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Metabolomics: an emerging frontier of system biology in marine macrophytes

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ABSTRACT

Metabolomics is a rapidly emerging discipline within functional genomics which is increasingly being applied to understand biochemical phenotypes across a range of biological systems. Metabolomics measures all (or a subset) metabolites in a cell at a specific time point, reflecting a snapshot of all the regulatory events responding to the external environmental conditions. Although metabolomics and system biology approaches have been applied to the study of terrestrial plants, few marine macrophytes have been examined using these novel technologies. Marine macrophytes (including seaweeds and seagrasses) are marine ecosystem engineers delivering a range of ecologically and economically valuable biological services; however they are under threat from a wide range of anthropogenic stressors, climate variation, invasive species and pathogens. Investigating metabolomic regulation in these organisms is crucial to understand their acclimation, adaptation and defence responses to environmental challenges. This review describes the current analytical tools available to study metabolomics in marine macrophytes, along with their limitations for both targeted and non-targeted workflows. To illustrate recent advances in system biology studies in marine macrophytes, we describe how metabolites are used in chemical defence to deter a broad range of invasive species and pathogens, as well as metabolomic reprogramming leading to acclimation or adaptive strategies to environmental and anthropogenic stresses. Where possible, the mechanistic processes associated with primary and secondary plant metabolism governing cellular homeostasis under extreme environments are discussed. Functional integration of metabolomics with the allied "omics" disciplines of transcriptomics and proteomics, as well as the emerging discipline of "fluxomics" are discussed in the context of developing biological system networks, the identification of unknown gene/protein functions and the analysis of metabolic pathways in marine plants exposed to stress. Finally, we provide a comprehensive overview of an in silico plant metabolome database that can be utilized to advance our knowledge from a system biology approach to marine macrophytes.

Contents

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1. Introduction

Marine macrophytes include marine macroalgae (or seaweeds) and marine flowering plants called seagrasses which are the dominant flora in coastal ecosystems worldwide. Both are considered as marine ecosystem engineers delivering a range of ecologically and economically valuable biological services such as nutrient cycling, carbon sequestration, sediment stabilization and habitat provision to a range of dependent marine fauna. Seaweeds have immense commercial importance for nutraceuticals, pharmaceuticals, human food, animal feed, soil conditioner, biofuels and hydrocolloids production [1] with annual market value US$7 billion [2]. Seagrass meadows cover an area of 30–60 million km^2 of the coastal oceans, supporting 50% of the world's fisheries and providing essential nutrition for almost three billion people. Their nutrient cycling value alone is estimated to be three times higher than tropical rainforests, worth US$2 trillion per year [3].

Marine macrophytes inhabit a unique aquatic environment and experience a diverse range of environmental fluctuations, anthropogenic stress and threats from invasive species and pathogens. Macrophytes experience chronic stresses including salinity fluctuations, light fluctuations, temperature, desiccation, eutrophication, light limitation from turbid water, hypoxia and/or anoxia, sediment discharge, heavy metal and industrial effluents, all of which affect the plant's health and thereby the ecosystem services that they provide [3–5]. To alleviate the stress imposed by extreme conditions and also in deterring their invasive species and pathogens, marine macrophytes must implement acclimation/adaptive strategies by re-programming their metabolite networks. The increasing amounts of transcriptomic based information on the acclimation of marine macrophytes to their extreme environment are being gathered [4]. However, very little is known about how transcriptomic changes translate into metabolite changes. Therefore, a global metabolomic profiling of marine macrophytes can provide a wide picture to understand the different biochemical pathways that maintain homeostasis under extreme perturbations within these plants [6,7].

Metabolomics is defined as the comprehensive and quantitative analysis of all (or a subset) metabolites in a biological system at a specific time point. Metabolomics is especially useful to understand how marine plants respond to highly dynamic environmental conditions that prevail in their unique ecological niches. Metabolites reflect the true integration of gene regulation and protein expression, whilst also incorporating the impact of the environment and/or other organisms. Therefore in a real sense, metabolites are a closer proxy of the phenotype, rather than mRNA transcripts or proteins alone, and metabolomics can provide an instantaneous snapshot of the physiological status of a biological system at any given time. A recent influx of genomic/transcriptomic resources of seaweeds (*Ectocarpus siliculosus*, *Chondrus crispus* and *Porphyridium purpureum*) and seagrasses (*Zostera marina*, *Z. noltii* and *Posidonia oceanica*) has dramatically altered the molecular horizons of marine macrophytes in studying their adaptive and tolerance strategies [4,8]. With the successful assembly and analysis of their complete genomes, several seaweeds species (*E. siliculosus*, *C. crispus* and *P. purpureum*) have already been considered as model organisms and are definitively more amenable for future molecular studies [9–11]. However, the decreased cost and increased sequencing efficiency of Next Generation Sequencing make possible to examine different species of marine macrophytes besides these model organisms, providing new opportunities for comparative genomics within the same group of seaweeds or seagrasses. In this respect, transcriptome-based analysis of marine macrophytes has provided novel insights into their successful propagation and adaptation to the highly dynamic marine environment [12–23]. Overall, these studies have inferred a complex regulation pattern for diverse gene networks; however, coherent explanation that connects these transcriptional responses to their functional phenotypic response has not yet been defined. The integration of metabolomics with a comprehensive transcriptomic and/or proteomic study can be used to validate the regulation of genes and/or proteins. Although, metabolite profiling in marine macrophytes has become a well-established discipline, most of the past work has been oriented towards examining the structural composition of cell wall matrices and identification of bioactive compounds for pharmaceutical purpose [7]. Relatively fewer investigations have examined the whole metabolomic response of marine macrophytes exposed to biotic and abiotic stress conditions [14–16,20,24–28].

Recent developments in analytical instrumentation and bioinformatics have advanced our capacity to measure numerous plant metabolites, evaluate metabolic changes in response to external stimuli and elucidate metabolic pathways. However, the analytical sensitivity and resolution required for the simultaneous separation and detection of 100 to 1000s of metabolites that are regulated under stress conditions have not yet been achieved [29]. Current studies are mostly restricted to targeted metabolomics, which focuses on amino acid and/or lipid metabolism [14,30,31]. Polyamines, oxylipins, floridosides and volatile organic compounds (VOCs) are other classes of targeted metabolites that are gaining interest in studies of the stress response in macrophytes [32–38]. Metabolomic database information generated for marine macrophytes is in its infancy [6,7] when compared to terrestrial plants where a range of metabolomic databases are available (Tables 1 and 2). The terrestrial plant KNApSAck database [39] for example, contains ~50,000 plant metabolite entries. In comparison, the seaweed metabolite database (SWMD; http://www.swmd.co.in), the only macrophyte metabolomics database available, contains only 500 metabolites entries and mostly from the red seaweed *Laurencia* [40]; and there is currently no metabolic library available for seagrasses. This review provides a comprehensive overview of the use and capabilities of diverse metabolomic analytical tools in a targeted and non-targeted metabolomics workflow in marine macrophytes. Recent advances in understanding the chemical defence mechanisms of marine macrophytes used to deter a broad range of invasive species and pathogens are discussed. Further, we summarize the metabolite re-programming of marine macrophytes in response to extreme environments that provide new insights on their adaptation and/or acclimation mechanism to different stressors. The stress metabolites that are described include: amino acids, sugars, sugar alcohols, oxylipins, polyunsaturated fatty acids, polyamines, organic acids and phenolic compounds. The integration of metabolomics with the allied 'omics disciplines of transcriptomic, proteomic and fluxomics approaches are discussed in the context of developing biological systems networks, identification of unknown gene/protein functions, and metabolic pathways in marine plants exposed to stress conditions. Finally, we provide a comprehensive overview of in silico plant metabolome database information that can be utilized for the interpretation of marine plant metabolomic data sets.

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Table 1
Web-based software packages for pre-processing, metabolite matching and analysing metabolomic datasets obtained from diverse analytical platforms.

Database programme/tools	URL	Data type	Content
ADMIS	http://www.amdis.net/	GC–MS	Designed to deconvolute coeluting metabolites
ADAP-GC.2.0	http://www.du-lab.org/	GC/TOF-MS	Designed to deconvolute coeluting metabolites
Analyzer Pro	http://www.spectralworks.com/	GC–MS	Data mining tool for targeted and non-targeted metabolomics
AtMetExpress	http://prime.psc.riken.jp/lcms/AtMetExpress/	LC-ESI-Q-TOF/MS	Phytochemicals dataset of *Arabidopsis thaliana*
BinBase	http://fiehnlab.ucdavis.edu/db	GC–MS	A platform for automated metabolite annotation
BioMagResBank	http://www.bmrb.wisc.edu	NMR	1 and 2 D ^{1}H and^{13}C NMR spectral repository for 1400 biomolecules
Birmingham	http://www.bml-nmr.org/	NMR	1D ^{1}H and 2D ^{1}H *J*-resolved NMR database of 208 standard metabolites
CAMERA	http://bioconductor.org/packages/	LC–MS	R-package to extract spectra, annotate isotope and adduct peaks
ChromaTOF	http://www.leco.com/products/separation-science/software-accessories/chromatof-software	GC × GC–MS	Designed to deconvolute coeluting metabolites and data processing
COLMAR	http://spin.ccic.ohio-state.edu/	NMR	A webserver to search chemical shift query for metabolite identification
DeviumWeb	https://github.com/dgrapov/DeviumWeb		Multivariate statistical data analysis and visualization tool
FiehnLib	http://fiehnlab.ucdavis.edu/db	GC-qTOF-MS	A MSRI based library for comprehensive metabolic profiling
GMD	http://gmd.mpimp-golm.mpg.de/	GC–MS	A MSRI based library containing EI spectra of ~2000 metabolites
GMDB	http://jcggdb.jp/rcmg/glycodb/	MALDI-TOF	Mass spectral database of O-or N- linked glycans and glycolipid glycans
KNApSAcK	http://kanaya.naist.jp/KNApSAcK/	FT/ICR-MS	An extensive species-metabolite database for plants
Lipid bank	http://lipidbank.jp/		A data base for natural lipids
LipidMaps	http://www.lipidmaps.org/		Database encompassing structures and annotations of lipids
MMCD	http://mmcd.nmrfam.wisc.edu/	NMR, MS	A hub for NMR and MS spectral data of small biomolecules.
MassBank	http://www.massbank.jp	LC, GC, CE–MS, MALDI-MS	MS database consist of 13,000 spectra from 1900 different metabolites
MassBase	http://webs2.kazusa.or.jp/massbase/	LC, GC, CE–MS	A mass spectral tag archive for metabolomics
McGill MD	http://metabolomics.mcgill.ca/		A metabolome database containing metabolite MS of organisms
MeltDB	https://meltdb.cebitec.uni-bielefeld.de/cgi-bin/login.cgi	GC, LC–MS	A platform for storage, sharing, standardization, annotation, integration and analysis of metabolomic dataset.
MetaboLights	http://www.ebi.ac.uk/metabolights	LC,GC–MS, NMR	An repository of >800 metabolite structures, reference spectra and their biological roles
MetaboloAnalyst	http://www.metaboanalyst.ca/	NMR, MS	Software for comprehensive visualization and data processing
Metabolome Express	https://www.metabolome-express.org	GC–MS	A web-tool for storage, processing, visualization and statistical analysis
MetaboMiner	http://wishart.biology.ualberta.ca/metabominer/	NMR	Tool for automatic peak processing and annotation and comprises of reference spectra of ~500 pure compounds.
MetaboSearch	http://omics.georgetown.edu/metabosearch.html	MS	A tool that enables simultaneous m/z search from HMDB, MMCD, Metlin and LIPID MAPS, and integrate the results.
METLIN	https://metlin.scripps.edu/	LC–MS, MS/MS	A Tandem MS Database repository with high resolution MS/MS spectra
MET-IDEA	http://www.noble.org/plantbio/sumner/met-idea/	GC, LC–MS	A platform for storage, sharing, standardization, annotation, integration and analysis of metabolomic dataset.
Molfind	http://metabolomics.pharm.uconn.edu/Software.html	HPLC/MS	A Java based software for identifying chemical structures in complex mixtures using HPLC/MS data.
Mzcloud	https://www.mzcloud.org/	MS/MS, MS^{n}	Database of HR tandem mass spectra for s structure, monoisotopic mass, precursor and peak (m/z) searches.
Mzmine2	http://mzmine.sourceforge.net/	LC–MS	A framework for data processing and visualizing
NIST	http://www.nist.gov/srd/nist1a.cfm	GC, LC–MS, MS/MS	A comprehensive MSRI library covering >200,000 EI spectra.
PMDB	http://www.sastra.edu/scbt/pmdb		A database of plant secondary metabolites of plants with 3D structures
PRIMe	http://prime.psc.riken.jp/	GC, LC, CE–MS NMR,	A Web-based RIKEN Metabolomic Platform for metabolomics and transcriptomics database analysis
PRIMe MS2T	http://prime.psc.riken.jp/lcms/ms2tview/ms2tview.html	LC-ESI-Q-TOF/MS	MS/MS spectral tag (MS2T) libraries for global metabolic profiling
ResPect	http://spectra.psc.riken.jp/	LC–MS	A tandem MS database for phytochemicals from >3500 metabolites.
Spin Assign	http://prime.psc.riken.jp/?action=nmr_search	NMR	A service from PRIMe that provides batch-annotations of metabolites
SDBS;	http://sdbs.db.aist.go.jp	EI-MS, FT-IR, ^{1}H-NMR, ^{13}C-NMR	It is an integrated spectral database for organic compounds, which includes 6 types of spectra including laser Raman spectrum and electron spin resonance (ESR) spectrum
VocBinBase	http://fiehnlab.ucdavis.edu/projects/VocBinBase	GC–TOF-MS	An automated peak annotation database system from 18 plant species.
XCMS and XCMS2	https://xcmsonline.scripps.edu/	LC,GC–MS, and MS2	A data analysis programme allows nonlinear retention time alignment, peak detection and matching without using internal standards.

2. Metabolomic platforms to identify metabolites in marine macrophytes

Three strategies have been used to date to analyse the metabolome of plants and marine macrophytes: 1) metabolite profiling; 2) targeted analysis; and 3) metabolic fingerprinting [41]. Metabolite profiling is semi-quantitative and medium throughput in nature allowing for the detection of a large set of both known and unknown metabolites. However, targeted analysis is an absolute quantitative approach that identifies specific metabolites involved in a particular pathway by utilizing specialized extraction protocols, as well as specialist separation and detection techniques. Finally, metabolic fingerprinting is the highest throughput procedure and generates fingerprints that characterize a specific metabolic state of a sample by non-specific and rapid analysis of crude metabolite mixtures.

2.1. Targeted and non-targeted metabolite profiling workflows

A targeted metabolomics (TM) approach measures and profiles a specific set of metabolites defined by prior knowledge of their chemical nature and structure. A specific TM workflow first requires optimization of the metabolite extraction procedure to maximise the recovery and overall sensitivity for detection [42]. Addition of internal standards into the solvent during extraction can be very informative in determining the extraction or derivatization efficiency, column degradation or contamination of detector source. The next step involves fine-tuning

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Table 2
Bioinformatics tools for metabolomics network and/-or pathway visualization.

Programme	URL
Arena3D	http://arena3d.org/
BioCyc	http://biocyc.org/
BioPath	http://www.molecular-networks.com/databases/biopath
BRENDA	http://www.brenda-enzymes.org/
Cytoscape	http://www.cytoscape.org/
ExPASy	http://www.expasy.org/
IPA	http://www.ingenuity.com/products/ipa
iPAD	http://bioinfo.hsc.unt.edu/ipad
iPath	http://pathways.embl.de/
KaPPA-Veiw	http://kpv.kazusa.or.jp/
KEGG	http://www.genome.jp/kegg/pathway.html
Lycocyc	http://pathway.gramene.org/gramene/lycocyc.shtml
MapMan	http://mapman.gabipd.org/web/guest/mapman
MarVis-Pathway	http://marvis.gobics.de
Marker View	http://sciex.com/products/software/markerview-software
MetaCrop	http://metacrop.ipk-gatersleben.de
MetaCyc	http://metacyc.org/
MetaSHARK	http://bioinformatics.leeds.ac.uk/shark
MetaTIGER	http://www.bioinformatics.leeds.ac.uk/metatiger/
Metabolonote	http://metabolonote.kazusa.or.jp/
MetExplorer	http://metexplore.toulouse.inra.fr
MetGenMAP	http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi
MetPA	http://metpa.metabolomics.ca/MetPA/
MetScape	http://metscape.ncibi.org/
Pathos	http://motif.gla.ac.uk/Pathos/
Pathguide	http://www.pathguide.org/
Paintomics	http://www.paintomics.org
Pathcase	http://nashua.case.edu/PathwaysMAW/Web/
PathwayExplorer	http://genome.tugraz.at/pathwayexplorer/pathwayexplorer_description.shtml
PaVESy	http://pavesy.mpimp-golm.mpg.de/PaVESy.htm
Pathvisio	http://www.pathvisio.org/biological-pathways-software/
PlantCyc	http://pmn.plantcyc.org/
ProMetra	https://prometra.cebitec.uni-bielefeld.de/cgi-bin//prometra.cgi?login=prometra
Reactome	http://www.reactome.org/
SMPDB	http://smpdb.ca/
VANTED	https://immersive-analytics.infotech.monash.edu/vanted/

the mass spectrometer (MS) to detect a specific set of metabolites with the use of authentic standards that define the retention time (RT) and mass parameters for quantification of the targeted analyte. In TM, the RT and masses are pre-determined by using authentic standards. Further in TM, the MS detector results data automatically at molecular level of a metabolite instead of m/z values and thereby this data reduction eventually improve the statistical robustness in TM work flow. Moreover, the chance of encountering analytical artefacts in the data matrix, such as variability in molecular features (m/z) for the same molecule, are less using TM, compared to non-targeted metabolomics (nTM). Given the advantages of TM work flows, one should consider that TM is time-consuming, labour intensive, requires authentic standards, and the development of a specific MS method for a targeted analyte is costly and requires calibration of the instrument on a regular basis to minimize the shift in RT [43].

Without prior knowledge of the metabolites that will fluctuate under stressful conditions, a TM approach runs the high risk of missing significant changes in the metabolome. In this case, the analysis would need to expand beyond the known targets with the inclusion of signals of unknown identity that can only be accomplished with non-targeted, unbiased, metabolite fingerprinting also known as global metabolomics. Often, a non-targeted approach has the ability to detect some of the metabolites (if not all) included in a targeted analysis [42]. Despite the obstacles related to instrument sensitivity and sample complexity, global metabolomics provides a panoramic view covering both primary (including sugars, amino acids and tricarboxylic acids involved in primary metabolic processes such as respiration and photosynthesis) and secondary metabolites (including alkaloids, phenolics, steroids, lignins, tannins etc) in a single run and has advantages of uncovering many novel compounds.

In the analysis of global metabolomics, samples are extracted in miscible organic/aqueous solvents and internal standards are used for data normalisation. Once the sample has been run on the analytical instrument, data extraction is the critical task, which relies on chemoinformatic procedures to assign metabolite identification to the MS data. Data mining from the acquired MS data involves several steps including noise filtering, baseline correction, centering, normalisation, peak picking, peak integration, RT alignment and deconvolution [41]. These steps can be performed using various computational platforms described in Section 3, and Tables 1 and 2. The resulting data is often presented as a matrix of molecular features in 3D space with m/z values, RT and signal intensity values relative to the normalisation procedure which can be further analysed using various statistical tools described briefly (see Section 3).

A range of analytical platforms have been established for both TM and nTM analyses which includes nuclear magnetic resonance (NMR), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and mass spectrometry (MS). MS-based metabolomics combines chromatographic separation with mass spectra and are available in multiple forms such as liquid chromatography (LC–MS), gas chromatography (GC–MS), capillary electrophoresis (CE–MS) and matrix-assisted laser desorption/ionization (MALDI-MS). However, due to a high degree of structural diversity, molecular weight and polarity of simple primary metabolites through to complex secondary metabolites, there is no single analytical platform capable of identifying and quantifying all possible metabolites in a single analysis [42]. Therefore, with the combination of different instrument platforms and techniques, a more complete metabolite profile can be revealed. Nevertheless, MS-based analytical approaches are favoured to investigate plant responses to environmental cues due to its sensitivity to low abundant molecules and the flexibility for detecting multiple classes of molecules [41]. For every type of MS-based metabolomics experiment, sampling and sample preparation are the critical steps in transforming the harvested samples into a solution that can be analysed to obtain a maximum coverage of metabolites. The following section first describes the sampling/sample preparation followed by the description on different analytical platforms currently used for marine macrophyte metabolomics studies.

2.1.1. Sampling and sample preparation

As mentioned before, metabolomics reflect a snapshot of all the regulatory events (at a specific time point) responding to the external environmental conditions. Therefore, a special care must be taken when plant samples are harvested for metabolite analysis. Ideally, all sampling should be performed within a very small time frame in order to minimize this biological variation and should be quenched as quick as possible. For quenching plant metabolism snap freezing in liquid nitrogen or freeze clamping can be performed to inactivate all endogenous hydrolytic enzymes. Frozen plant tissue samples can be stored at −80 °C until extraction or freeze-dried for longer storage. The next crucial step in sample preparation is the extraction of metabolites prior to their separation and detection. A range of homogenisation and extraction procedures with a mixture of solvents of varying hydrophilicity/hydrophobicity have been described for this purpose [44]. Being, metabolites present in different compartments of cells, the disruption of those cells or their protective covering (such as thick cell wall and cuticle layers) can maximise the extraction of metabolites. Therefore, plant tissues/cells need to be ground to a fine powder prior to extraction by either manual grinding with a mortar and pestle (this is still the gold standard for metabolomics) or using automated technique such as cryo-grinding aiming to disrupt matrices and/or particle size reduction. Homogenization can also be performed in acidic conditions using trichloroacetic acid (TCA) or perchloric acid (PCA) [44]. Next to homogenization, the samples can be extracted by solvent extraction, supercritical fluid extraction, sonication and solid phase extraction. Most

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commonly use solvent extraction methods involve acetonitrile–water, methanol, ethanol, methanol–water, and methanol–chloroform–water. However, no comprehensive extraction technique exists for the recovery of all classes of compounds with high reproducibility and robustness. Recently, Yuliana et al. [45] developed a unique solvent gradient extraction method to recover almost all metabolites in a single protocol. In this method, the grounded plant tissue powder was packed with kieselguhr (an anti-clogging agent). A gradient of increasing polarity of solvents is then pumped through the column and fractions were collected at different time points. All metabolites examined from all collected fractions represented the total metabolome of plant tissue. The greatest advantage of this method is the ability to extract a wide range of metabolites in conditions that preserve their integrity. Moreover, since plant organs are heterogeneous, many different cell types will be present and hence any analysis of a whole organ is an 'averaged' composition rather than one that reflects the metabolome of a particular cell type. Recent developments in using laser micro-dissection technology to excise particular cell types and their subsequent analysis by conventional metabolomics approaches or using in situ mass spectrometry for metabolite imaging across a particular tissue proving to beneficial if the aims are cell or tissue specific metabolomics oriented [46].

2.1.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS is the most commonly used technique to separate low molecular weight metabolites (such as amino acids, amines, sugars, organic acids, fatty acids, long-chain alcohols and sterols of molecular weight ~ 500 Da), which are either volatile or can be converted into volatile and thermally stable compounds via derivatization [47]. Chemical derivatization first involves the conversion of all the carbonyl groups into corresponding oximes using O-methyl-hydroxylamine hydrochloride or alkoxyamines reagent that stabilise sugars into their open-ring conformation (*syn* and anti-stereoisomers). This step is followed by silylation for amino acid analysis using N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)/N-methyl-(trimethylsilyl)-trifluoroacetamide (MSTFA) or N-methyl-N-tertbutyl-(dimethylsilyl)-trifluoroacetamide (MTBSTFA) in the presence of 1% trimethylchlorosilane (TMCS) which acts as a catalyst for this reaction. These silylation reagents replace active hydrogen from polar functional groups –OH, —COOH, —NH and –SH of specific metabolites with [—$Si(CH_3)_3$] and converts them into volatile, thermally stable and less polar trimethylsilyl (TMS)-ethers, TMS-esters, TMS-amines, or TMS-sulphides groups, respectively [47]. GC with an electron impact ionization (EI) detector coupled to a single quadrupole (Q) mass analyser is the oldest and most developed analytical platform with high sensitivity, resolution, robustness and reproducibility, but suffers from slow scan rates and lower mass accuracy (~50–200 ppm). Therefore, GC with a time-of-flight mass analyser (TOF-MS) with high mass accuracy (~1–5 ppm), faster acquisition time, improved deconvolution for complex mixtures has become the method of choice over ion trap (TRAP) or triple QUAD (QqQ) based mass analyzers in metabolomics studies [41].

An innovative method of GC × GC with TOF-MS or QqQ-MS has recently been introduced offering very high acquisition rates (500 Hz), higher resolution and sensitivity. Still, GC based analytical tools are limited to the analysis of thermally stable volatiles (500–800 Da), thus providing a fairly comprehensive coverage of primary metabolites but not of secondary metabolites. Further, the derivatization step that demands extra care and time in sample handling using an anhydrous atmosphere, may vary according to the metabolite of interest. In recent years, studies using GC–MS, GC-FID (flame ionization detector) and GC-TOF-MS with marine macrophytes such as *Ectocarpus*, *Porphyra* and *Zostera*, have provided a comprehensive understanding of the metabolic networks linked to glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway (PPP) and photorespiration pathways involved in the acclimation to external perturbations [14,16,20,27,28].

2.1.3. Liquid chromatography mass spectrometry (LC–MS)

LC–MS is another important tool for metabolomic analysis, widely used for the analysis of secondary metabolites with large molecular weight (>500 Da). LC–MS separates metabolites which are dissolved in a liquid mobile phase on the basis of their interaction with a solid stationary phase before their passage into a mass analyser. LC–MS can be used with several different ionization techniques and selection of specific separating columns based on the chemical properties of both mobile and stationary phases [29]. Electron spray ionization (ESI) and atmospheric ionization (API) are the most commonly used ionization methods for LC–MS [48]. These techniques are suitable for metabolites with chemically unstable structures, heat-liable functional groups, high vapour points and high molecular weights such as non-polar metabolites like phospholipids, fatty acids, sterols and steroids. However, using an API source with soft ionization provides only limited structural information of molecules [48]. Further fragmentation of ions using collision induced dissociation (CID) on tandem MS instruments allows two or more sequential stages of mass spectrometric analysis i.e. tandem MS^n can provide detailed structural information [47]. Generally, two configurations of the tandem MS^n analytical instruments are available with LC–MS based approach: tandem-in-time and tandem-in-space. Tandem-in-time instruments use ion-trap MS such as quadrupole ion traps (QIT-MS), orbitrap and FT-ICR-MS. Whereas, tandem-in-space instruments use triple QqQ, TOF and qTOF with selective/precursor/multiple reaction monitoring analysis performance. Details on the workflows of these mass analyzers, and their compatibility with HPLC/UHPLC systems for MS or MS^n based metabolite profiling, are given in reviews [47,49]. The combination of LC with various kinds of mass analyzers, allows identification of diverse array of metabolites; however, flexibilities in the methods also cause difficulty in establishing large mass spectral libraries for peak identification because RT and mass spectra are instrument-specific [29].

Advanced LC–MS based techniques such as HPLC/ESI-CID-MS/MS (triple quadrupole instrument) and HPLC/ESI-$ITMS^n$ (quadrupole ion trap instrument) have been employed to uncover differences in the spectra of positional isomers of sulphated oligosaccharides [50]. Reverse phase liquid chromatography (RP-HPLC), using C18 or a similar medium particle size column (3–5 μm) with a silica based stationary phase, enables the separation of a diverse classes of phyto-natural products such as photosynthetic pigments, lipids and oxylipins [30]. Recently, a defence metabolite named thalassiolin B has been purified and characterized using RP-HPLC and NMR from the seagrass *Thalassia testudinum* which is effective in inhibiting pathogenic protist *Labyrinthula* sp. [51]. Furthermore, RP-HPLC has been used to isolate and purify a natural bioactive compound named rosmarinic acid from *Z. marina* and phenyl thioketone from *Cymodocea serrulata*, both had high antioxidant, anti-carcinogenic and anti-microbial properties [52,53]. In recent years, ultra-high performance liquid chromatography (UHPLC) is the method of choice for metabolomics research rather than RP-HPLC, because it provides greater resolution, peak capacity and lower detection limits with the use of small particle size columns (1.7 μm). However, it does require high pressure (10,000–15,000 psi) compared to 6000 psi in RP-HPLC [49]. Furthermore, RP-based chromatography can separate semi-polar and non-polar analytes; however, highly polar analytes are not retained on these columns.

With the development of hydrophilic interaction liquid chromatography (HILIC), it is now possible to separate polar metabolites. HILIC is orthogonal to RP-HPLC in using silica or derivatized silica, but similar to normal phase liquid chromatography (NP-LC) in that it uses large amounts of water and water-miscible polar organic solvent in the mobile phase [47]. Recently, HILIC–ESI-MS has been employed to characterize the polar lipid composition (glycolipids, glycol/glycerol-sphingolipids, betaine lipids and phytyl derivatives) in red seaweed *Chondrus crispus* [31]. This lipidomic-based approach using an advanced metabolomics analytical platform has been very promising in the study

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of lipid metabolism by marine macrophytes in response to fluctuating environmental conditions.

UHPLC–HILIC–ESI-MS or UHPLC–QqQ-MS (tandem MS) based analytical platforms have also been used to identify a diverse range of phlorotannins with varying degree of polymerization in seaweeds [54,55]. Phlorotannins are phenolic compounds involved in the chemical defence, protection from UV radiation, and are integral components of cell walls of brown seaweed [56]. Further, UHPLC-QTOF-MS analytical platform has successfully implemented in identifying phosphatidyl choline, lysophosphatidylcholine and sphingaine phospholipids as potential biomarkers that have undergone major structural changes at different developmental stages of *Pyropia haitanensis*, reflecting their role not only as structural components of cell membranes, but also as a cofactors for membrane enzymes and signalling molecules [57]. Although various metabolites can be analysed by LC–MS platforms, peak annotation is still troublesome with the shortage of mass spectral libraries for API-MS. Thus, more efforts are required for comprehensive metabolite profiling through the production of additional reference compounds and the generation of more comprehensive databases [48].

2.1.4. Capillary electrophoresis and Fourier transform ion cyclotron resonance-based mass spectrometry (CE–MS and FT-ICR-MS)

CE–MS is a powerful analytical tool to analyse a broad range of ionic metabolites based on charge and size ratio. CE–MS metabolite coverage largely overlaps with that of GC–MS, but does not require any derivatization, therefore saving time and consumables. Similar to LC–MS, API is the most suitable ionization source of ionization for CE–MS. CE is performed in a silica capillary tube where the ends are dipped in buffer solutions and across which a high voltage (20–30 kV) is applied. Unfortunately, CE–MS has inherent limitations of low sensitivity, poor migration time reproducibility, and a lack of reference libraries; therefore it is the least suitable platform for analysing complex biological samples [47]. FT-ICR-MS is a direct injection high-resolution mass spectroscopy (DIHR-MS) with a powerful ion-trapping system to acquire estimates of empirical formulae of detected analytes with very high resolution, highest mass accuracy, and very low detection limits. It is also compatible with MS^n analyzers. However, the high magnetic field associated with this equipment, complex ion-ion interactions and the high cost of the instrument have impeded its widespread application and use in metabolomics research [47]. Structural analysis of κ-carrageenan sulfated oligosaccharides (>2 kDa) was successfully obtained using the nano-ESI-FTICR-MS/MS platform [58].

2.1.5. Matrix-assisted laser desorption ionization mass spectrometry (MALDI)

MALDI is an ionization-based mass spectroscopy imaging (MSI) technique used to achieve spatial analysis of metabolites present in a biological system. This technique involves coating the tissue surface with a thin layer of a matrix comprising either 2,5-dihdroxybenzoic acid, sinapinic acid, α-cyano-4-hydroxycinnamic acid or 1,8-bis (dimethylamino)naphthalene (DMAN). A laser beam is injected across the matrix-coated tissue to acquire a mass spectrum at each point analysed [47]. After acquiring all spectra, m/z values need to be selected to build-up an ion image that portrays the distribution of ions within the tissue. The major limitations of MALDI-TOF include ion suppression, variation in ionization efficiencies of different compounds with similar chemical nature, saturation of the MS detector and overlapping peaks for different compounds with similar masses [47]. Various instrumental platforms for MSI analysis can be used such as MALDI-TOF, MALDI-TOF/TOF, MALDI-qTOF and MALDI-FT-ICR. Recently, MALDI-TOF based analytical tools have been used in marine macrophytes to identify proteins (e.g. pigment proteins, metabolic enzymes/proteins, signalling peptides and ion transporters), which regulate differentially in response to external perturbations [59]. Furthermore, the use of MALDI-TOF/TOF-MS confirmed the presence of novel fucoidan-CF2 and SgF in the brown seaweeds *Coccophora langsdorfii* and *Saccharina gurjanovae* respectively [60,61]. Other ionization techniques commonly used for MSI involve secondary ion mass spectrometry (SIMS), desorption electrospray ionization (DESI) and laser ablation electrospray ionization (LAESI) [62]. Recently, surface-associated bromophycolides and callophycoic acids have been identified using DESI-MS enabling chemical defence against *Lindra thalassiae* mediated fungal infection (a major pathogen for seaweeds and seagrasses) [63].

2.1.6. Nuclear magnetic resonance (NMR)

NMR is another profiling technique to identify metabolites based on the spectral behaviour of the atomic nuclei with either an odd atomic number (^1H) or odd mass number (^{13}C) under a strong magnetic field. NMR has lower sensitivity than MS, but still is a widespread tool among researchers because it does not require any chromatographic separation, derivatization, and sample preparation is simple, with high throughput and rapid analysis. However, NMR is pH sensitive and thereby, buffered solutions are mostly required to stabilise the pH. A mixture of methanol and aqueous phosphate buffer (pH 6.0, 1:1 v/v) or ionic liquids such as 1-butyl-3-methylimidazolium chloride have been considered best in providing a good overview of both primary and secondary metabolites [64]. ^1H NMR has been the dominant profiling method because it is fast and simple but suffers from signal overlap in the complex spectra of plant extracts. However, a 2D NMR spectroscopy approach can overcome this by spreading the resonances in a second dimension, although the acquisition time can take longer (20 min for 2D *J*-resolved spectroscopy), compared to <5 min for 1D NMR. Other useful 2D NMR methods for metabolite identification include heteronuclear single quantum coherence spectroscopy (HSQC), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMBC) and nuclear overhauser effect spectroscopy (NOESY) [64]. Among, NMR techniques, high-resolution magic angle spinning (HRMAS) spectroscopy technology is especially suited to analyse a small volume of intact tissue while avoiding any chemical extraction, which is necessary for both MS and liquid state NMR practises. In HRMAS, tissue sample is rapidly spun (at ~2–5 kHz) at a magic angle of 54.7° relative to B_0 manageticfield that reduces the line broadening (caused by heterogeneity in samples and anisotropic NMR parameters such as chemical shift anisotropy and dipole interaction) [65]. The MAS technique combined with high magnetic field (typically 11.7 T or 500 MHz for ^1H), results in improved spectral resolution. The key advantage of HRMAS over conventional NMR is that after spectral acquisition the tissue sample remains intact and thus the same sample can be utilized for gene expression studies and their direct comparison to metabolic profile. HRMAS based metabolomic analysis has proven to be a promising tool in identifying chemomarker in *Turbinaria conoides* [66], in examining the effects of salt stress on carbohydrate and nitrogenous reserves in *Solieria chordalis* [67] and also in defining the geographical differences of European spread brown seaweed *Sargassam muticum* [68].

NMR-based platforms are the best tools to resolve structural complexity of cell wall matrices of diverse seaweeds and providing details on monosaccharide components, linkages, anomeric configurations, branching positions and sulfations [69]. Structural elucidation of sulfated galactones in the seagrass *Ruppia maritima* using NMR approaches has recently been documented [70]. These galactans are unique and differ from seaweeds as both *a* and *b* units of D-galactose are not distributed in an alternating order in the seagrass. Recently, apiose-rich cell wall polysaccharides (called apiogalacturonan, AGU) such as zosterin have been characterized in the seagrass species, *Z. marina* and *Z. caespitosa*, using ESI-CID-MS^2 and NMR spectroscopy. These AGUs comprise α-1, 4-d-galactopyranosyluronan backbone linked to apiofuranose and apiose residues in the ratio 4:1 [71,72]. Metabolic studies using NMR in *P. haitanensis* exposed to high temperature has shown acclimation features with elevated levels of laminitol, isofloridoside and phenylalanine among others [25].

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3. In silico data analysis tools in hand for system biology

Vast amounts of metabolic data are generated by a diverse range of analytical instruments which need to be archived, managed, interpreted and finally integrated. Sophisticated bioinformatics tools are designed for raw data processing, mining, integration, statistical analysis, management, as well as for mathematical modelling of metabolomic networks. Once the data is acquired, it needs to be pre-processed to reduce background noise, adjusted for baseline shifts and machine drift, peaks detected and aligned, and finally the mass spectra deconvoluted. A range of bioinformatics tools for effective in silico data pre-processing have been designed for this purpose including AnalyzerPro, AMDIS (Automated Mass Spectral Deconvolution and Identification system), XCMS, and many more (Table 1) [73]. These software detects component peaks in the chromatograms and calculates the relative concentration by integrating peak area relative to the unique m/z of internal standards. For global metabolomic profiling, the construction of MS/MS data resources and databases are critical for metabolite identification. MS/MS libraries facilitate the detection of precursor ions by using metabolite-specific fragment ions (product ions) for structural elucidation. MS/MS data have recently been made available via web databases such as the Human Metabolome Database (HMDB), Golm Metabolite Database (GMD), FiehnLib, NIST, BinBase, METLIN, LipidMaps, Madison Metabolomics Consortium Database (MMCD) and MassBank (Table 1).

The Plant Metabolome Database (PMDB; http://www.sastra.edu/scbt/pmdb) is a structurally and functionally annotated database of plant secondary metabolites including alkaloids, phenolics, terpenoids, phytohormones and others with their 3D structure. The NIST-MS database (http://www.nist.gov/srd/nist1a.cfm) represents the largest commercial library for metabolite identification. It is based on EI-MS, tandem MS/MS and retention index (RI) data that also includes integrated tools for GC–MS deconvolution with AMDIS (version 2.72; http://chemdata.nist.gov/mass-spc/amdis). The Golm Metabolome Data-base (GMD) provides GC-EI-MS and RI (MSRI-mass spectral and retention index) libraries that use both alkanes and fatty acid methyl esters (FAMEs) for RI calculation whereas FiehnLib, a commercial MSRI library, uses FAMEs rather than alkanes. HMDB (http://www.hmdb.ca/) is a freely assessable database for all detectable metabolites found in the human body and used for metabolomics, clinical chemistry and biomarker discovery. Further, the Adams library, Terpenoids Library 5, and VOC BinBase are GC-specific MSRI libraries for volatile compounds. Recently, ReSpect (RIKEN tandem mass spectral database), an API-MS^n based library for the identification of phytochemicals has been developed by PRIMe, a platform from RIKEN metabolomics [74]. Another, freely accessible metabolic database is METLIN (https://metlin.scripps.edu). It is a repository for tandem mass spectrometry data with >10,000 distinct metabolites collected by ESI-QTOF. KNApSAcK represents the largest phytochemical database with 50,897 metabolites and recently has been upgraded with the addition of KNApSAcK Metabolite Activity DB and KNApSAcK-3D directory that define triplet relationships (metabolite–biological activity–target species) with three-dimensional structures of all the stored metabolic compounds [39]. Plant Metabolomics.org and the Medicinal Plant Metabolomics Resource (MPMR) are also excellent MS databases for amino acid, fatty acids, organic acids, phytosterols, isoprenoids, lipids and secondary metabolites. Plant specific metabolomic databases such as ARMeC (for *Arabidopsis* and potato), KOMICS (tomato), MeKO@PRIME (*Arabidopsis* mutants), MotoDB (tomato) and SoyMetDB (soya bean) are freely accessible web portals for pre-processing, mining, and visualization of metabolomics dataset [73].

To draw a conclusive notion on different biochemical processes that underwent regulation under specific stress conditions from large amounts of metabolomic data, several databases are available for decoding metabolic pathways and networks, thereby facilitating our understanding of transcriptomic and metabolomic data. KEGG and MetaCyc are the most commonly utilized bioinformatics-based data analysis tools for genomics, metagenomics, metabolomics and other omics studies, as well as modelling and simulation in systems biology. KEGG and MetaCyc are the largest and most comprehensive databases available contains information of metabolites, enzymes, diseases, drugs and genes, as well as providing a graphical representation of metabolic pathways and networks derived from various biological processes. Both have been frequently integrated into most interpretation platforms available for metabolomic pathway analysis. Similarly, KaPPA-View (http://kpv.kazusa.or.jp/; cover 10 plant genomes) is another metabolic pathway analysis tool that simultaneously displays transcripts and metabolites with their respective enzymatic reactions on the same map. Apart from these, many other bioinformatics tools are available for metabolomic network visualization and/—or pathway visualization (see Table 2). For detailed information on these and other tools, refer the link http://www.plantcyc.org/ [73,75,76]. Chemical compound databases such as the Chemical Abstract Service (CAS), PubChem, ChEBI, Chemspider and DAIOS are also freely available to retrieve chemical structures of small molecules. Recently, the USA National Science Foundation (NSF) launched the Plant, Algae and Microbial Metabolomics Research Coordination Network (http://pamm-net.org/) [77]. Similar efforts have also been initiated by the European Framework Programme for the 'coordination of standards in metabolomics' (COSMOS) [78]. The purpose of both platforms is to develop national and international metabolomics database/repositories to facilitate metabolite annotation, identification, mapping, metabolite distribution and exploration of the interaction of multiple species (and their metabolome) in context to marine ecology and biodiversity perspective. It is not possible in the scope of the present review to provide detailed descriptions for each piece of software available for metabolomics data processing, integration and network analysis. Thus, various software packages and tools available have been listed in Tables 1 and 2 with their URL and short description.

To perform the extensive data analysis of a metabolome, the entire data matrix needs to be analysed with multivariate statistical tools such as hierarchical cluster analysis (HCA) and principle component analysis (PCA) which basically reduce the data dimensionality and provide a surface view of class separation, clustering and outliers. Generally, PCA is followed by partial least square discriminate analysis (PLS-DA) or orthogonal partial least square discriminate analysis (O-PLS-DA) in order to enhance the separation between groups of observation and to understand which variables are responsible for such separation. These types of statistical and other metabolite enrichment analyses can be performed by a variety of software such as DeviumWeb, MetaGeneAlyse, MetaboAnalyst, IMPaLA, MetaMapR, MPEA, MSEA and MBRole (see http://csbg.cnb.csic.es/metab_rev/) [73].

4. Application of metabolomics to the study of marine macrophyte system biology

4.1. Chemical defence strategy of marine macrophytes to natural enemies

There is a growing body of evidence that marine flora are under constant threat from a diverse range of natural enemies (including pathogenic bacteria, fungus, herbivores, competitors and epiphytes) contributing to the major decline in their distribution [79]. More frequent incidences of bleaching, rotting, wasting, ice–ice, and gall formation diseases in marine plants, caused by microbial infection, have been suggested being due to warming oceans [79,80]. Marine macrophytes produce chemical defence metabolites that enable them to deter a broad range of natural threats. In general, the defence metabolites produced by marine flora has been categorized as anti-bacterial/anti-fungal/anti-fouling compounds [81], quorum sensing inhibitors (QS inhibitors) [82,83], pathogen-induced defence metabolites such as oxylipins and halogenated compounds [84–86], and others belonging to flavonoids, sterols, and phenolics which protect against herbivory and competitors (Table 3).

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Only few studies on the production of antibacterial compounds by marine macrophytes have documented the ecological role of these compounds. For example, a bioactive metabolite named poly-brominated 2-heptanone (PBH; 1,1,3,3-tetrabromo-2-heptanone), isolated and characterized using HPLC, ^{1}H and ^{13}C NMR and EI-MS from surface-localized glands cells, was shown to inhibit bacterial colonization in the red alga *Bonnemaisonia hamifera* [87,88]. Other studies have demonstrated the chemical defence potential of PBH against grazers [89] and native competitors [90]. The photosynthetic pigment fucoxanthin analysed by RP-HPLC appears to function as a surface-associated antimicrobial agent preventing the settlement of bacteria on the surface of brown macroalga *Fucus vesiculosus* [91]. However, unlike PBH, fucoxanthin is less selective and act as general inhibitor of bacterial attachment, rather specifically inhibiting bacterial growth and impacting on community composition. Other metabolites such as DMSP, proline and alanine, common metabolites identified in seaweeds and seagrasses, have also been shown to preferentially inhibit surface attachment of the bacteria *Cytophaga* sp., while promoting the other bacteria *Rheinheimera baltica* [92]. A cyclic lactone-lobophorolide characterized from the seaweed *Lobophora variegata* using 1D and 2D NMR and HRQTOF-MS showed strong activity against pathogenic and saprophytic marine fungi, suggesting that seaweeds use targeted antimicrobial chemical defence strategies [93] (Table 3).

Accumulation of phenolic compounds such as *p*-hydroxybenzoic acid and *p*-coumaric acid have been reported in the seagrasses *T. testudinum* and *Halodule wrightii*, whether infected with *Labyrinthula* sp. (a causal organism of wasting disease in seagrasses) or grazed by the sea urchin *Lytechinus variegatus* [94,95] (Table 3). Recently, the presence of flavone glycoside-Thalassiolin B in significant concentrations from *T. testudinum* (partially characterized using ^{1}H NMR and LC–MS) was reported which was capable of inhibiting the growth of *Labyrinthula* sp. [51]. Furthermore, various flavonoids such as luteolin, apigenin, luteolin-3-glucoronide, and luteolin-4-O-glucoronide have also been isolated and characterized by HPLC/MS^{n} and NMR from *T. testudinum* and *Enhalus acoroides* with anti-feedent and anti-larval activities against *Spodoptera litura* and *Bugula neritina* larvae [96]. Luteolin from leaf tissues of *T. testudinum* has also been shown to inhibit the settlement of motile zoospores of the protist *Schizochytrium aggregatum* [97]. Fatty acid methyl esters (FAMEs, C18-C22: 9,12-octadecandienoic acid; 1,2-benzenedicarboxylic acid; oleic; and erucic acid) identified using GC–MS were major bioactive compounds from two seagrasses *Syringodium isoetifolium* and *C. serrulata* with anti-fouling and toxic properties against bacterial and fungal pathogens [98] (Table 3). Furthermore, brominated compounds such as bromoform and dibromoacetic acid from *Asparagopsis armata* [99], and bromo-phycocollides from *Callophycus serratus* [63] have been identified using GC–MS and DESI-MS with potential antibacterial and antifungal activities.

Other metabolites known as QS inhibitors (QSI) are produced either by marine macrophytes or the associated microbes that interfere with microbial communication networks, their gene regulation and thus inhibit bacterial colonization. These QSI includes: halogenated furanones, hypobromous acid, betonicine, floridoside and isethionic acid isolated and characterized from red and brown seaweeds (Table 3). The process of QS inhibition is mediated mostly by N-acyl homoserine lactones (AHLs), which require functional AHL synthase and LuxR homologue proteins [83]. The first QSI named (5 Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone was isolated from the red seaweed *Delisea pulchra*, that supressed bacterial colonization [100] and interfered with AHL signals [83]. Such a chemical defence might be used by macroalgae to suppress the expression of specific pathogenic traits, while not necessarily killing the pathogen, thus averting disease without promoting resistance traits of bacteria. Three new AHL antagonist namely D-galactopyranosyl-(1–2)-glycerol (a floridoside), betonicine and isethionic acid in red seaweed *Ahnfeltiopsis flabelliformis* have also been discovered recently [82]. Recently, QSI named 2-dodecanoyloxyethanesulfonate using FT-ICR/MS was identified in seaweed *Asparagopsis taxiformis* (Table 3) [101].

A novel metabolite 8-hydroxy-4E, 6E-octadien-3-one along with other secondary metabolites (Table 3) was discovered from *Gracilaria lemaneiformis* using 1D, 2D NMR and HRESI-TOF-MS with potential allelopathic effects against *Skeletonema costatum* [102]. Allelopathy has also been observed with significant accumulation of phenolics named ferulic acid and the methyl 12-acetoxyricinoleate in adult leaves of *P. oceanica* when it interacts with the competitor *Caulerpa taxifolia* [103]. A range of terpenes, belonging to the dictyol class such as pachydictyol A, dictyol B/E/H, have been isolated from the brown seaweed *Dictyota* sp., which act as anti-feeding metabolites against herbivores including fishes and sea urchin [104]. Few distinct diterpenes have been found to deter grazers includes (6*R*)-6- hydroxydichotoma-3,14-diene-1,17-dial from *Dictyota menstrualis*, which inhibits herbivory from the amphipod *Parhyale hawaiensis*, dolabellane from *D. pfaffii* and dolastane from *Canistrocarpus cervicornis* against the sea urchin *Lytechinus variegates* [104,105] (Table 3). Further, the production of lipid-soluble metabolites such as 4β-hydroxydictyodial A and others by offshore populations of *D. menstrualis* were found to successfully deter herbivorous amphipods *Ampithoe longimana*, in contrast to inshore populations of the same species [106]. Elatol, a halogenated sesquiterpenol characterized from *Thalassia* sp. was found to be effective against feeding by reef fishes and sea urchin *Diadema antillarum* [107]. Further, a sterol named stigmast-5, 24-dien-3-ol (fucosterol) from *Sargassum tenerrimum* with potential anti-feedant properties against fishes has been isolated [108]. Recently, a pentachlorinated monoterpene originated from mevalonate pathway in *Plocamium brasiliense* was shown to exhibit chemical defence by preventing settlement of mussels, inhibiting range of fouling microalgae and bacteria [109]. Further, phlorotannins which are phenolic compounds formed by the oligomerization of 1,3,5-trihydroxybenzene (also known as phloroglucinol), exclusively produced by brown seaweeds and have been reported with potential antibacterial, antifungal, antifouling, antifeeding and allelopathic activities among others [110–113]. Recently, a gene *CYP73A* involve in phenol synthesis was found to be strongly up-regulated in *Z. marina* plants when infected with protist *Labyrinthula zosterae* [114].

Pathogen-mediated accumulation of secondary metabolites has been found to function as a defence strategy in seaweeds and seagrasses. For example, exposure to lipoteichoic acid/lipopolysaccharides (LPS)/alginate oligosaccharides triggers the release of reactive oxygen species (ROS) that modify algal protein expression with the accumulation of secondary metabolites such as diverse oxylipins (see Section 4.2.2) [84,115]. A significant activation of vanadium bromoperoxidases and thus the accumulation of halogenated organic compounds with ROS scavenging potentials have also been demonstrated in red and brown seaweeds during pathogen infection [116].

4.2. Metabolic rearrangements in the adaptation and acclimation of marine macrophyte in responses to environmental cues and anthropogenic pressure

Marine macrophytes are exposed to highly dynamic conditions and sometimes, extreme environments and anthropogenic stress. The impact of all these stresses tends to be similar, because they all directly or indirectly exert considerable pressure on the redox balance of cells, leading to the perturbation of various physiological functions at all levels of organisation and eventually affecting the productivity of these aquatic ecosystems [4]. Cellular responses to such stresses include adjustments of membrane systems, modification of cell wall architecture, changes in cell cycle and cell division, production of compatible solutes, enzymatic and non-enzymatic mediated ROS detoxification, differential regulation of diverse genes, as well as proteins, transporters, transcription factors and protein kinases among others [4]. The following section describes recent findings on metabolic adjustments to stress response using advance metabolomics response.

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Table 3
Defence metabolites produced by marine macrophytes to deter their natural enemies.

Metabolites	Marine organisms	References
Antibacterial/Antifungal/Antifouling/Antiepiphytes		
1,1–3,3-tetrabromo-2-heptanone	*Bonnemaisonia hamifera*	[81,87]
Fucoxanthine	*Fucus vesiculosus*	[91]
DMSP, aminoacids	*F. vesiculosus*	[92]
Cyclic lactone-lobophorolide	*Lobophora variegata*	[93]
Phenolics — p-hydroxybenzoic acid, p-coumaric acid and vanillin	*Thalassia testudinum*	[94,95]
Flavonoids — thalassiolin B; luteolin, apigenin, lutelin glucoronide	*T. testudinum*; *Enhalus acoroides*	[51,96]
Fatty acids — 9, 12-octadecandienoic acid; 1, 2-benzenedicarboxylic acid; oleic and erucic acid	*Syringodium isoetifolium* and *Cymodocea serrulata*	[98]
Phlorotannin — 1,3,5-hydroxybenze derivatives	*Ascophyllum nodosum* and *Sargassacean species*	[111,113]
Brominated compounds — bromoform and dibromoacetic acid	*Asparagopsis armata*	[99]
Bromophycocollides	*Callophycus serratus*	[63]
Quorum sensing inhibitors		
(5 Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	*Delisea pulchra*	[100]
D-galactopyranosyl-(1–2)-glcerol, betonicine and isethionic acid	*Laminaria digitata*	[82]
2-dodecanoyloxyethanesulfonate	*Asparagopsis taxiformis*	[101]
Allelopathic and antifeeding metabolites		
8-hydroxy-4E, 6E-octadien-3-one; 3b-hydroxy-5α, 6α-epoxy-7-megastigmen-9-one; N-phenethylacetamide, squamolone and 2-ethylidene-4-methylsuccinimide	*Gracilaria lemaneiformis*	[102]
Ferulic acid and methyl 12-acetoxyricinoleate	*Posidonia oceanica*	[103]
Terpinoids — Pachydictyol A, dictyol B/E/H, (6R)-6- hydroxydichotoma-3,14-diene-1,17-dial 10–18- diacetoxy-8-hidroxy-2,6-dolabelladiene	*Dictyota* sp. *D. menstrualis* *D. pfaffii*	[104]
Dolastane diterpenes	*Canistrocarpus cervicornis*	[105]
Sterols — Stigmast-5, 24-dien-3-ol (fucosterol)	*Sargassum tenerrimum*	[108]
Sesquiterpenol — Elatol	*Thalassia* sp.	[107]
Phlorotannin — 1,3,5-hydroxybenze derivatives	*Fucus vesiculosus*	[110]

4.2.1. Rearrangements in amino acids, organic acids, sugar and polyols

Marine plants sense and respond to unfavourable conditions by regulating their physiological processes, specifically carbon and nitrogen metabolism, which leads to alterations in various metabolic networks, linked to amino acids (AAs) and organic acids. Among various AAs, glucogenic AAs (amino acids that can be converted into glucose through gluconeogenesis: glutamate, aspartate, serine, glycine, proline, asparagine and glutamine) and aromatic amino acids (phenylalanine, tyrosine and tryptophan) are the major central organic nitrogenous compounds (Fig. 1, green boxes 1–5) that are involved in the storage and transport of nitrogen, and that are also precursor molecules for diverse metabolic pathways under stress conditions [117]. A common response of marine macrophytes exposed to abiotic stress is the accumulation of either of AA groups or both followed by a reduction in AAs upon recovery from the stress. This accumulation process could be linked to AA synthesis and inter-conversion, stress mediated reduction in protein synthesis, and/or stress-induced protein degradation. Moreover, AA metabolism under stress conditions act in close association with the organic acid pool involved in glycolysis within the cytoplasm and the Krebs tricarboxylic acid (TCA) cycle in mitochondria, in order to avoid the reduction in cellular energy budget and organic acid biosynthesis. Besides AAs, sugars (sucrose, fructose, trehalose, ribulose) and sugar alcohols (mannitol, *myo*-inositol, ribitol) are other important groups of metabolites with osmoregulatory and antioxidant properties (Fig. 1). An elevated level of these metabolites is equally important for energy production, stabilization of cellular membranes, maintenance of turgor, vitrification of cytoplasm and signalling in cells under stress [117].

Recently, an investigated on the metabolomic regulation of *Z. marina* to the diurnal effects of anoxia within root and leaf tissues using GC-TOF-MS analysis was conducted [28]. In this study, among a total of 69 metabolites identified, the most significant metabolites that had undergone regulations were those involved in glycolysis, TCA cycle and nitrogen metabolism. In leaves, a significant increase in alanine, asparagine, 2-oxoglutarate (or α-ketoglutarate), γ-aminobutyric acid (GABA), lactate, pyruvate, galactonic acid, succinate and glycerol, with a concomitant decrease in aspartate, glutamate and glutamine, was observed. In roots, the regulation of most of these metabolites was similar; however the level of α-ketoglutarate was constant with only a moderate increase in succinate and aspartate. Metabolic regulations was further examined using a metabolic pathway analysis software (MetPa), which confirmed the presence and activity of the alanine-GABA shunt in both leaves and roots (Fig. 1, grey box 5); however, the α-ketoglutarate shunt operated only in leaves during anoxic conditions [28]. It was suggested that the function of the alanine-GABA shunt was to mitigate the toxic effects of lactate or ethanol produced during anoxic condition, by providing an alternative route for pyruvate metabolism. Also, alanine and GABA accumulation allowed C and N storage during anoxia and this would provide energy for metabolism upon re-establishment of normoxic conditions. The α-ketoglutarate provides energy, which yields additional ATP under anoxia and also mitigates cell acidosis by alanine accumulation during anoxia.

In another study, the adaptive metabolic processes in association with heat stress were examined in *Z. marina* and *Z. noltii* under laboratory conditions using GC-TOF-MS analysis [20]. Both species inhabit warm regimes (26 °C) and exhibited significant accumulation of sucrose, fructose and *myo*-inositol. This process became apparent with a parallel study of their transcriptomes, wherein the expression of genes encoding the enzymes involved in the breakdown of sucrose and fructose were down-regulated, and thus resulted in their accumulation. On the contrary, genes encoding enzymes involved in *myo*-inositol synthesis were up-regulated. Given the osmoprotective function of sugars, the accumulation of *myo*-inositol (Fig. 1) was suggested to act as a substrate to generate protein stabilising osmolytes such as di-*myo*-inositol phosphate, which has also been observed to accumulate in thermal extremophiles in response to heat stress [20]. These metabolomic findings, when integrated with transcriptomic data, showed that the TCA cycle, glycolysis and pentose-phosphate pathways were responsible for heat attenuation. In another study, the adaptive features of seaweed *P. haitanensis* strains (SF1 and SF2) to high temperature were found to be linked to elevated levels of betain, betain aldehyde, laminitol (a methylated product of *myo*-inositol), isofloridoside, taurine, isothionate, alanine, glutamate, tyrosine, phenylalanine, uridine and adenosine with lower levels of malate, citrate, fructose, floridoside

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and choline-O-sulphate [25]. These compounds were identified with NMR in conjunction with AMIX and SIMCA-P^{+} software for metabolite annotation and statistical analysis. Most of the elevated metabolites belonged to different metabolite classes such as amino acids, sugar, sugar alcohols and low molecular weight carbohydrates, but were eventually linked to osmoregulation, indicated a complex strategy for osmotic adjustment in *P. haitanensis* strains to withstand high temperature stress. Additionally, the presence of isofloridoside (a galactoside based organic solute, Fig. 1, grey box 3) in both strains, when compared to wild types, was suggested to be a biological marker for thermal tolerance [25].

Recently, Zou et al. [26] examined the metabolomic regulation to acute and chronic levels of copper (Cu) stress in the seaweed *Sargassum fusiforme* using both NMR and GC-TOF-MS. The most regulated metabolites were amino acids, organic acids, polyols and sugars. Under acute stress wherein levels of alanine, glutamate, glutamine, proline, malate, aspartate, phenylalanine, mannitol, phosphocholine, dimethylglycine and trimethylamine decreased, the branched and aromatic amino acids, such as tyrosine and tryptophan increased by >1.5 fold under acute stress (Fig. 1, green box 2). However, in chronic stress, the level of most amino acids especially glutamine, trimethylamine, and aspartate, together with malate, increased to several fold with a significant decrease in phenylalanine. These metabolomic regulations suggested that the shikimate pathway is upregulated (Fig. 1 green box 2), phenolic compounds are synthesized and differential regulation of nitrogen assimilation via the glutamine oxoglutarate aminotransferase (GOGAT) pathway occurs under acute (Fig. 1, green box 5), but not under chronic stress. Additionally, *S. fusiforme* preferred trimethylamine and mannitol, as osmoprotectant/protein stabilizers/reducing equivalents and antioxidant, rather than proline and dimethylglycine under chronic stress. Similarly, the levels of aromatic AAs (phenylalanine and tyrosine) together with branched chain AAs (valine, leucine and isoleucine) were increased significantly under Cu stress (Fig. 1, green boxes 2–4), as a result of higher protein catabolism in *E. siliculosus* [27]. An extensive metabolite profiling study with UHPLC and GC–MS with DSQII quadrupole-MS in *E. siliculosus* upon exposure to hyposaline, hypersaline and oxidative stress conditions, identified mannitol and proline as the preferred candidates for osmoregulation under hypersalinity stress. However, the level of mannitol, together with other detected organic acids and sugars, such as glycolate, glycerol, succinate, fumarate, malate, citrate, isocitrate, urea, trehalose and glucose, did not change significantly with other stresses. Among the AAs, arginine, proline, threonine and GABA were responded significantly with their accumulation in hypo- and/or hyper- salinities [14]. This study provided the first indication that GABA synthesis in *E. siliculosus* may occur from a polyamine shunt in the absence of a GABA shunt (Fig. 1, grey boxes 5, 6) [14].

A global metabolite profiling using GC-FID and UPLC-DAD, across the two light–dark cycles in *E. siliculosus* evident three major metabolite clusters aggregating around: 1) alanine; 2) mannitol; and 3) glutamate using MestReNova statistical software [16]. The first cluster consisting of alanine together with glutamine, threonine, methionine, glycine, serine and citrate reached their highest concentrations during the light phase and not dark phase. The second cluster consisting of mannitol together

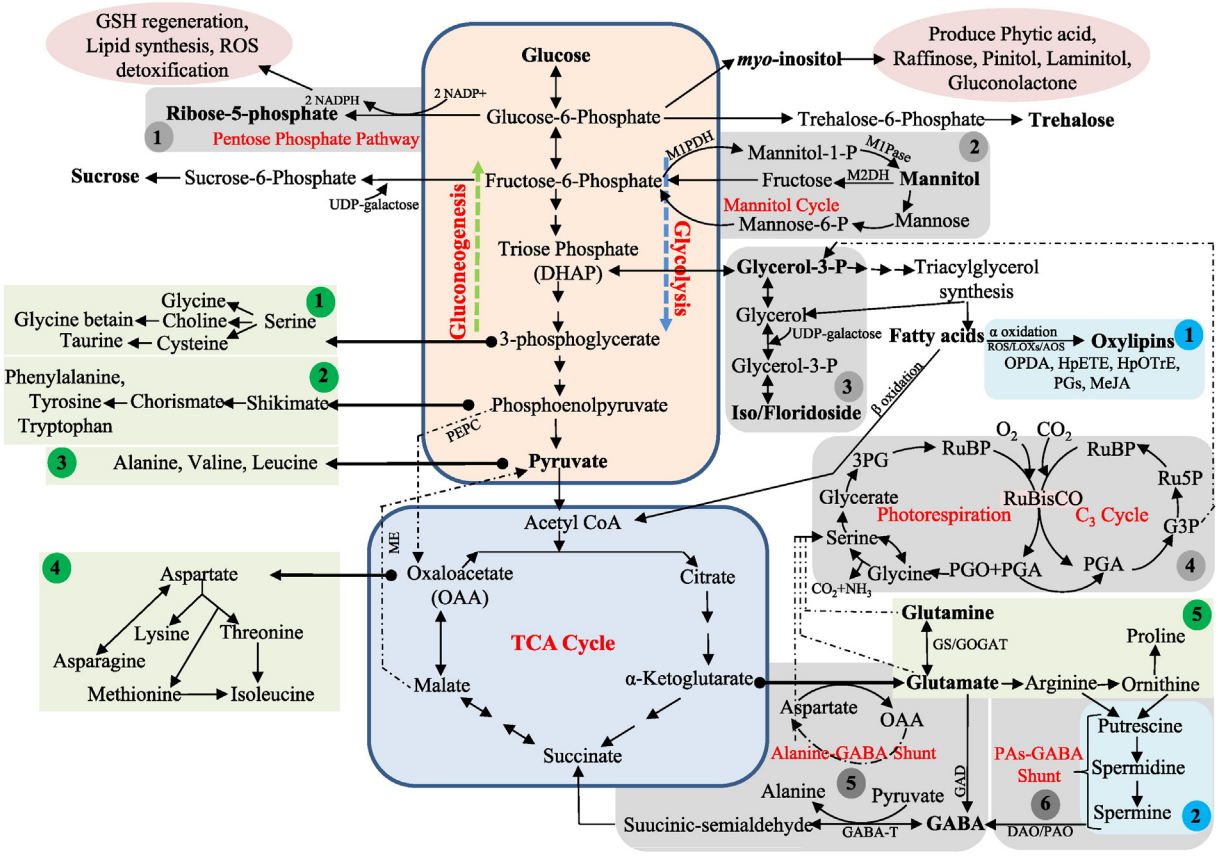

Fig. 1. Schematic representation of metabolic rearrangements in marine macrophytes under extreme environmental and/or-anthropogenic stress conditions. Major influenced pathways include glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle and other linked associated pathways such as pentose phosphate pathway (PPP), mannitol cycle, floridoside synthesis, photorespiration and C3-cycle, alanine-GABA (γ-aminobutyric acid) and polyamine (PAs) shunts (represented in grey boxes numbered 1 to 6). Green box (numbered 1 to 5) represent the amino acids synthesized from various metabolites of glycolysis and TCA cycle. Blue boxes (1 and 2) represent the biosynthetic pathways of oxylipins and PAs respectively. Dotted lines represent the interconnected pathways of metabolites. PGA-phosphoglycerate; PGO-phosphogluconate; RuBP-ribulose1,5-bisphosphate; Ru5P-ribulose-5-phosphate; G3P-glycerol-3-phosphate; DAO/PAO-diamine/polyamine oxidase; GAD-GABA dehydrogenase; GABAT-GABA transaminase; ME-malic enzyme; PEPC-phosphoenolpyruvate carboxylase; M2DH-mannitol-2-dehydrogenase; M1PDH-mannitol-1-phosphate dehydrogenase; M1Pase-mannitol-1-phosphatase.; DHAP-dihydroxyacetone phosphate; GHD-gutathione; ROS-reactive oxygen species; LOXs-lipoxygenase; AOS-allene oxide synthase; OPDA-12-oxo-Phytodienoic acid; HpETE-12-hydroperoxy-eicosatetraenoic acid; HpOTrE-13-hydroperoxy octadecatrienoic acid; PGs-prostoglandins; MeJA-methyl jasmonic acid; GS/GOGAT-glutamine synthetase/glutamine-2-oxyoglutarate aminotransferase.

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with valine, lysine, leucine, arginine, asparagine and aspartate, reached their highest concentrations at the transit phase from light to dark. However, the third cluster consisting of glutamate and isoleucine was predominantly found in the dark phase. These findings provide evidence of the involvement of the glycolate-based photorespiratory pathway as a result of inorganic carbon depletion at the end of the light period with serine and glycine accumulation (Fig. 1, grey box 4). Also, the accumulation of alanine during the light phase reflected its association with carbon assimilation similar to C4 plant metabolism, which is generally regulated by the availability of inorganic carbon such as from pyruvate. Together these examples suggest that glycolysis, the TCA cycle, PPP (Fig. 1, grey box 1) and the photorespiratory pathways (Fig. 1, grey box 4) are the most responsive metabolomic processes in marine macrophytes when exposed to external perturbations (Fig. 1).

4.2.2. Rearrangements in oxylipins, polyamines, fatty acids and others

Oxylipins are lipid metabolites derived from unsaturated fatty acids either enzymatically or by chemical auto-oxidation. Marine plants possess octadecanoid and/or eicosanoid oxylipin biosynthetic pathways, which emanate from C18 and/or C20 polyunsaturated (PUFAs). These pathways involve various lipoxygenases (LOXs), dioxygenases, allele oxide synthase/cyclase (AOS/AOC) and cyclooxygenase (COX), which lead to the formation of a range of oxylipins (fatty acids hydroperoxides, hydroxy-, oxo-, epoxy-, or keto fatty acids), divinyl ethers, volatile aldehydes, phytohormone jasmonic acid (JA), methyl jasmonic acid (MeJA), prostaglandins (PGs) and leukotrienes (Fig. 1, blue box 1). Marine seaweeds are well documented to contain a variety of oxylipins with pharmacological interest; however, their biological functions as signalling molecules, mediating stress response and chemical defence against epiphytes, grazers and pathogens has only recently been recognized and appears to be an active area of research [27,33,85,118–120].

The involvement of oxylipins as a stress response to heavy metal (copper, Cu) was first shown in the seaweeds species *Laminaria digitata* [33] and *E. siliculosus* [27] using LC-LTQ-Orbitrap MS. In both studies, the exposure of seaweeds to Cu stress induced the synthesis of oxylipins through an enzymatic mechanism that involved LOX and COX. Accumulation of several complex oxylipins, and cyclopentenones from C18 and C20 fatty acids were observed such as 9-hydroperoxy octadecatrienoic acid (9-HpOTrE), 13-hydroperoxy octadecatrienoic acid (13-HpOTrE), 13-HpODE (13-hydroperoxy-9Z,11E-octadecadienoic acid), 13-hydroxy-octadecatrienoic acid (13-HOTrE), 13-HODE (13-hydroxy-9Z,11E-octadecadienoic acid), 12-oxo-Phytodienoic acid (12-oxo-PDA), oxo-ETE (oxo-eicosatetraenoic acid), 18-hydroxy-17-oxo-eicosatetraenoic acid, MeJA, phytoprostanes (cyclic C18 A1-phytoprostanes) and various PGs (Fig. 1, blue box 1). Changes in the level of oxylipins was thought to be involved in the regulation of a detoxification process including up-regulation of genes coding detoxifying enzymes such as cytochrome P450, UDP-glucose transferase, the ABC-transporter, as well as heat shock proteins. Accumulation and/or induction of volatile organic compounds (VOC) was observed in *P. haitanensis* (extracted with SPME and analysed by GC–MS) in response to desiccation stress including: 3-octanone; 1-octen-3-ol; 1,8-octanediol; 5-octen-1-ol; 1-octanol; *E,E*-2,4,octadien-1-ol; and 1,4-dimethoxy-benzene [37]. The majority of these VOCs derived from C20 PUFAs via LOXs catalysis, and were thought to act as pheromones for the communication between thalli or transmission of information to other parts of the thallus during desiccation. Recently, the occurrence of natural bioactive oxylipins, such as phytoprostanes (autoxidation products of α-linolenic acid: F1t-phytoprostanes and L1-phytoprostanes) have been reported and validated using the UHPLC–QqQ-MS/MS method in various seaweed species [121].

Oxylipins provide an innate immunity against pathogens and were first demonstrated in the red seaweed *C. crispus*, wherein oxylipins were identified by using the LC-APCI analytical tool [118]. Their findings showed that when *C. crispus* was challenged by the pathogen *Acrochaete operculata*, it produced both C20 and C18 oxylipins (12-hydroperoxy-eicosatetraenoic acid (12-HpETE), 13-HpODE and JA), and activated the LOXs, shikimate dehydrogenase and phenyl ammonia-lyase, two key enzymes of the defence metabolism in higher plants (Fig. 1, blue box 1). Similarly, when the brown seaweed *L. digitata* was challenged with bacterial lipopolysaccharides, there was an accumulation of the oxylipins, 13-HOTrE and 15-hydroxy-eicosapentaenoic acid (15-HEPE) [32]. However, whether their generation was due to an oxidative burst, or the involvement of LOXs, was not clear.

MeJA triggered oxidative cascades of PUFAs leading to the synthesis of PGs, 13-HODE, 13-oxo-9Z, and 11E-octadecadienoic acid (13-oxo-ODE) has been demonstrated in *C. crispus* using RP-LCMS equipped with APCI [122]. Similarly, MeJA triggered the generation of ROS and synthesis of hydroxy-oxylipins with an up-regulation of the 13-LOX pathway. MeJA also caused the accumulation of phenolic compounds and the up-regulation of enzymes involved in secondary metabolism, such as polyphenol oxidase, shikimate dehydrogenase, and phenyl ammonia-lyase. These were evident in a shift towards secondary metabolism as a defence strategy in *Gracilaria dura* to combat MeJA-induced oxidative stress [30]. Furthermore, the defence responses of red seaweed *Gracilaria vermiculophylla* against the herbivore *Idotea baltica* [24], and of *G. chilensis* to the epiphytes *Acrochaetium* sp. and *Ceramium rubrum* [119] using UPLC-QTOF and NMR, were identified as 8-HETE (8-hydroxy-ecosatetraenoic acid); 7,8-di-HETE; PGA_2; 15-keto-PGE_2; and PGE_2 oxylipins with possible induction of LOXs and phospholipases [85].

Recently, invasive and non-invasive *G. vermiculophylla* and *G. chilensis* respectively, were found to defend themselves against the herbivorous sea snail *Echinolittorina peruviana* through the production of 7,8-di-HETE (examined with LC-ESI-MS), whereas the PGs and other oxylipins were inactive against herbivores [120]. In contrast, the lipid metabolic defence pathway of *P. haitanensis* in response to agaro-oligosaccharides, involved C20 fatty acid metabolism were directed to VOCs such as 1-octen-3-ol and 1,5-octadien-3-ol, but were not oxidized to oxylipins [57]. Despite recognizing the importance of oxylipins in the innate and induced defence systems in seaweeds, the existence and biological relevance of such oxylipins has not been studied in seagrasses to date.

Polyamines (PAs) are aliphatic amines (diamine putrescine and cadaverine [Put, Cad], triamine spermidine [Spd], tetramine spermine [Spm] and thermospermine [tSpm] produced in marine macrophytes in free and conjugated forms bound to small molecules such as hydroxycinnamic acid, or to larger molecules such as proteins or nucleic acids (Fig. 1, blue box 2). These secondary metabolites have been analysed in marine plants using a targeted metabolite approach with a RP-HPLC based system coupled with fluorescence detection after derivatization of plant crude extract with either dansylchloride or benzoylchloride [36,123]. In marine plants, the function of PAs has been examined in cell division, maturation of reproductive structures and callus induction [124]; however, their function as stress alleviators against salinity, desiccation stress and metal toxicity has only recently been recognized [34–36,123,125–128]. For example, a hyposaline shock to *Grateloupia turuturu* has been shown to accumulate free Put, Spd and Spm together with a significant decrease in transglutaminase activity and an increase in arginine-dependent PA synthesis [125]. Furthermore, pre-treatment of *Ulva lactuca* with Spd or Spm, in contrast to Put, resulted in constantly higher expression of protein disulphide isomerase (UfPDI) when exposed to hypersalinity, suggesting the potential of PAs to alleviate salinity stress and restore the growth rate [127]. Further, the elevated expression of methionine adenosyltransferase (MAT; catalyses spermine synthesis from methionine) in *Undaria pinnatifida* under hypersalinity and desiccation stress further suggested the role of PAs as stress alleviators [129]. Recently, Spm was found to regulate the stabilization of DNA methylation by reducing cytosine demethylation, which alleviated the

cadmium-induced stress in *G. dura* and *U. lactuca* [34,36]. PAs have been suggested to protect cells by forming binary or tertiary complexes anions, such as the phospholipid polar heads that impede auto-oxidation of Fe^{2+} and phospholipids, and subsequently reducing the generation of ROS [4].

The red seaweed *Pyropia cinnamomea* compensates for UV stress by increasing PA levels, especially bound-soluble and bound-insoluble PAs via the arginine-dependent PA synthesis pathway [123]. Similarly, the higher ratio of Put/Spd, together with a high level of phenolics in *Sargassum cymosum* when exposed to UVA + UVB radiation suggests that their binding to phenolic molecules results in stabilising the primary cell wall by cross-linking it to cellular components such as polysaccharide-bound phenols [128]. Further, accumulation of GABA possibly from Put degradation via diamine and/or polyamine oxidase activities (DAO and/or PAO) during hypersalinity stress in *E. siliculosus* has also been documented (Fig. 1, grey box 6) [14]. Endogenous and/or exogenous PAs have been shown to modulate stress triggered ROS homeostasis and oxidative damage by enhancing the antioxidant enzyme activities and pools of non-enzymatic antioxidants [34,36]. In addition, stabilization of PSII proteins in the thylakoid membranes (D1, D2, ctyb6/f) with their covalent binding to PAs catalysed by transglutaminases and/or by electrostatic interaction owing to their poly-cationic nature has also been suggested [4]. So far, *Cymodocea nodosa* is the only seagrass examined for the presence of PAs, wherein the total PA level was shown to decrease significantly in the embryonic stage, but increased during the seedling development stages [126,130]. Further, exogenous application of Spm has been shown to protect *C. nodosa* from hyposalinity stress with significant accumulation of Cad, which maintains the photosynthetic apparatus under long-term hypo-osmotic stress [126].

Lipids play crucial roles in adaptation and acclimation to a diverse range of biotic and abiotic stresses. The ability to adjust membrane fluidity by modulating the level of PUFAs with enhanced activity of fatty acid desaturases is a feature of stress acclimation in marine plants [14, 34,35,38]. Fluidity in membranes is required to maintain the diffusion of lipophilic compounds and the activity of membrane-bound enzymes. Many targeted and non-targeted GC–MS based metabolomic approaches have revealed fluctuations in fatty acid composition in marine plants exposed to various biotic and abiotic stresses. An enhanced proportion of oleic acid (C18:1, n-9) and linoleic acid (C18:2, n-6) with a parallel decrease in palmitoleic acid (C16:1, n-9) was observed in *G. corticata* at hypersalinities in contrast to hyposalinity [38]. This fluctuation in PUFAs was attributed to the induced activation of Δ9 fatty acid desaturase. Higher PUFAs could be an adaptive strategy to maintain greater membrane fluidity, to stabilise the protein complexes of PSII and to control the physicochemical properties of membranes, such as the increased activity of the Na^+/H^+ antiporter system of the plasma membrane in order to cope with hypersalinity stress [38]. Moreover, shift from n-3 to n-6 PUFAs was observed at higher salinities with the induction of two Δ12 and two Δ15 desaturases in *E. siliculosus* [14]. Similarly, enhanced levels of n-6 PUFAs such as arachidonic acid (C20:4, n-6, AA) and dihomo-γ-linolenic acid (C20:3 n-6), with a possible induction of elongases and Δ8 and Δ5 desaturases, was observed in a higher UFA/SFA ratio in *G. corticata* desiccated for 2 h [35]. A considerable increase in C18 (C18:1, C18:2, C18:3) and C20 (C20:5, C20:5) UFA, together with an increase in VOCs was observed in *P. haitanensis* after treatment with agaro-oligosaccharides, suggested their role in defence [57]. Increases in linolenic acid, AA and eicosapentaenoic acid (C20:5) in *E. siliculosus* (during Cu stress) and *L. digitata* (during Cu stress and bacterial lipopolysaccharide infection) correlated well with the occurrence of octadecanoid and eicosanoid oxylipins, together with the upregulation of the allene oxide synthase gene (*AOS*). These metabolic rearrangements could suggest the possible involvement of PUFAs in oxidative stress-mediated signals of acclimation [27,32,33].

Photosynthetic products such as floridoside and isofloridoside (galactosylglycerols) have also been shown to contribute to osmotic acclimation of red algal cells [37]. Furthermore, regulation of glycerol-3-phosphate (G3P, responsible for floridoside synthesis, Fig. 1 grey box 3) metabolism under heat stress in *P. haitanensis* suggested that floridoside are involved as in a conserved defence response across angiosperms as well as algae [131]. Phenolic compounds in seagrasses are integral components of cell walls and are also involved in defence against grazing [56]. Recently, a marked decrease in phenolics (proanthocyanindins, syr + 4-HBA, vanillin, acetovillinone, coumaric acid and ferulic acid) observed by RP- HPLC in the seagrasses *Ruppia maritima*, *C. nodosa*, and *Z. capricorni* in response to ocean acidification under laboratory conditions suggested their high vulnerability to futuristic oceanic conditions [132].

5. Integration of metabolomics with sister omic platforms

Integrated "omics" analysis centred on metabolomics can be a powerful technique to identify novel genes and discover new metabolic pathways that enable plants to combat range of biotic and abiotic stresses. In the omics era, with the availability of whole genome sequences and advanced bioinformatics tools, the application of functional genomics is progressively attaining the promised impact by integrating omics information. Functional genomics is providing novel insights into how cells and organisms respond to stress with a systems biology approach. System biology, as a holistic approach, is the integration of data from various disciplines including physiology, genomics, transcriptomics, proteomics, and metabolomics into numerical models that aim to simulate the physiology of the whole organism. Thus, system biology on one hand analyses the topology of biochemical and signalling network-based approaches involved in stress response, and on the other hand it also captures the dynamics of these responses [133]. Various network-based approached used in system biology include gene to metabolomic network, gene to protein, and protein to metabolite interaction studies. Because of the accessibility of full genome and the characterization of the key genetic pathways driving their metabolism, model organisms such as *E. siliculosus*, *C. crispus*, *P. purpureum* and *Z. marina* (whose genome sequence anticipated to be released in early 2016) are promising candidates for system biology studies in marine macrophytes.

In terrestrial plants, the integrative system biology approach is relatively simple and mature with the availability of knock-out and knock-down lines, over-expressing lines used to clarifying the correlation between gene expressions and metabolite accumulation. Some examples include the identification of the genes encoding anthocyanin and flavonoid biosynthesis, glycosyl-transferase; leucine biosynthesis, and induction of phenylpropanoid pathways under a stress environment [7]. However, only a handful of reports on marine macrophytes have examined gene-metabolomic co-expression, which explains their adaptive/acclimation responses to external perturbations. For example, a combination of genomic data, together with transcript and metabolite profiles has provided an integrated view of changes under abiotic stress in the model brown seaweed *E. siliculosus* [14]. It was shown that hypersaline stress induced more changes in metabolite content compared to hyposalinity and oxidative stress, whereas mannitol and proline were the prominent osmo-protectants, but not urea and/or trehalose. The most striking result was the increase of GABA under hypersalinity stress, despite the fact that the GABA shunt is absent in *Ectocarpus.* This combination of omics data suggested that this ubiquitous signal molecule could be synthesized when salt stress induced the Put degradation pathway in this model brown seaweed, but in the absence of *GAD* (glutamate decarboxylase) and *GABA-T* (GABA transaminase) genes which are responsible for the functioning of the GABA shunt (Fig. 1, grey box 5) [14]. However, the expression analysis for the genes involved in the metabolism of PAs needs to be further explored to confirm or disprove this assumption. Based on their observations, three putative *M1PDH* genes (named *EsM1PDH1*, *EsM1PDH2* and *EsM1PDH3*), the gene encoding mannitol-1-phosphate dehydrogenase [134], two putative

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M1Pase genes (named *EsM1Pase1* and *EsM1Pase2*) encoding mannitol-1-phosphatase and GDP-mannose dehydrogenase (*GMD*) involved in mannitol metabolism (Fig. 1, grey box 2) were sequenced, cloned and characterized with their high expression during a diurnal cycle, short-term saline treatment and oxidative stress [135].

In another study by the same research group, it was shown that *Ectocarpus* impregnations of genomic alterations at the metabolite level were able to stabilise the transition of evolutionary colonisations of alga from freshwater to the marine habitat [15]. Similarly, few candidate genes have been identified in seagrasses involve in photosynthesis, metabolic pathways and translation machinery that underwent positive selection and enabled the seagrasses to adopt marine environments from their ancestral terrestrial life [136]. Later, characterization of mannitol-2-dehydrogenase gene in *Saccharina japonica* (*SjM2DH*) confirmed that it is a new member of the polyol-specific long-chain dehydrogenases/reductase (PSLDR) family and play an important role in mannitol metabolism (Fig. 1, grey box 2) under salinity, oxidative and desiccation stress [137]. Transcriptomic and metabolomic analysis of Cu stress acclimation in *E. siliculosus* is yet another example of a gene-metabolite co-expression study in seaweeds. This study highlighted the activation of oxylipins and repression of inositol (*myo*-inositol) signalling pathways, together with the regulation of genes encoding for several transcription associated proteins. A significant accumulation of 12-OPDA with no change in MeJA together with up-regulation of genes belonging to the CYP74 family (an interesting candidate for allene oxide synthase-AOS activity involved in the synthesis of oxylipins) suggested that 12-OPDA mediates its signals independently of MeJA by activating general detoxification and stress responses including HSPs and ABC transporters [27]. However, the high number of yet uncharacterized brown algal specific genes induced in response to Cu stress provides novel lines of enquiry into the molecular interaction of this model organism to metal stress.

Recently, an integrated metabolomic–transcriptomic investigation conducted on thermal stress response of two seagrass species, *Z. marina* and *Z. noltii*, highlighted a process of metabolic heat attenuation by modulating the synthesis of carbohydrates namely sucrose, fructose and *myo*-inositol, together with up-regulation of ATPase, HSPs and carbohydrate metabolic enzymes [20]. Elevated expression of genes encoding glyceraldehyde-3-phosphate dehydrogenase, sucrose phosphate synthase, ribulose-phosphate-3-epimerase, ribose-5-phosphate isomerase, β-fructofuranosidase, fructose-bisphosphate aldolase and inositol-3-phosphate synthase involved in sugar metabolism, together with the accumulation of different sugars, suggested that the calvin cycle, glycolysis, PPP and neighbouring associated pathways are the main candidate metabolic process for heat attenuation with an increase in primary productivity in response to thermal stress (Fig.1).

A recent combined transcriptomic-physiology study in *P. oceanica* using cDNA-microarrays examined the light-adaptation strategies to different light environments [138]. Their findings suggested that photoacclimation (with upregulation of RuBisCO, ferredoxin, chlorophyll binding proteins) and photoprotection (with up-regulation of xanthophyll cycle related genes named *PSBS*, *ZEP* and homogentistate phytiltransferase (*HTP1*, tocopherol biosynthesis related gene) together with physiological responses of high xanthophyll cycle pigment pools, high numbers of reaction centres, lower antenna size, and high *Fv*/*fm* values, are common plant responses to adaptation/acclimation. Furthermore, the same research group integrated transcriptomic and proteomic approaches using Suppressive Substractive Hybridization (SSH) and Universal Soft Ionization Source (USIS) MS coupled with 1D-SDS electrophoresis, supported the above findings for the acclimation and adaptive responses of *P. oceanica* inhabiting shallow and deep water regimes [22]. Stress proteins such as zinc fingers, metallothionein-like, cytochrome P450, caffeoyl-CoA O-methyltransferase, aquaporin PPI2 and S-norcoclaurine synthase, were the most abundant in a deep water (low light) plant library; however, heat shock cognate 70 kDa, ketol-acid reductoisomerase, acyl-CoA-binding proteins, proteasome subunit alpha, E3 ubiquitin, and the ATP-dependent Clp protease were most abundant in a shallow (high light) plant library. Among peptide sequences, chloroplastic ATP synthase subunits (active candidate in the Calvin cycle), glyceraldehyde-3-phosphate dehydrogenase (glycolytic candidate), RuBisCO activase A, and a 14–3-3 like protein (mitochondrial protein bound to phosphorylated proteins for modulating their functions) were most abundant in shallow plants exposed to high light. However, mitochondrial ATP synthase subunits (mostly involved in respiration) were down-regulated in low light. This integrative study suggests that plants from deep meadows are more sensitive to oxidative stress than shallow plants due to the higher investment by the former in maintaining basal metabolism and consequently lower resources available for cell defence and repair.

All of these examples show the potential of system biology approaches to provide knowledge on all the elements of a system (i.e. genes, proteins and metabolites), and to determine the relationship between these elements, and key alterations of networks when influenced by biotic and abiotic stresses. By integrating the information from different levels, a system biology approach allows us to examine the behaviour of a system using computational algorithms, thus allowing us to make predictions of the system under different perturbations. However, the success of this approach depends on a multidisciplinary participation from biologists, bioinfomaticians, mathematicians, bio-statisticians and chemists for a thorough interpretation of datasets in order to draw meaningful conclusions from detailed studies. The availability of advanced analytical tools for metabolomics studies, has allowed researchers to identify more metabolites with more precision, which has prompted the needs to examine their relationship to each other in intra- and inter- related biochemical pathways (metabolic networks). Adavnced bioinformatics tools (originally developed for terrestrial plants) has accelerated this task and has allowed correlations to be made between datasets generated from different omics platforms (see Section 6 and Tables 1 and 2 for more details).

6. Metabolic flux analysis in marine macrophytes

Quantifying precisely the plant metabolism remains a challenging but promising frontier to better understand the functioning of marine macrophytes in connection to their stressful environment. Metabolic fluxes that define the rates at which metabolites are converted or transported between compartments in a network of biochemical reactions are crucial for the quantitative interpretation of how environmental factors translate into a healthy or stressed phenotype [6]. In this context, an isotope labelling based metabolic flux analysis (MFA) has been proven to be a powerful methodology for tracing fluxes of nutrients from single cell to ecosystem level in land plants. Therefore, MFA holds great promise to precisely quantify metabolic fluxes and to determine metabolic pathways at a steady state rate in marine macrophytes and to provide a direct link to their ecosystem health. Stable isotope labelling based MFA generally involves two main steps: 1) exposing an organism or environmental sample to stable isotope-enriched substrates at steady state (i.e. isotopes entering and leaving the cell remain constant with time) and subsequently 2) analysing the labelled biomarkers as tracers to quantify and model biochemical fluxes [139]. In the second step, the labelled biomarkers can be analysed by measuring the difference in concentration between naturally occurring and the stable isotope at elemental, metabolite or cellular level.

MFA at the elemental level involves the combination of stable isotope labelling with combustion of plant material followed by mass spectrometry. This technique can provide information on assimilation or translocation of elements such as carbon or nitrogen within different tissues of seagrass when incubated in seawater enriched with stable isotope labelled-bicarbonate ($NaH^{13}CO_3$) and –ammonium ($^{15}NH_4Cl$) [140]. Results obtained using this technique highlighted the importance of translocation from mature to young ramets in order to acquire

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resources, and for seagrass colony expansion [140]. However, MFA at the metabolite level involves the combination of stable isotope labelling with a chromatography analytical platform (GC/LC) or NMR coupled with mass spectrometry. In this case, incorporation of stable isotopes can be determined in specific metabolites that is based on the shift of individual detection peaks (m/z values), depending on the number of labelled carbon atoms incorporated. To our knowledge, MFA studies at the metabolite level using a MS-based approach are scarce in marine macrophytes [6]. Recent studies have investigated the flux of photosynthetic carbon into the biosynthesis of low-molecular weight carbohydrates such as digeneaside and floridoside with the stable isotope ^{13}C from $NaH^{13}CO_3$ using ^{13}C and ^{1}H NMR spectroscopy in the red seaweed species, *Grateloupia turuturu* and *Solieria chordalis*, when exposed to hypo-and hyper-salinities [141,142].

The development of the Secondary Ion Mass Spectrometry Instrument (NanoSIMS) has revolutionised the way metabolite fluxes are measured, and it is now possible, even at a cellular level, within different tissues and cellular compartments in terrestrial plants. NanoSIMS can provide an image with precise quantification of up to 7 different isotopes at a micro to nanometre scale (up to 50 nm). In the marine environment, MFA using NanoSIMS have been applied to a broad range of organisms such as phototrophic bacteria, cyanobacteria, blue-green algae and more recently in dinoflagellate symbionts in coral tissue, to measure relative metabolic contribution and functional diversity [143]. Furthermore, applications of NanoSIMS and SIMS in marine macrophytes holds great potential not only to quantify and spatially resolve metabolic fluxes between different tissues and the cellular compartments of these plants, but also to explore the metabolic activity of microbes associated within the surrounding rhizosphere. This innovative combination of techniques could therefore improve our understanding on the functioning of marine macrophytes and related ecosystems such as seagrass meadows.

7. Conclusion

Despite being a relatively new approach in marine plant biology, metabolomics alone or in combination with other omics disciplines is becoming the major tools in revealing new knowledge on diverse metabolites produced by marine plants to deter broad range of natural enemies, and also on their metabolomic reprogramming for acclimation to extreme perturbations. From a technological perspective, MS based metabolomics (GC−/LC–MS) have been the most preferred in either of targeted and non-targeted work flow conducted on quadrupole, ion trap, TOF, Q-TOF mass spectrometers. Among, all the metabolic responses, alterations in the primary metabolism are the most evident, however, changes in secondary metabolism have not been explored much in response to abiotic stress. Nevertheless, various kinds of secondary metabolites such as phenolics, sterols, flavonoids, fatty acids, and terpenoids have been identified in marine macrophytes enabling them to deter broad range of natural enemies. While metabolomics is considered as an emerging facet of system biology, the metabolite annotation and accurate identification remains the greatest challenge in global metabolomic studies. In addition, the combination of LC with various kinds of mass analyzers, allow the identification of diverse array of metabolites; however, flexibilities in the methods also cause difficulty in establishing large mass spectral libraries for peak identification because RT and mass spectra are instrument-specific. Therefore, the future of marine biology metabolomics as an omic tool not only depends on technology advances but also requires a community wide effort to generate "Cyc" like freely assessable and user friendly mass spectral databases. These databases should be centred especially to marine model organisms for which either genome are sequenced (seaweeds namely *E. siliculosus*, *C. crispus* and *P. purpureum*) or global transcriptome data resources are available (seaweeds such as *P. umbilicalis* and *P. haitanensis*; seagrasses such as *Z. marina*, *Z. noltii* and *P. oceanica*). This joint effort will allow marine researchers to identify large numbers of metabolites from various marine organisms and this information could eventually be applicable to metabolomic engineering, genome editing and system biology research of marine macrophytes. Further, metabolomics coupled to metabolomic flux analysis with the use of labelled stable isotopes should be undertaken to provide better insights on the activity of metabolic pathways which is not available when metabolite levels alone are measured. It is highly likely that integrating the results of metabolomics with metabolomic flux analysis, transcriptomic and proteomics will further deepen our understanding on system biology and to discover new biomolecules that are crucial for survival process of marine macrophytes under extreme perturbations. It is also relevant to undertake validation approaches to test whether metabolites/pathways are genuinely involved directly to mediate the response of marine macrophytes to external perturbations. Such efforts will deliver a proof of concept for biomarker discovery and metabolic engineering of marine macrophytes under the scenario of global climate change. Obtaining such information is highly important for environmental managers and stakeholders to undertake effective management and conservation strategies that will protect marine macrophytes ecosystem without running the risk of causing their long term damage.

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