as average (mg/g dry weight of algae) ± SD or as ratio σ-6/σ-3 and PUFA/SFA ± SD.

Fatty acids	Axenic callus		Axenic callus + AGMPFs		Adult stat. culture + microbiome	
C14:0	0.00646[a]	± 0.00218	0.02438[a]	± 0.00402	0.027[a]	± 0.01397
C15:0	n.d.		0.00571[a]	± 0.00058	0.01491[a]	± 0.00514
C16:0	1.31617[a]	± 0.26428	2.76984[b]	± 0.66392	1.65633[b]	± 0.31063
C16:1	n.d.		0.21611[a]	± 0.05064	0.05344[a]	± 0.04288
C16:2 (n-6)	0.05789[a]	± 0.00672	0.39272[b]	± 0.08172	0.14969[a]	± 0.11327
C16:3 (n-3)	0.0634[a]	± 0.00723	0.2177[b]	± 0.05772	0.06961[a]	± 0.05266
C16:4 (n-3)	0.73092[a]	± 0.11999	1.54103[a]	± 0.36437	0.86213[a]	± 0.63602
C18:0	0.01529[a]	± 0.00603	0.02774[a]	± 0.00406	0.02962[a]	± 0.01587
C18:1	0.76466[a]	± 0.32436	1.63278[b]	± 0.36724	0.6572[a]	± 0.29051
C18:2 (n-6)	0.46362[a]	± 0.10480	2.43615[b]	± 0.50653	1.04674[a]	± 0.61905
C18:3 (n-6)	0.0271[a]	± 0.00223	0.11103[b]	± 0.02246	0.03381[a]	± 0.01277
C18:3 (n-3)	0.63055[a]	± 0.36848	1.20488[a]	± 0.85326	1.1115[a]	± 0.99644
C18:4 (n-3)	0.34509[a]	± 0.08936	0.97218[b]	± 0.25410	0.70692[ab]	± 0.20540
C20:3 (n-3)	0.0015[a]	± 0.00005	0.01103[ab]	± 0.00268	0.01449[b]	± 0.00961
C20:4 (n-6)	0.01584[a]	± 0.00610	0.07215[b]	± 0.02009	0.05805[b]	± 0.01522
C20:5 (n-3)	0.00173[a]	± 0.00065	0.00365[a]	± 0.00374	0.03014[b]	± 0.01260
C22:0	0.01181[a]	± 0.00292	0.04318[b]	± 0.00988	0.02724[c]	± 0.00623
C22:1 (n-9)	0.01239[a]	± 0.01882	0.0048[a]	± 0.00363	0.01771[a]	± 0.02166
TFA	4.46441[a]	± 1.25686	11.68705[b]	± 2.16106	6.56655[a]	± 3.2779
PUFA	2.33764[a]	± 0.68446	6.96252[b]	± 1.29417	4.08308[ab]	± 2.62511
SFA	1.34973[a]	± 0.26078	2.87084[b]	± 0.68223	1.75511[a]	± 0.33019
ω6	0.56446[a]	± 0.10778	3.01205[b]	± 0.62974	1.2883[a]	± 0.75893
ω3	1.77318[a]	± 0.57674	3.95047[b]	± 0.86119	2.79479[a]	± 1.88974
ω6/ω3	0.32696[a]	± 0.03989	0.77305[b]	± 0.16814	0.47201[a]	± 0.0969
PUFA/SFA	1.71098[a]	± 0.16398	2.46962[a]	± 0.42276	2.19339[a]	± 1.06381

Figure S1

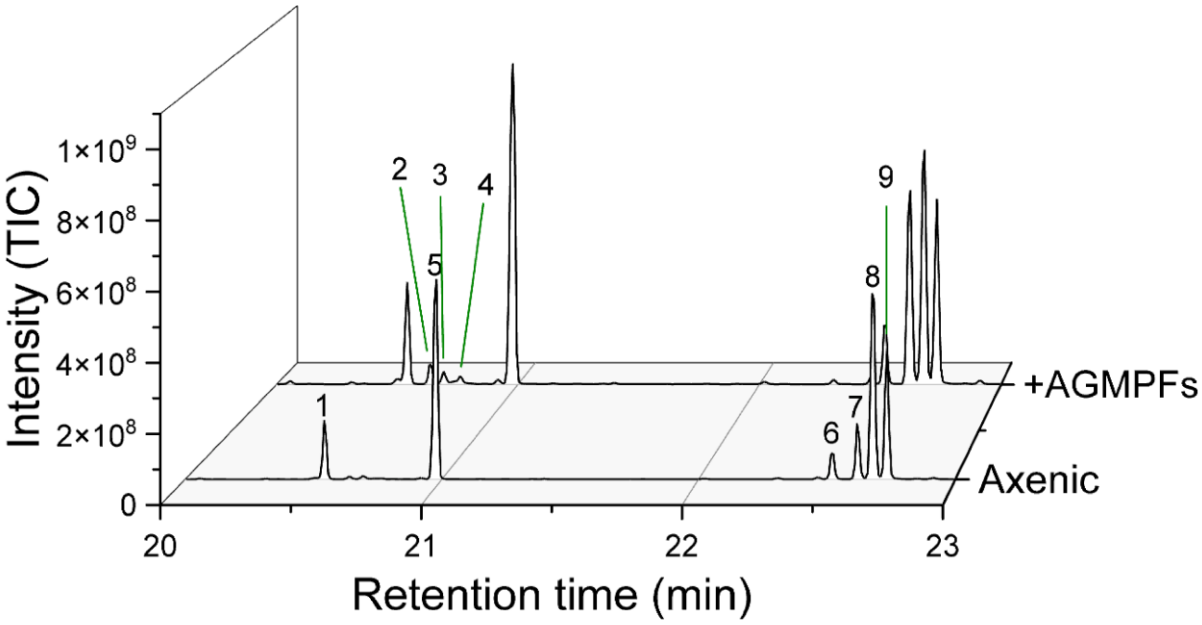

Figure S1: Comparison of representative GC-MS chromatograms of the total ion current (TIC) for fatty acid analysis in axenic calli of *Ulva mutabilis*: axenic control sample vs. AGMPF-treated sample after 14 days of inoculation. The assignment of major the fatty acid methyl esters (FAME) is as follows: **1**: C16:4 n-3, **2**: C16:2 n-6, **3**: C16:3 n-3, **4**: C16:1 n-7, **5**: C16:0, **6**: C18:4 n-3, **7**: C18:2 n-6, **8**: C18:3 n-3, **9**: C18:1. Traces of minor FAME C14:0 and C15:0 (18 – 20 min) and C20- and C22- fatty acids (24 – 27 min) are not shown. Separation of the FAME was performed on a Phenomenex Zebron ZB-SemiVolatiles column.

Materials and Methods

Cultivation of *Ulva*. *Ulva mutabilis* (sl-G[mt +]; morphotype 'slender'; *locus typicus*: Ria Formosa, Portugal, strain FSU-UM5-1) was grown in an *Ulva* culture medium (UCM) at 18°C ± 2 °C with a light/dark cycle of 17/7 h (Califano and Wichard, 2018; Stratmann et al., 1996). The light intensity was 40–80 µmol photons m^{-2} s^{-1}. Algae from stationary stock cultures were utilised to generate gametes, and separation from bacteria was conducted using the phototactic movement of gametes according to an established protocol (Califano and Wichard 2018). Axenic gametes were cultivated for six weeks in a UCM in sterile tissue culture flasks equipped with filter caps (Sarstedt, Nürnbrecht, Germany). The cultures were merged and allocated into six culture flasks under sterile conditions (**Fig. 1**, main text). Three culture flasks contained 40 ml of UCM, while the remaining three flasks contained a mixture of 10 ml UCM, 30 ml *Roseovarius* sp. supernatant (UCM with 1% glycerol added, obtained at an OD_{620} = 0.1 and sterile filtered), and (−)-thallusin (2×10^{-8} mol l^{-1} in medium). Following a two-week cultivation period with consistent microscopy observation (Leica DM IL LED, Leica Microsystems, Aschaffenburg, Germany), the complete biomass of each culture was harvested after being rinsed in ultrapure water and subsequently frozen in liquid nitrogen. Samples were lyophilised overnight at -50 °C (Christ Alpha 1-2 LD Plus, Osterode, Germany), and the weight of each sample was subsequently measured. The biomass was pulverised using a Qiagen Tissuelyser II (Venlo, Netherlands), and the samples were preserved at -70°C until the extraction of metabolites was performed. The cultivation and sample preparation of adult *Ulva* specimens in the stationary phase were conducted similarly.

Maintenance of the *Ulva* callus. *Ulva* calli were cultured in a plastic tissue culture flask under standard conditions in the UCM. Due to the slow growth, the medium was changed every two

months. The calli could be moved from one cell culture flask to another using a plastic scraper while growing on the plastic foundation.

Extraction procedure. Compounds were extracted using a commercially available kit (MAK338, Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions for 40 mg biomass (dry weight). The algae samples were vortexed with 1.5 ml of extraction solution for one minute. Then, 0.25 ml of aqueous buffer was added and vortexed again for one minute. The resulting emulsion was mixed using the kit's syringe–filter combination and filtered into a new 2 ml reaction vessel. The aqueous phase was filtered off, yielding a clear, green solution. The exact volume was determined, and samples were aliquoted into 1.5 ml amber glass vials (WICOM, Heppenheim, Germany) and stored at -25°C. The samples were applied for metabolomics (UHPLC-MS) and fatty acid profiling (GC-MS).

Metabolomics. Aliquots of the samples were evaporated in vacuo and resolved in 30 µl of Methanol (MeOH). The quality control (QC) sample was prepared by mixing 5 µl of each sample. The platform used C_{18} reversed-phase chromatography (Dionex UltiMate 3000 RS UHPLC system; Accucore® RP-C_{18}, 100 × 2.1 mm; 2.6 µm) coupled with a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany).

For chromatography, the column oven was maintained at 25°C and the samples at 10°C. Eluent A – a mixture of UHPLC grade water, 2% (v/v) UHPLC grade acetonitrile, and 0.1% (v/v) formic acid – was used. Eluent B consisted of pure UHPLC-grade acetonitrile. Three microliters of each sample were injected. Separation was performed using gradient elution with a flow rate of 0.4 ml/min: 0-0.2 min 0% B; 0.2-8.0 min 0-100% B; 8.0-11.0 min 100% B; 11.0-11.1 min 100-0% B; and 11.1-12.0 min 0% B.

Mass spectrometry utilised the positive/negative polarity switching mode for ionisation. The parameters were the following: Two full-scan modes were selected: Polarity: positive; scan

range: 100 to 1500 *m/z*; resolution: 70,000; AGC target: 3×10^6; maximum IT: 200 ms. General settings: sheath gas flow rate: 60; auxiliary gas flow rate 20; sweep gas flow rate: 5; spray voltage: 4.0 kV; capillary temperature: 360 °C; S-lens RF level: 50; auxiliary gas heater temperature: 400 °C; acquisition time frame: 0.5 - 11.5 min.

For the negative mode, all values were kept beside the spray voltage, which was set to 2.5 kV. In the end, data-dependent analyses were recorded for the QC samples to generate MS/MS data. Additionally, the following MS parameters were applied: Scan range: auto, resolution: 17,500; AGC target: 1×10^5; maximum IT: 50 ms, loop count = 5, preferred charge state = 1, dynamic exclusion: 20 sec, Isolation window: ± 0.2 *m/z,* stepped collision energies: 15, 30, 45 NCE. Peak picking, alignment, deconvolution and metabolite identification were performed using the Compound Discoverer 3.3 (CD, Thermo Fisher Scientific). Features identified in the blank samples (≥20% of the feature area) were excluded from the analysis. Pooled quality control samples (QC) were utilised to correct for time-dependent batch effects. The raw data was normalised based on the dry weight of the sample. Using default settings (Template: "Untargeted Metabolomics with statistics, detect unknowns with ID using online databases and mzlogic") with minor adjustments (detect compounds: minimum intensity 50000, signal to noise ratio set to 3 in respective nodes, limitation of specific unlikely ion adducts), the raw data was compared within CD with Thermo Fisher data repositories like mzcloud, lab intern data repositories and open data repositories, such as ChemSpider and KEGG. The generated peak list was statistically analysed using Metaboanalyst 6.0 with the univariate and multivariate methods. The data was processed by log transformation and auto-scaling. The volcano plots illustrate the univariate analysis results for each metabolite feature with $p < 0.1$ and a fold-change greater than two.

Derivatisation of fatty acids. The aliquoted samples were evaporated entirely using a nitrogen stream (Biotage-TurboVap® LV, Uppsala, Sweden) and subsequently methylated. The

methylation solution comprised 19 ml of methanol and 1 ml of acetyl chloride (Lepage and Roy 1984). For the derivatisation, 500 µl of methylation solution and 600 µl of *n*-hexane were added to the evaporated samples (Wichard et al. 2007). The samples were heated at 95°C for ten minutes and then cooled on ice. The samples were combined with 500 µl of ultrapure water and vortexed for one minute. The upper hexane phase was transferred to a new 1.5 ml short-thread bottle (ND9; LABSOLUTE®) preloaded with six spatula tips of anhydrous sodium sulfate. Subsequently, 200 µl of solution was aliquoted into new 1.5 ml vials and stored at -25°C.

Targeted fatty acid analysis. The approach used gas chromatography (Trace 1300 with AS3000 autosampler, Thermo Fisher, Bremen, Germany, equipped with Phenomenex Zebron ZB-SemiVolatiles column, 30 m, 0.25 mm I.D., 0.25 µm film thickness, Torrance, California, United States) coupled with mass spectrometry (ISQ single quadrupole, Thermo Fisher, Bremen, Germany). One microliter was injected in a splitless mode at 260°C. The oven temperature was initially set at 60°C for five minutes, raised at a rate of 10 °C/min to 300°C and sustained for an additional one minute. The temperature of the MS transfer line was set to 250°C. Helium was used as a carrier gas at a 1.2 ml per minute consistent flow rate. Electron impact ionisation occurred at 70 eV and a temperature of 280°C. Each measurement sequence started with two air blanks and concluded with assessing the external standards.

The Thermo Xcalibur 3.0.63 Qual Browser was used to examine the chromatograms. The external standard mix, Supelco 37 FAME (fatty acid methyl ester), was aliquoted and diluted 1:20 in dichloromethane (v:v). A second external standard of cis-4,7,10,13-Hexadecatetraenoic acid (C16:4 n-3) was prepared (0.0217 g l^{-1} after derivatisation). The standards were measured at the end of every sequence. The external standards were employed to identify the chromatographic peaks corresponding to the fatty acid methyl esters. Characteristic masses for fatty acid methyl esters were utilised (Mjøs 2004). The characteristic mass-to-charge ratios (*m/z*) observed were *m/z* = 74 for the saturated fatty acid methyl esters, *m/z* = 55 for the

monounsaturated, *m/z* = 67 for the di-unsaturated, and *m/z* = 79 for the polyunsaturated fatty acid methyl esters, as determined from the extracted ion chromatograms (EIC).

The fatty acids were identified by comparing the retention time, molecular ion and fragmentation pattern with reference standard mixtures. The signals found in the algal analysis matched the external standards. C18:4(n-3), C16:2(n-6), and C16:3(n-3) were identified in the algal samples by the molecular ion and fragmentation pattern only.

Fatty acids quantification. After identification, the integrated peak area of the individual FAME was determined in the total ion or the extracted ion current mode (for characteristic fragments only, in case of peak overlapping) using the ICIS-Peak-Integrations-Algorithm (Baseline window: 5; area noise factor: 100; peak noise factor: 1; peak height: 5%; tailing factor: 9). The concentration of the analyte was calculated through the response factor. It is the ratio between the signal (area) produced by the analyte, and the quantity of analyte which produces the signal using the peak area of the external FAME standard. C18:4(n-3), C16:2(n-6) and C16:3(n-3), not present in the standards, were evaluated on the peak area of similar fatty acids provided by the FAME standard to estimate the concentration. For C18:4(n-3), the peak area of C16:4(n-3) was employed, while the peak area of C16:1(n-7) was used to estimate isomers of C16:1, C16:2(n-6) and C16:3(n-3). Data was normalised by the dry mass of the analysed algal tissue before statistical analyses were performed. Biological triplicates were employed to calculate the mean ± standard deviation. Significant differences between means of treatments were determined by the one-way ANOVA with a Fisher Post-hoc-test ($p < 0.05$).

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