



Epigenetic Regulation Links Bacterial Signaling to Growth and Reproductive Development in the Green Macroalga *Ulva compressa* (Chlorophyta)

Janine F. M. Otto¹ · Hermann Holbl¹ · Nico Ueberschaar² · Georg Pohnert¹ · Thomas Wichard¹

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Abstract

DNA (deoxyribonucleic acid) methylation is a key epigenetic mechanism that regulates gene expression and developmental transitions in eukaryotes. Interestingly, algal growth- and morphogenesis-promoting bacteria can reduce global DNA methylation levels in the green seaweed *Ulva* (Chlorophyta), highlighting DNA methylation as a dynamic and environmentally responsive epigenetic mechanism in marine macroalgae. We hypothesized that DNA methylation serves as a rapid and essential epigenetic regulatory mechanism during gametogenesis in the model organism *Ulva compressa* (cultivar *U. mutabilis* slender). We further propose that this process is controlled by sporulation inhibitors - extracellular compounds long known to regulate reproduction in *Ulva*, yet whose molecular mode of action has remained elusive. Using ultra-high-performance liquid chromatography-mass spectrometry (HPLC–HRMS), we quantified global levels of 5-methylcytosine (5mC) and *N*6-methyladenine (6mA) across defined life-cycle stages of *Ulva*, including gametogenesis induction and gamete release. We observed a rapid and pronounced increase in 5mC immediately following removal of sporulation inhibitors, consistent with active epigenetic reprogramming. In contrast, 6mA was detected only in mature thallus (vegetative tissue of macroalgae) and gametes, and not throughout gametogenesis, indicating a distinct yet minor role for this DNA modification in *Ulva* development. Re-addition of sporulation inhibitors prevented both gametogenesis and the characteristic methylation dynamics, indicating that these not-yet-characterized inhibitors act as epigenetic modulators. Together, our findings identify DNA methylation as a key regulatory layer linking bacterial cues, sporulation control, and reproductive development in *Ulva*, with direct implications for the epigenetic management of growth and reproduction in marine algal biotechnology.

Keywords DNA methylation · Epigenetics · Gametogenesis · Life cycle regulation · Sporulation · *Ulva mutabilis*

Introduction

Macroalgae form the backbone of marine coastal ecosystems because they are important primary producers, provide habitats for other aquatic species, and are essential for the health of marine habitats (Kuba Gabrielle et al. 2021). Nevertheless, the molecular mechanisms governing their

complex life cycles remain largely unexplored. Epigenetic modifications, particularly DNA methylation, have emerged as key regulators in adaptation to changing environmental conditions and the development of land plants (Bartels et al. 2018; Hemenway and Gehring 2023). However, the role of DNA methylation in marine algae remains largely unknown, particularly during critical developmental stages. While the epigenetic regulation of development is well-studied in higher plants and animals (Bartels et al. 2018; Hemenway and Gehring 2023; Messerschmidt et al. 2014; Molaro et al. 2011; Smith et al. 2025), much less is known about these mechanisms in marine algae (Ferrari et al. 2023). The level of DNA methylation often corresponds with the complexity of organisms and their life cycles (Bird, 2002). Many prokaryotes and some unicellular eukaryotes exhibit

✉ Thomas Wichard
thomas.wichard@uni-jena.de

¹ Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany

² Mass Spectrometry Platform, Friedrich Schiller University Jena, Humboldtstr. 8, 07743 Jena, Germany

lower levels of developmentally relevant DNA methylation when compared to most multicellular organisms with more complex life cycles (Lopez et al. 2015; Molaro et al. 2011). Nevertheless, the brown algae *Saccharina japonica* and *Ectocarpus siliculosus* have relatively low levels of genome-wide cytosine methylation, at 1.4% and <0.035%, respectively (Cock et al. 2010; Fan et al. 2020). The green seaweed *Ulva* spp. is one of the most abundant algal species in coastal benthic environments worldwide, and can form green tides (Fletcher 1996; Smetacek and Zingone 2013). Its DNA is known for its highly methylated CpG content and high global methylation level (Gupta et al. 2012, 2015). Although *Ulva* undergoes a complex diplohaplontic life cycle similar to that of other macroalgae (Wichard 2015), the role of DNA methylation during these processes remains unknown. *Ulva* requires algal growth and morphogenesis-promoting factors (AGMPFs) released by two distinct bacterial groups for normal development (Fig. 1). *Maribacter* spp. produces the heterocyclic compound thalusin, which induces cell differentiation in *Ulva* (Alsufyani et al. 2020; Dhiman et al. 2022). Another unknown bacterial factor essential for cell division and, thus, algal growth is produced by *Roseovarius* sp. (Wichard 2023). Together, the two bacteria and the alga form a tripartite community in which the organisms communicate through chemical signals and influence each other (Spoerner et al. 2012; Wichard 2023). Under such standardized conditions, *U. compressa* ‘slender’ develops a tubular-like thallus and a rhizoid (Løvlie 1964). In contrast, only undifferentiated calli with visible cell wall protrusions develop in the absence of the natural microbiome, especially in the absence of *Maribacter* sp. strain MS6 and *Roseovarius* sp. strain MS2. While the developmental role of AGMPFs is well established (Spoerner et al. 2012; Ghaderiardakani et al. 2017), their impact

on the underlying molecular and epigenetic regulatory processes is only beginning to be understood. Initial studies have demonstrated altered gene expression patterns in *Ulva* correlated with the presence of AGMPF-releasing bacterial symbionts (Kwantes and Wichard 2022). Recently, we demonstrated significant differences in the global DNA methylome of *Ulva* grown in the presence of AGMPF-releasing bacteria (Otto et al. 2025). Under axenic conditions, the relative amount of 5-methylcytosine (5mC) was significantly higher than in xenic-grown adult *Ulva* specimens, whereas N6-methyladenine (6mA) levels remained below the limit of detection. These differences suggest that bacterial associations modulate the global methylation landscape; however, such environmentally induced epigenetic states may need to be reset during key life-cycle transitions.

In *Ulva*, gametogenesis represents a major developmental switch and is tightly regulated by two sporulation inhibitors: a putative glycoprotein (SI-1) and a low-molecular-weight compound (SI-2), which are released into the surrounding medium and accumulate between the bilayered thallus layers of the alga (Kessler et al. 2018a, b; Stratmann et al. 1996; Vesty et al. 2015). These inhibitors prevent the formation of gametes in mature algae (Alsufyani et al. 2017). When both SI-1 and SI-2 are removed or degraded, cells enter gametogenesis, a process that triggers major transcriptional and morphological changes (He et al. 2019; Katsaros et al. 2017; Liu et al. 2022). Once the “point of no return” is reached, approximately 36 h after induction, the developmental program leading to a gametangium cannot be reversed. The determination phase is followed by the differentiation phase, during which a vegetative blade cell develops into a gametangium (Stratmann et al. 1996; Kessler et al. 2017). This transition requires large-scale reprogramming of gene expression, mediated by changes in DNA methylation in many eukaryotes. Finally, a low-molecular-weight swarming inhibitor released by *Ulva* thalli prevents discharging premature gametes upon gametogenesis has been completed and must be diluted below a threshold concentration before gamete discharge occurs (Wichard and Oertel 2010).

Here, we demonstrate that bacterial interactions profoundly shape the DNA methylation landscape in *Ulva* and that these epigenetic states are not permanent but dynamically and reproducibly reset during each reproductive cycle. Therefore, we investigated the dynamics of the methylome during gametogenesis in detail. Using high-resolution mass spectroscopy, we analyzed the global levels of 5mC and 6mA as potential key life cycle regulators, aiming to elucidate dynamic methylation during the crucial step of gametogenesis in *Ulva compressa* under the control of sporulation inhibitors (Kessler et al. 2018b; Stratmann et al. 1996; Vesty et al. 2015). Understanding changes in DNA methylation during critical life-cycle transitions will

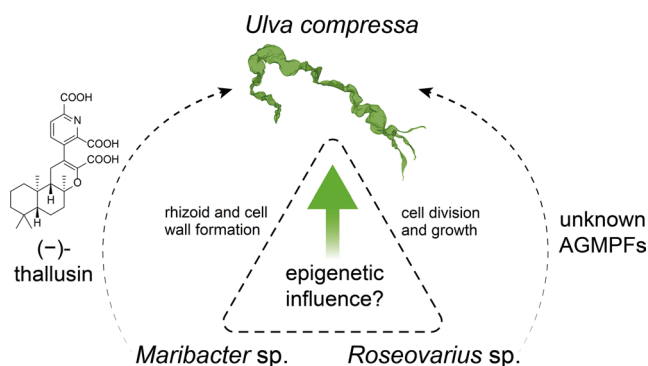


Fig. 1 *Ulva compressa* inhabits a tripartite community. *Maribacter* sp. and *Roseovarius* sp. produce algal growth- and morphogenesis-promoting factors (AGMPFs) that are necessary for the normal growth and development of *Ulva compressa* and may also influence gene regulatory or epigenetic processes in the algae (Spoerner et al. 2012; Ghaderiardakani et al. 2017)

advance our knowledge of algal reproductive biology and provide broader insights into epigenetic regulation in non-vascular plants.

Materials and methods

Reagents

The following reagents were dissolved in LC-MS-grade water (Chemsolute, Th. Greyer GmbH & Co. KG, Reinnigen, Germany) and used as internal standards (ISs): 2'-deoxy-*N*-6-methyladenosine- d^3 (6mAd 3), 2'-deoxycytidine- $^{13}C_1$, $^{15}N_2$ (C- $^{13}C_1$ $^{15}N_2$), 2'-deoxy-5-methylcytidine- $^{13}C_1$, and $^{15}N_2$ (5mC- $^{13}C_1$ $^{15}N_2$). These reagents were purchased from Toronto Research Chemicals Inc. (North York, Canada), while 2'-deoxyadenosine- $^{15}N_5$ (A- $^{15}N_5$) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, US).

Algal culture conditions

U. compressa Føyn morphotype 'slender' (mt+, FSU-UM5-1, cultivar *Ulva mutabilis*) was cultivated with the core microbiome, including the essential AGMPFs-releasing bacteria *Roseovarius* sp. MS2 and *Maribacter* sp. MS6 (Hardegen et al. 2025) or under axenic conditions in *Ulva* culture medium (UCM) (Califano and Wichard 2018; Stratmann et al. 1996). Cultures were maintained at 18 ± 2 °C, with a 17:7 h light-dark cycle and 40–80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Bacteria grew at 50:50 marine broth/UCM. Axenic cultures were obtained by gamete separation from bacteria using their phototaxis according to Califano and Wichard (Califano and Wichard 2018).

Induction of gametogenesis

Approximately 3 g of adult thalli were chopped and washed three times (15 min) in Instant Ocean medium (Aquarium Systems, France) to remove sporulation inhibitors. The algal fragments (1–4 mm 2) were transferred into UCM and incubated for 72 h (Wichard and Oertel 2010). After removal of the swarming inhibitor, accumulating in the growth medium, through medium exchange at 72 h, gametes were released and collected 20–40 min later and either used to prepare axenic cultures or as a seed stock for algal cultivation.

Preparation of sporulation inhibitor extracts

SI-1 was extracted from axenic culture medium by phenol extraction and acetone precipitation, whereas SI-2 was obtained from the intercellular fluid of chopped thalli by Tris-buffer extraction; activity of both inhibitors was

verified using a bioassay (see Supporting Information for details). The activity of the sporulation-inhibitor fraction was determined using serial dilution. One unit of the SI-1 and SI-2 is hereby defined as the minimal amount of the factor that inhibits differentiation of a blade cell into a gametangium (=gametogenesis), with an inhibitory rate > 50% in 1 mL UCM (i.e., 200 1–4 mm 2 fragments) within 3 days under previously described conditions (Kessler et al. 2018a).

Sampling and DNA extraction

Fragmented thallus samples of *Ulva* were collected at 0.5, 24, and 72 h following induction of gametogenesis. Control samples were either chopped without washing or washed without chopping (see Supplementary Information). For SI-treated cultures, purified SI-1 and SI-2 were added immediately after the last washing step at a final concentration of 100 units mL $^{-1}$ each, and samples were collected at the same time points. In all treatments, collected fragments were rinsed to remove residual bacteria and stored at -80 °C. Microscopy images were recorded at each time point. Freeze-dried thalli were homogenized with metal beads, and genomic DNA was extracted using the GenElute™ Plant Genomic MiniPrep Kit (Sigma-Aldrich, Germany).

Gamete DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Germany). RNase I was added during lysis, and DNA concentrations were measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Germany). Bacterial DNA was isolated from 2-week-old stationary cultures using the Bacterial DNA Preparation Kit (Jena Bioscience, Germany). DNA samples were stored at -20 °C until chemical analysis.

DNA hydrolysis, HPLC-HRMS analysis, and data processing

For DNA hydrolysis (Otto et al., 2025), approximately 50 ng of DNA was mixed with internal standards and 8% HCl to a final volume of 40 μL , and the mixture was hydrolyzed in a sealed tube at 120 °C for 3 h. After freezing, samples were dissolved in water and analyzed via high-performance liquid chromatography coupled with mass spectrometry (HPLC-HRMS). All samples were run in triplicates. Analyses were performed on a Thermo UltiMate 3000 UHPLC system coupled to a Q Exactive Plus™ Orbitrap MS (Thermo Fisher Scientific, Germany) and separated on a Phenomenex Synergi™ Fusion-RP column using a 5-min gradient at 0.4 mL min $^{-1}$ (Phenomenex, CA, US). Full-scan positive-mode data (80–800 m/z) were acquired at a resolution of 35,000 (Otto et al. 2025). Data processing was performed using Thermo FreeStyle and Xcalibur Quan Browser. Peaks were extracted via XIC, detected using the ICIS algorithm (with smoothing set to 9), and quantified

with a mass tolerance of ± 8 ppm (see Supporting Information, Fig. S1–S3). For time-course methylome analyses, one-way ANOVA with Tukey's post hoc test ($p < 0.05$) was performed in Origin 2023 (OriginLab, MA, US) after verifying equal variances across groups. F-tests and *t*-tests were used for pairwise comparisons (further details in Supporting Information).

Results

Building on the link between bacterial signaling and *Ulva* development, we examined how global DNA methylation patterns shift during key life-cycle stages. We connected methylation differences during reproductive transitions with microbial presence across three experiments, as outlined below.

- (i) To exclude that the observed differences could be caused by bacterial DNA contamination in the xenic thallus samples, we analyzed DNA from 4-week-old *Roseovarius* sp. and *Maribacter* sp. cultures. Both strains exhibited substantially lower 5mC levels than *Ulva*, and only small amounts of 6mA were detectable (Fig. 2), making a significant contribution of methylated bacterial DNA to the measured methylation levels of xenic *Ulva* samples highly unlikely.

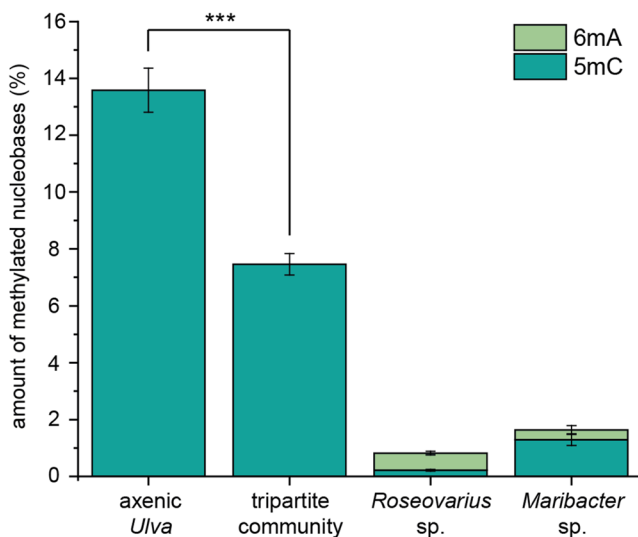


Fig. 2 Bacterial DNA methylation patterns differ between *Roseovarius* sp. MS2, *Maribacter* sp. MS6, and the genomic DNA of *Ulva compressa*. Relative amount of 5-methylcytosine (5mC) and 6-methyladenine (6mA) in *Ulva* grown with and without (axenic) their associated bacteria and in both bacterial species. *Roseovarius* sp. and *Maribacter* sp. exhibit low but detectable levels of 5mC. DNA from axenic and xenic *U. compressa* exhibits significantly higher levels of 5mC. *Roseovarius* sp. exhibits the highest proportion of 6mA, followed by *Maribacter* sp. No detectable 6mA was found in both axenic and xenic *U. compressa*. Error bars represent mean $\pm$ standard deviation. $n = 1$ –3 biological replicates, with three technical replicates each

- (ii) To assess whether DNA methylation changes during *Ulva*'s development and gametogenesis (Fig. 3A), we extracted and analyzed DNA from gametes collected immediately after their release. Interestingly, these gametes exhibited 5mC levels ($13.2\% \pm 0.7\%$) comparable to those of axenic calli ($13.6\% \pm 0.8\%$) (*t*-test, $p > 0.05$; not significant; Fig. 3B). As the gametes developed into germlings over the following weeks, the 5mC level declined to approximately 6% (*t*-test, $p < 0.001$, ****). The 6mA level in the gametes was near the limit of detection and increased by approximately 0.8% in mature *Ulva* (Fig. 3D).
- (iii) Finally, to test whether global DNA methylation returns to higher levels during gametogenesis, we induced gametogenesis in *Ulva* and collected thallus samples directly after washing (0.5 h), 24 h, and 72 h to quantify 5mC and 6mA (Fig. 3B and D). Induced blade cells showed a rapid increase in 5mC immediately after the removal of sporulation inhibitors, reaching levels ($12.9\% \pm 0.3\%$) comparable to axenic-grown *Ulva*. In contrast, non-induced control samples maintained consistently low methylation levels (mature *Ulva*: $t < 0$ h, $5.8\% \pm 0.5\%$).

Following this initial rise, 5mC levels declined slightly during the determination phase (24 h, $10.6\% \pm 0.4\%$) and increased again shortly before gamete release at 72 h ($12.1\% \pm 0.3\%$). After removing the swarming inhibitor after sampling at 72 h, released gametes displayed a 5mC level of $12.8\% \pm 0.2\%$, which was not significantly different from that of the first analyzed gametes (*t*-test, $p > 0.05$, not significant). Slightly higher methylation levels of released gametes were detected when we compared them to the chopped thallus sample collected after 72 h (*t*-test, $p < 0.05$; *). However, microscopic examination revealed a mixture of residual undifferentiated blade cells (i.e., low methylation levels) and fully developed gametangia (i.e., high methylation levels) in this sample (Fig. S4), in contrast to the homogeneous population of discharged gametes with high methylation levels.

To determine whether an early surge in 5mC is related to the initiation and regulation of gametogenesis, extended amounts of purified SI-1 and SI-2 (100 Units/mL) were added to the chopped thalli immediately after washing, and samples were collected at 0.5 h, 24 h, and 72 h (Fig. 3C and E). As expected, no gametogenesis occurred in the presence of the SI (see images of Fig. 3C and 3E). Although the 5mC analysis again showed a slight increase immediately after washing, the values remained significantly lower ($9.4\% \pm 0.4\%$) than in cultures without sporulation inhibitors (ANOVA; see Fig. 3B and C). During the determination phase, 5mC levels returned to pre-induction values ($5.8\% \pm 0.4\%$) and declined further to $1.0\% \pm 0.5\%$ by 72 h. Therefore, the re-addition of SI-1 and SI-2 prevented both

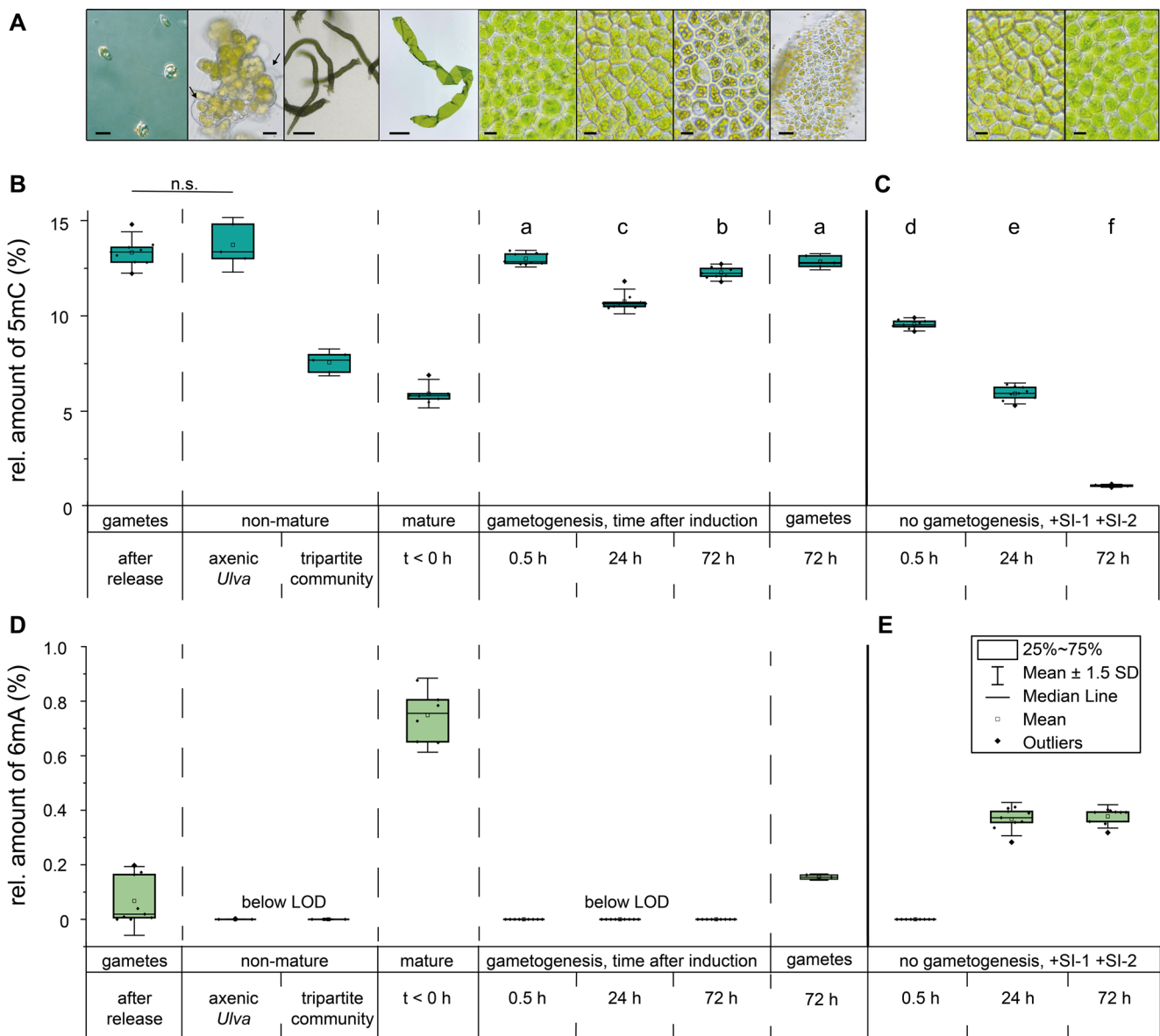


Fig. 3 Dynamic changes in the global 5mC level of *Ulva* during its lifecycle. **A** Microscopic images of the different stages in the *Ulva compressa* slender lifecycle. Microscopic pictures and scale bars from left to right: gametes: 10 μ m; axenic callus with cell wall protrusions (arrow): 20 μ m; germlings (tripartite community): 100 μ m; mature alga: 1 cm; blade cells develop into gametangia upon induction: 20 μ m; gametes during release: 60 μ m. **B** Relative amount of 5mC in genomic *U. compressa* DNA. Induction of gametogenesis leads to a rapid increase in 5mC levels. Dynamic changes in the 5mC level were detected during gametogenesis. **C** The gametogenesis process could be interrupted by adding sporulation inhibitors (SI-1 and SI-2) to the chopped thalli, which led to a massive decline in the cytosine methylation.

gametogenesis and the characteristic methylation rebound, with excess sporulation inhibitor producing the lowest methylation levels observed.

In the same experiments, we quantified the relative amount of 6mA. Mature *Ulva* (8 weeks old) exhibited an

elevated 6mA level of $0.7\% \pm 0.1\%$, while 6mA values remained below the limit of detection (0.056%) throughout gametogenesis. In samples treated with sporulation inhibitors, slightly increased 6mA levels (to 0.4%) were detected at 24 and 72 h, respectively (Fig. 3E).

To gain a more detailed understanding of the temporal progression of DNA methylation during gametogenesis, we repeated the experiment with additional sampling points at 6, 12, and 48 h (Fig. S5). Following gametogenesis induction and the initial strong increase in methylation, a decline in global 5mC during the determination phase was observed and persisted until 48 h. During the differentiation phase, 5mC levels rose again, although they did not return to the high values observed early in the determination phase (0.5, 6, and 12 h), which can be again correlated to a higher number of undifferentiated blade cells observed under the microscope. Overall, gametes were discharged with a reset global methylation level before encountering their associated bacteria, such as *Maribacter* sp. and *Roseovarius* sp., which induce growth and morphogenesis.

Discussion

In this study, we show that global DNA methylation, particularly 5mC, is dynamically regulated during gametogenesis and early developmental stages in the slender green seaweed *U. compressa* (Fig. 4). In higher plants, it

has previously been demonstrated that bacterial symbionts can induce alterations in DNA methylation patterns (Grandbastien 1998; Yu et al. 2013). Gupta et al. (2012) observed methylation polymorphisms associated with morphogenesis using a Methylation Sensitive Amplification Polymorphism approach. Supporting this hypothesis, a previous study (Otto et al. 2025) reported significant differences in DNA methylation between young *Ulva* germlings grown with or without their bacterial symbionts, releasing AGMPFs, which are well investigated as drivers of algal development (Spoerner et al. 2012; Ghaderiadekani et al. 2017; Ulrich et al. 2026). However, the molecular pathways leading to morphogenic regulation remain largely unknown (Yamamoto et al. 2018; Alsufyani et al. 2020; Wienecke et al. 2024).

A plausible explanation for our results is that the observed reduction in 5mC represents developmental epigenetic remodeling during thallus formation, rather than a direct demethylating action of AGMPFs. In diverse eukaryotic systems, shifts in DNA methylation are tightly linked to major developmental transitions and are considered key facilitators of differentiation-specific gene expression programs (Suelves et al. 2016; Kyono et al. 2020; Hunt et al. 2024). In this context, global DNA demethylation in xenic

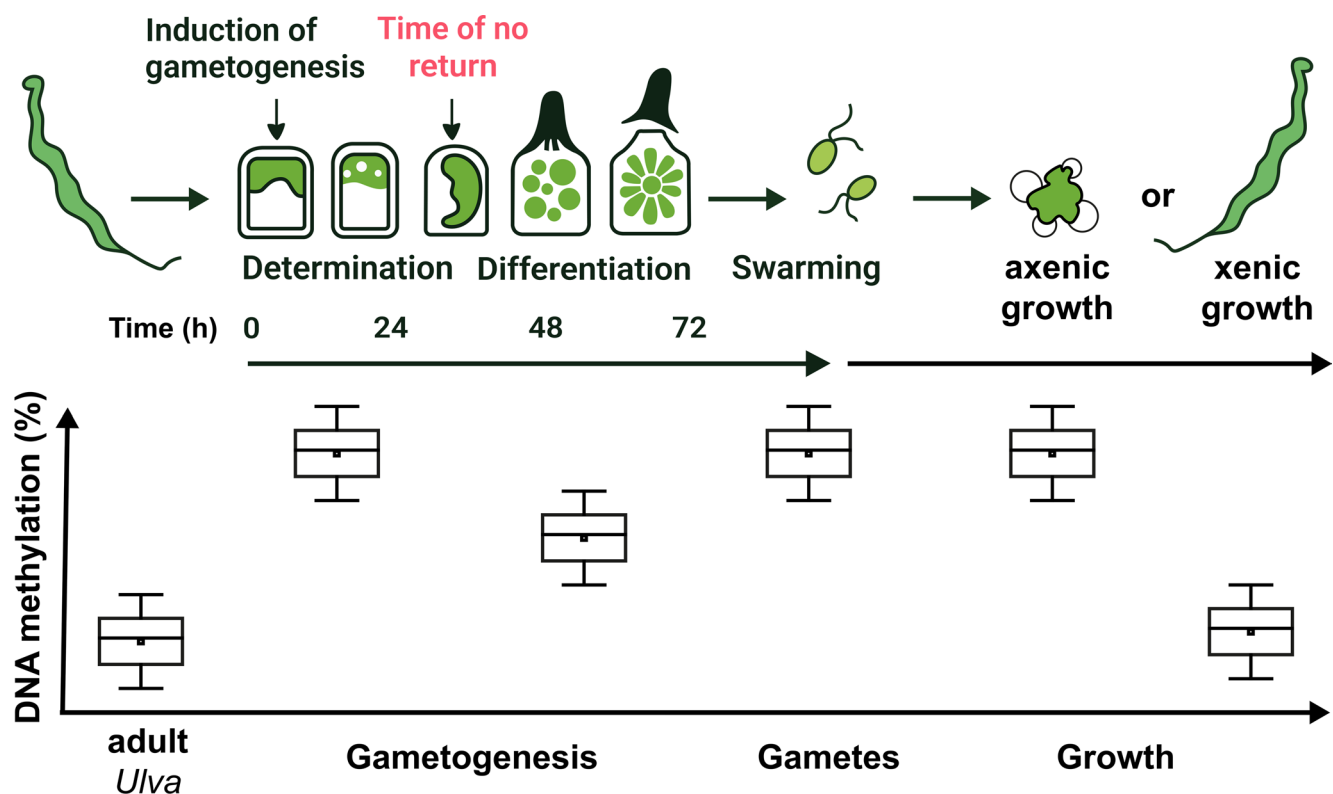


Fig. 4 Overview of the temporal physiological and epigenetic dynamics of gametogenesis. After induction, the system undergoes phases of determination and differentiation, culminating in the formation of functional gametes, which are discharged. In parallel with gametogenesis, the global DNA methylome, dominated by 5mC is changing.

Sporulation inhibitors (SI-1 and SI-2) inhibit the process when re-added before the “point of no return” (Wichard and Oertel 2010). Only the presence of associated bacteria leads to physiological development following gamete release, coupled with a decrease in the global 5mC level

Ulva thalli may represent an epigenetic state that enables morphogenetic gene expression in response to bacterial signals. Nevertheless, whether AGMPFs directly modulate DNA methylation machinery or whether epigenetic changes arise as a downstream consequence of morphogenesis remains an open question and will require further studies.

Furthermore, the mechanistic role, dynamics, and reversibility of DNA methylation during defined life-cycle transitions remained unresolved. Here, we demonstrated that freshly discharged gametes exhibit methylation levels similar to those of axenic germlings, indicating that epigenetic imprinting is reprogrammed during both gametogenesis and early development in *Ulva*, as observed in higher plants. In particular, male sperm cells of *Arabidopsis thaliana* exhibit elevated expression of methyltransferases (Borges et al., 2008) and are hypermethylated relative to vegetative cells (Calarco et al. 2012). Notably, we focused on parthenogenetic reproduction in *Ulva* through unmated gametes (Hoxmark 1975). Sporulation inhibitor removal revealed a rapid increase in methylation content of approximately 7% within 1 h after gametogenesis induction. These findings align with the observation that approximately 45% of the *Ulva* genome is differentially expressed during life cycle changes (Liu et al. 2022). We argue that the combination of the sporulation inhibitors SI-1 and SI-2 not only regulates reproductive timing in *U. compressa* but also serves as a practical tool for controlling and studying epigenetic dynamics.

The 7% increase in 5mC levels represents a remarkably rapid epigenetic response in a multicellular eukaryote. This dynamic methylation appears biochemically feasible in *U. compressa*, given its compact genome and potential upregulation of multiple DNA methyltransferases (De Clerck et al. 2018). Although the exact DNA methyltransferase complement of *Ulva* remains uncharacterized, the required methylation rate could be achieved through the coordinated activity of several enzymes, possibly including bacterial-derived methyltransferases acquired via horizontal gene transfer (De Clerck et al. 2018). The intracellular *S*-adenosylmethionine (SAM) pool, supported by sulfur and methyl metabolism in *Ulva* (Han et al. 2025) and the availability of alternative methyl donors such as DMSP (dimethylsulfoniopropionate) produced by *Ulva* in elevated amounts (Kessler et al. 2017), likely provides sufficient methyl groups to sustain this process. Together, these findings suggest that *Ulva* possesses an efficient and adaptable methylation machinery that mediates rapid epigenetic responses during key developmental transitions and can also respond to stress stimuli. Although global demethylation during sexual reproduction in plants has been reported (Baroux et al. 2011; Jullien and Berger 2010), we observed globally elevated methylation here. We can exclude that the methylation represents an

exclusive stress response to thallus cutting since no increase in 5mC content was observed in cut, unwashed, and thus sporulation inhibitor-containing thalli.

Upon the initiation of gametogenesis, a cell must transition from vegetative to reproductive growth (Fig. 4). Therefore, genes responsible for growth and cell wall synthesis, for example, are silenced. Hypermethylation, particularly in promoter regions, is often associated with lower transcriptional activity because most transcription factors cannot bind methylated DNA (Qiao et al. 2024). Better genomic stability could be another factor contributing to the rapid increase in methylation levels. At the onset of gametogenesis, chromatin-opening processes occur to increase DNA accessibility for transcription (She and Baroux, 2014) and to enable transposable elements (TEs) to become active (Slotkin et al. 2009). A sudden burst of methylation might maintain TE silencing during cell-fate changes, thereby reducing genomic plasticity.

Following the initial surge in DNA methylation, the successive phases of gametogenesis displayed distinct 5mC levels (Fig. 4), suggesting that multiple rounds of epigenetic reprogramming occur during this developmental transition. The re-addition of sporulation inhibitors to chopped and washed thalli effectively interrupted gametogenesis (Nahor et al., 2021; Stratmann et al. 1996). The pronounced decline in 5mC levels over time in these samples indicates active demethylation or methylation inhibition following DNA replication, suggesting that sporulation inhibitors may function as epigenetic modulators of DNA methylation. At least one sporulation inhibitor may interfere with DNA methylation regulation in *Ulva*, potentially by binding the essential cofactor SAM, thereby blocking its access to DNA methyltransferases, or by promoting demethylation. Identifying the chemical nature and mode of action of these inhibitors will be crucial to test these possibilities.

In addition to 5mC, *N*6-methyladenine (6mA) has recently emerged as a potential epigenetic mark in several eukaryotes, including plants and algae (Bochtler and Fernandes 2021; Karanthamalai et al. 2020; Zhang et al. 2023; Fu et al. 2015). In this study, we first quantified the 6mA level in a macroalga and examined changes throughout its life cycle. In contrast to 5mC, no clear dynamic trend was observed throughout gametogenesis. Nevertheless, the development of gametes into mature *Ulva* results in a significant increase in the 6mA level. According to its genome-wide distribution, the role of 6mA in plant development, tissue differentiation (Liang et al. 2018), gene expression (Fu et al. 2015), and mitochondrial replication (Fedoreyeva and Vanyushin 2002) is discussed (Karanthamalai et al. 2020). In *Ulva*, the accumulation of 6mA in mature thalli may therefore reflect epigenetic stabilization after morphogenesis. This potentially supports stable cell function via mitochondrial replication and gene expression regulation. However, the exact role of 6mA in green algae remains largely unknown and will require further analyses.

Conclusions

DNA methylation in *U. compressa* is dynamic and closely linked to both microbial interactions and developmental transitions. The higher 5-methylcytosine levels observed under axenic conditions, together with the rapid methylation changes during gametogenesis, suggest that epigenetic reprogramming serves a key role in coordinating the algal life cycle and acts as a molecular switch that initiates reproductive development. In contrast, subsequent demethylation occurs during gamete differentiation (i.e., germination), when AGMPF-releasing bacteria are present following gamete discharge from the gametangium. DNA methylation is thus a pivotal regulatory mechanism in *Ulva*, integrating environmental and bacterial cues with developmental control.

Importantly, our results suggest that sporulation inhibitors do not merely block reproductive development but also function as epigenetic modulators that can suppress or reverse methylation dynamics associated with gametogenesis. By modulating the SIs, it is possible to experimentally trigger or suppress the associated methylation and to alter the downstream gene and metabolic expression profiles reported by Liu et al. (2022). This establishes sporulation inhibitors as valuable tools for elucidating the causal relationships among environmental cues, DNA methylation, and developmental processes in marine algae. The insight provides a mechanistic bridge between classical physiological studies on sporulation control and modern epigenetic regulation. From a marine biotechnology perspective, it is highly relevant: spontaneous sporulation poses a major challenge in *Ulva* aquaculture, and our findings indicate that sporulation inhibitors (or potentially other methylation inhibitors) could be harnessed to stabilize vegetative growth via epigenetic control, thereby reducing economic risks in biomass production systems.

Ulva, as an emerging non-vascular model for epigenetic regulation, complements higher-plant systems and underscores the importance of microbial–algal interactions in shaping developmental epigenomes. By demonstrating that epigenetic states are reset during each reproductive cycle, our study also provides a conceptual framework for interpreting epigenetic variability in field-collected macroalgae, a topic of broad relevance to marine biotechnology research.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10126-026-10595-5>.

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Author Contributions JO performed the sample preparation, DNA extraction, MS data analysis, and drafted the manuscript. HH assisted

with the cultivation of **U. compressa*, supported the gametogenesis experiments, and edited the manuscript. TW, together with JO, NU, and GP, conceptualized the study. GP, NU, and TW revised the manuscript. All authors contributed to and approved the final version of the manuscript.

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Data Availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Competing Interests The authors declare no competing interests.

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