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Thukral, Monica
Allen, Andrew E
Petras, Daniel

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Progress and challenges in exploring aquatic microbial communities using non-targeted metabolomics

Monica Thukral [1,2], Andrew E. Allen [1,2] and Daniel Petras [3,4 ✉]

Advances in bioanalytical technologies are constantly expanding our insights into complex ecosystems. Here, we highlight strategies and applications that make use of non-targeted metabolomics methods in aquatic chemical ecology research and discuss opportunities and remaining challenges of mass spectrometry-based methods to broaden our understanding of environmental systems.

INTRODUCTION

The field of aquatic microbiology has been very successfull in implementing and using molecular biological techniques such as nucleic acid sequencing and cloning/conjugation to study individual microorganisms and ecosystem function [1, 2]. In the last decades, tools used in the field of molecular biology have constantly evolved and expanded, including emerging omics tools such as transcriptomics, genomics, and proteomics [3]. While there are conflicting viewpoints on whether molecular biology is defined by the level of life at which biology is studied, omics tools have been revolutionary to studying biology on the molecular level and are now widely used in many molecular biology studies [4–6].

Mass spectrometry (MS)-based metabolomics methods, both targeted and non-targeted, are becoming widely used tools to study aquatic ecosystems [7–9]. Targeted metabolomics typically refers to detecting and quantifying a specific metabolite or set of metabolites [10, 11]. Non-targeted metabolomics on the other hand, aims to detect all metabolites within a sample, typically used as a discovery approach to generate hypotheses about the identity, origin, function and effects of small molecules in biological systems. The study design, including whether to use targeted or non-targeted metabolomics depends on whether the driving question and nature of the study is to investigate already known molecules or pathways, or whether it is to characterize the total chemical diversity of a biological system. Natural product chemistry studies on the other hand have a long-standing tradition of isolating organic compounds from isolated organisms or biomass and elucidating their structures with multi-modal approaches such as MS and nuclear magnetic resonance (NMR) spectroscopy and assess their biological activity, mainly in the context of pharmaceutical properties [12, 13]. While these methods inform organismal presence/absence as well as molecular insights and functionalities, the field has only scratched the surface of understanding chemical exchange in complex microbial community dynamics [14, 15]. Although there has been great progress in microbial and chemical ecology studies, with many new molecular insights into different ecosystems, we are far from fully capturing and understanding how microorganisms affect each other through the multitude of other metabolites they produce (Fig. 1).

Non-targeted metabolomics promises to provide information of which molecules are present, exchanged, and modified in a given (eco)system, which may illuminate the black box of organismal and community metabolomes. The field of lipidomics is often considered to be a specialized sub-field of metabolomics, and both targeted and non-targeted lipidomics methods have contributed significantly to our understanding of aquatic metabolomes [16, 17]. Over the past 30 years, significant advances have been made in the fields of non-targeted metabolomics as well as nucleotide sequencing. Within the last years, long-read-sequencing has emerged as new sequencing strategy, which is particularly useful for the assembly of metagenomes [18], that include repetitive gene sequences in mega-synthetases of specialized metabolites, such as non-ribosomal peptide synthetases (NRPS, e.g. Microcystins) or polyketide synthetases (PKS, e.g. Brevetoxin) [19, 20].

Technological advances in nucleic acid sequencing and in mass spectrometry have occurred concurrently and are both key to enabling chemical ecological discoveries (Fig. 2A). Gas Chromatography- Mass Spectrometry (GC-MS) was developed in the late 50 s and has been widely used to identify and quantify metabolites [21–24]. LC- and GC-MS-based metabolomics, high-resolution MS-based metabolomics, peak detection and alignment software, and ultra-performance liquid chromatography became widely accessible in the early 2000s [25–28]. In the mid 2000s, the orbitrap mass spectrometer entered the market and the first tandem mass spectrometry databases were developed, concurrent with the introduction of next generation sequencing [29–33]. These advances were followed by RNA-seq and ion mobility mass

[1]University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA, USA. [2]J. Craig Venter Institute, Microbial and Environmental Genomics Group, La Jolla, CA, USA. [3]University of Tuebingen, CMFI Cluster of Excellence, Tuebingen, Germany. [4]University of California Riverside, Department of Biochemistry, Riverside, CA, USA. ✉email: daniel.petras@uni-tuebingen.de
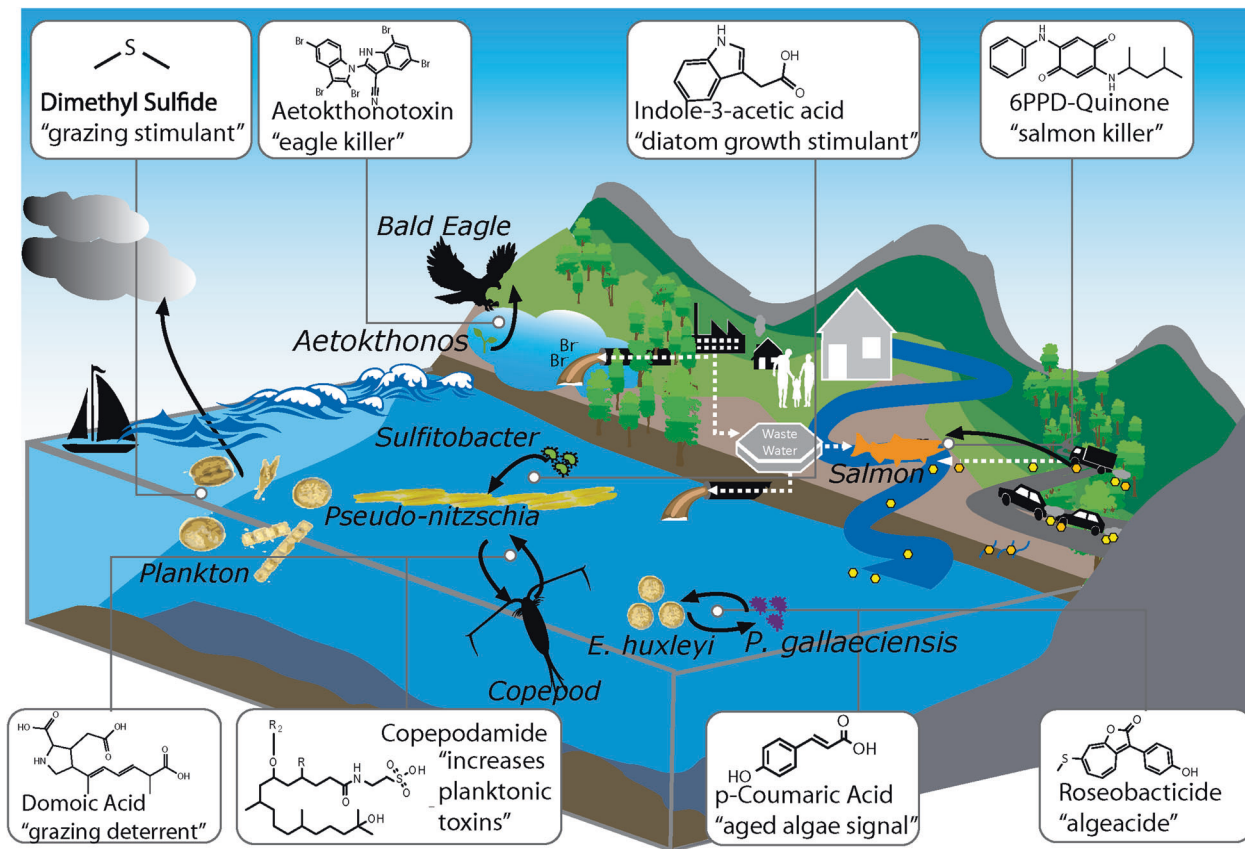
Fig. 1 Small molecules mediate microbial community function. Depicted includes photosynthetic microalgae producing dimethyl sulfide inducing both cloud formation and grazing by zooplankton, and on the other hand *Pseudo-nitzschia*'s production of domoic acid as a grazing deterrent. In addition, *Sulfitobacter's* production of indole-3-acetic acid increases planktonic toxins like domoic acid from *Pseudo-nitzschia*. Furthermore, when *an E. huxleyi* bloom ages, it produces p-coumaric acid, with response to which *P. gallaeciensis* begins to produce algaecide roseobacticide. In freshwater nearby, *Aetokthonos* cyanobacteria colonizes a freshwater invasive plant *Hydrilla* and produces aetokthonotoxin which bioaccumulates up the food web to top predator bald eagles, causing neurological disease. On a road nearby, cars' rubber tires contain 6PPD which runs off into freshwater creeks and is transformed to 6PPD-quinone, causing salmon mortality.

spectrometry by 2009 [34, 35]. Over the last decade, sensitivity, resolution, and scan speed of both orbitrap and Q-ToF-based platforms have significantly increased [36]. Other recent advances include molecular networking and long read sequencing, and finally the contemporary revolution of machine learning tools and artificial intelligence [37–42].

In parallel to the decreasing cost of nucleic acid sequencing, the cost of acquiring an equivalent amount of MS data has significantly decreased as well (Fig. 2B). However, despite the technological advances, decreasing costs, and wider accessibility of metabolomics tools, major bottlenecks remain. For example, currently only 5–10% of spectra in most non-targeted metabolomics experiments can be annotated as known molecules: the remaining 90–95% of spectra are unknown and considered as "dark matter" which contains vast unknown knowledge space [14]. In addition to providing examples of the successful use of metabolomics tools to study aquatic ecosystems, we will discuss key bottle necks and emerging solutions in this article.

SUCCESS STORIES OF DECIPHERING CHEMICAL MEDIATORS IN AQUATIC CHEMICAL ECOLOGY
Metabolomics tools enable the study of community dynamics of bloom-forming organisms
Metabolomics techniques have rapidly enhanced the study of cosmopolitan bloom-forming organisms: cyanobacteria, diatoms, dinoflagellates and haptophytes (Table 1, Fig. 1, Fig. 3). Targeted metabolomics has been used to determine how commonly used

algaecides affect toxin production of cyanobacteria *Microcystis aeruginosa* microcystins [43]. Here, targeted metabolomics led to the discovery of how algaecides alter the total metabolome. For example, exogenous addition of copper sulfate decreased metabolites associated with oxidative stress, whereas hydrogen peroxide and sodium carbonate peroxidase increased those oxidative stress metabolites. This work established fundamental data that can be leveraged by policy-makers in deciding how to treat *Microcystis* blooms while managing toxin production. In addition to benefiting policymakers addressing cyanobacterial blooms through targeted metabolomics, non-targeted metabolomics has been used in cyanobacterial research to discover new compounds.

For example, when the cyanobacterial genus *Trichodesmium*, an open-ocean bloom-forming organism that fixes nitrogen gas into organic nitrogen was interrogated with non-targeted metabolomics using MS/MS-based molecular networking, three cytotoxic compounds were discovered in the bloom: smenamide A, smenamide B, and smenothiazole A [44]. The discovery of these novel cytotoxic compounds by using non-targeted metabolomics allows for further probing of the ecological roles of these molecules in blooms in addition to a wealth of novel data of secondary metabolites of understudied yet globally abundant organisms such as *Trichodesmium*.

Diatom microbial ecology studies too have benefited from metabolomics methods. The genes and enzymes involved in domoic acid's biosynthetic pathway were identified using methods including transcriptomics, MS, and NMR (Fig. 3a) [45].
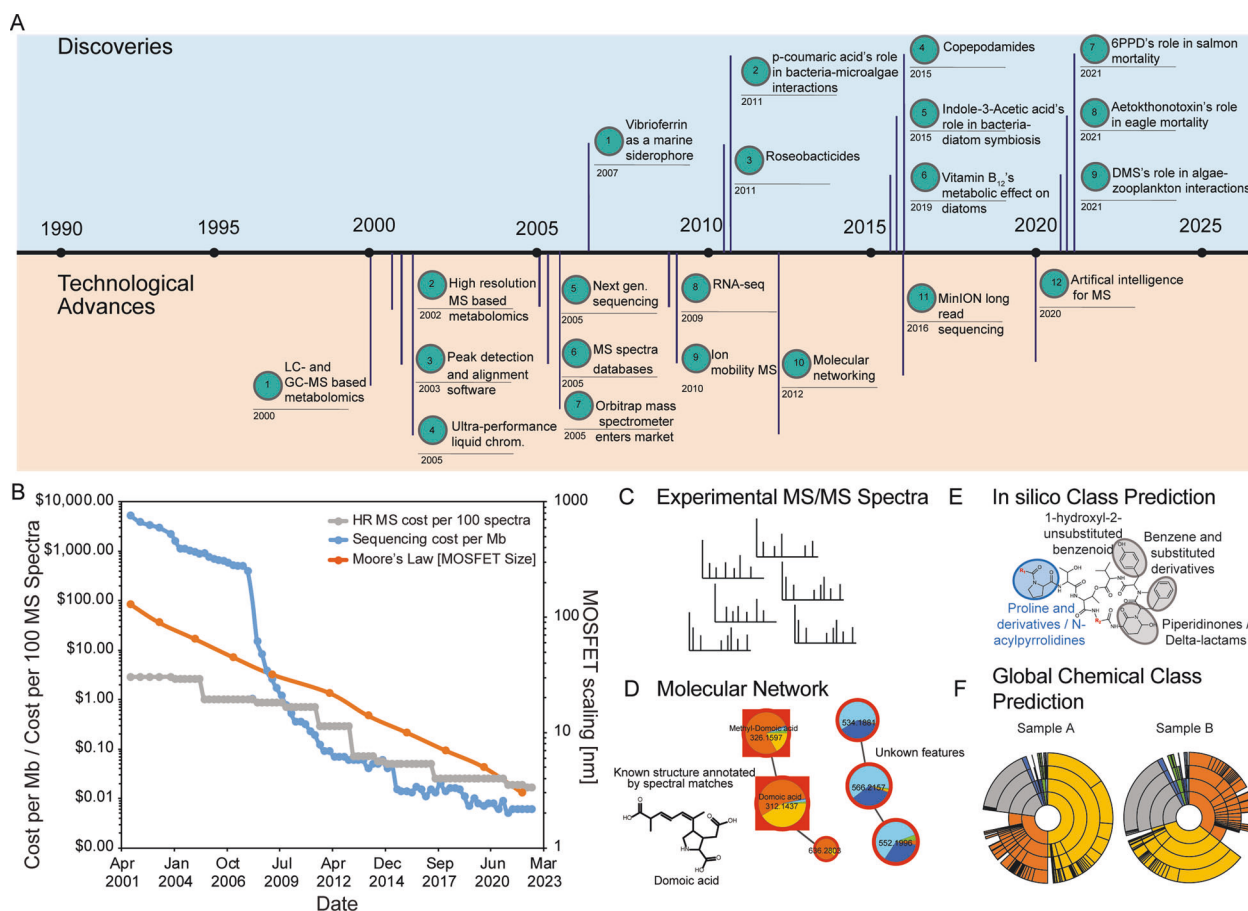
Fig. 2 Technological advances drive discoveries in Harmful algae bloom research. Panel (**A**) shows a timeline of, in our opinion, important discoveries of metabolites and/or their roles in aquatic ecosystem function as well as technological advances that were made and will be important for new molecular insights. Panel (**B**) shows the development of cost of MS and sequencing analysis in comparison to Moore's law shown as logarithmic decrease of transistor size (MOSFET scaling), updated from previous work [160] and assuming 25€/h instrument run time according to Deutsche Forschungsgemeinschaft (DFG) Guidelines for Instrumentation Usage Costs and Core Facilities. Panels **C** through **F** show examples of new metabolite data annotation and visualization methods through molecular networks and class level annotation of metabolites [47, 109, 115]. Panel **D** shows an example of a GNPS molecular network of metabolomes from Pseudo-nitzschia cultures that annotated domoic acid by spectral matches in addition to unknown features [47]. Panel **E** shows rivulariapeptolide [115], for which structures were annotated using new tools like CANOPUS [109].

The *Pseudo-nitzschia* genus is well studied due to its capability to produce the domoic acid neurotoxin which can cause mass mortality to mammals and fish in addition to significant economic downturns in coastal regions dependent on fisheries. This genus only exhibits haploid gametes during the understudied sexual phase when it encounters another cell of the opposite mating type [46]. Non-targeted metabolomics on each of the two mating types of *Pseudo-nitzschia multistriata* in addition to mixed mating types revealed characteristic metabolites of mating types: higher levels of the fatty acid oleamide in the mixed culture and higher levels of the bacterial osmoregulation-compound ectoine in the MT- mating type culture. Recent work discovered distinct metabolomes and microbiomes among species of *Pseudo-nitzschia* [47]. These conclusions are bases for future work studying the economically and ecologically relevant diatom genus *Pseudo-nitzschia*, and can support ongoing work of sexual reproduction of *Pseudo-nitzschia* in the laboratory.

Allelopathy, or production of small molecules that disrupt growth or kill competing species is a common competitive regulatory mechanism within aquatic microbial communities. *Karenia brevis* is a dinoflagellate that exhibits allelopathy, disrupting competing phytoplankton such as the diatoms *Asterionellopsis glacialis* and *Thalassiosira pseudonana* [48]. MS was used to elucidate the metabolic pathways involved in the

dinoflagellate's allelopathy and in the resistance of the affected phytoplankton using metabolomics and proteomics. Exposure to allelopathic molecules impacts the evolution of resistance: *A. glacialis* was more metabolically robust and exhibited higher resistance to exposure than *T. pseudonana* did, likely because of regular exposure to *K. brevis* blooms. In the sensitive, less "immune" *T. pseudonana*, exposure affected several metabolic pathways, leading to decreased photosynthetic capacity, reduced ability to osmoregulate, suppression of lipid synthesis, and increased cellular oxidative stress [48, 49]. Supplementing this conclusion, the allelopathic capacity of the exudates of five strains of *K. brevis* on *A. glacialis* were interrogated, determining that higher concentrations of uncharacterized fatty acid-derived lipids and aromatic/polyunsaturated compounds led to higher allelopathy of a strain [50]. These studies reveal the utility of metabolomics to gain a snapshot of the effects of chemical allelopathic effects of one microorganism on another, revealing dynamics and predictions of microbial community structure. Predicting how microorganisms will interact chemically is especially important as global change leads to encounters of species not historically exposed to one another.

In addition to characterizing dynamics of ecologically-relevant organisms, metabolomics has recently been utilized to typify dissolved organic matter (DOM) in the environment that the

Table 1. Examples of chemical cue characterization over time.

Molecule or discovery	Functional level (ecosystem, lab mesocosm, organism, in vitro)	Year of characterization	Select techniques in characterization
Smenamide A and B, and Smenthiazole A as cytotoins in a *Trichodesmium* bloom [44]	Ecosystem	2018	LC-MS/MS, NMR.
Domoic Acid Biosynthesis [45]	In Vitro	2018	Transcriptomics, MS, NMR.
Azelaic Acid and Rosmarinic Acid's role in a diatom modulating bacterial community [60]	Lab mesocosm	2020	Transcriptomics, MS.
Roseobacticides [61]	In Vitro, Lab Mesocosm	2011	NMR, LC-MS.
p-coumaric Acid's role in bacterial-microalgal interactions [62]	In Vitro, Lab Mesocosm	2011	NMR, LC-MS.
Indole-3-Acetic Acid's role in symbiosis between bacteria and diatoms [66]	Lab Mesocosm.	2015	Growth experiments, Genomics, Transcriptomics, MS.
Vitamin B12's metabolic effect on diatoms [70]	Ecosystem, Lab mesocosm	2019	Growth Experiments, MS.
Vibrioferrin as a marine siderophore [73]	In Vitro	2007	HPLC, NMR, MS.
Copepodamides [79]	Ecosystem, Lab mesocosm	2015	Degradative chemical experiments, NMR, MS.
DMS's role in algae-zooplankton interactions [84]	Ecosystem, Lab Mesocosm	2021	Genetic Transformation, GC-FPD, Growth experiments.
6PPD's role in salmon mortality [89]	Ecosystem, Lab mesocosm	2021	LC-MS/MS, NMR.
Aetokthonotoxin's role in eagle mortality [90]	Ecosystem, In Vitro Gene/Protein	2021	AP-MALDI-MSI, LC-MS/MS, NMR, Crystallography, Imaging,
Rivulariapeptolides as serine protease inhibitors [115]	In Vitro	2022	Native metabolomics, MS, NMR.

Thirteen discoveries, alongside the functional level of the molecule or discovery, the year of characterization, and selected techniques used in the characterization.

microorganisms inhabit [51]. Building upon stoichiometric ratios that are traditionally used to characterize organic matter in rivers and streams, non-targeted metabolomics was utilized to identify higher levels of flavonoids along a creek as it moved downstream. Another study described patterns and differences in river surface water metabolomes of various sizes and ecosystems using a community-science approach to amalgamate global data [52]. This data is important to understanding the sources and sinks of carbon transport, and to characterizing spatial and temporal dynamics of metabolites in the environment that microorganisms and higher-trophic organisms experience, consume, and produce.

Metabolic "Hot Spots" reveal algae-bacteria interactions
To understand ecosystem dynamics, the phycosphere has been explored in both fresh water and marine aquatic environments through multiple-omics methods, revealing how bacterial consortia interacts with bloom-forming microalgae. The physcophere describes the region directly surrounding the algal cell in which algal extracellular exudates support bacterial growth .

Dissolved organic molecules diffusing from the algal phycosphere attract heterotrophic bacteria, and bacteria benefit from these organic molecules in exchange for cofactors that the microalgae depend on for their growth. Metabolites are exchanged at the phycosphere, and act as signaling compounds to communicate between phytoplankton and bacteria, whether in a mutualistic, antagonistic, or parasitic manner [53]. Cirri and Pohnert proposed the concept of "metabolic hotspots" around algal cells, where resource exchange occurs, and which bacteria have evolved to utilize for their metabolic needs [54]. In both terrestrial and marine aquatic environments, bacteria that inhabit algal phycospheres are not highly abundant outside that region, reflecting their specialization to inhabit the phycosphere niche [53, 55–57]. Non-targeted metabolomics breaks free from examining single microorganism-molecule interactions, and rather captures the broad chemical dynamics in the total community.

For example, heterotrophic bacteria that grow in the physcosphere of freshwater cyanobacteria *Microcystis aeruginosa* and on its DOM drew down more dissolved organic carbon than non-phycosphere-inhabiting-heterotrophs, particularly removing more lipids, organic acids and organoheterocyclic molecules [58]. The phycosphere-heterotrophs also had larger genome sizes, reflecting the wider resource pool of DOM available in the phycosphere to maintain larger genomes [59].

Diatoms have some control over their microbiome: they attract beneficial bacteria and dispel the harmful ones through unknown cellular mechanisms and metabolic signaling pathways [60]. They change their transcriptional activity when they encounter certain microbial communities, and as a result release central and secondary metabolites. In response, bacteria that are attuned to the diatom's exudate respond transcriptionally to these metabolites with varied speeds and intensities and may consume these central metabolites. For example, azelaic acid and rosmarinic acid produced by diatom *Asterionellopsis glacialis* were found to modulate bacterial behavior and growth to simultaneously promote diatom symbionts and demote diatom opportunists [60].

In addition to symbiosis, bacteria have been shown to act as pathogens of microalgae. Roseobacter *P. gallaeciensis* blooms along blooming haptophyte *Emeliania huxleyi*. When an *E. huxleyi* bloom ages, it produces p-coumaric acid, with response to which *P. gallaeciensis* begins to produce potent algaecides called roseobacticides (Fig. 3b) [61, 62]. This dynamic interaction of the algae and bacteria encompasses mutualism and parasitism as the blooms boom and bust. P-coumaric acid and other phenolic acids, are common plant metabolites that can be released in plant exudates as well as through the breakdown of lignin. Hence, there is the possibility that *P. gallaeciensis* could also respond
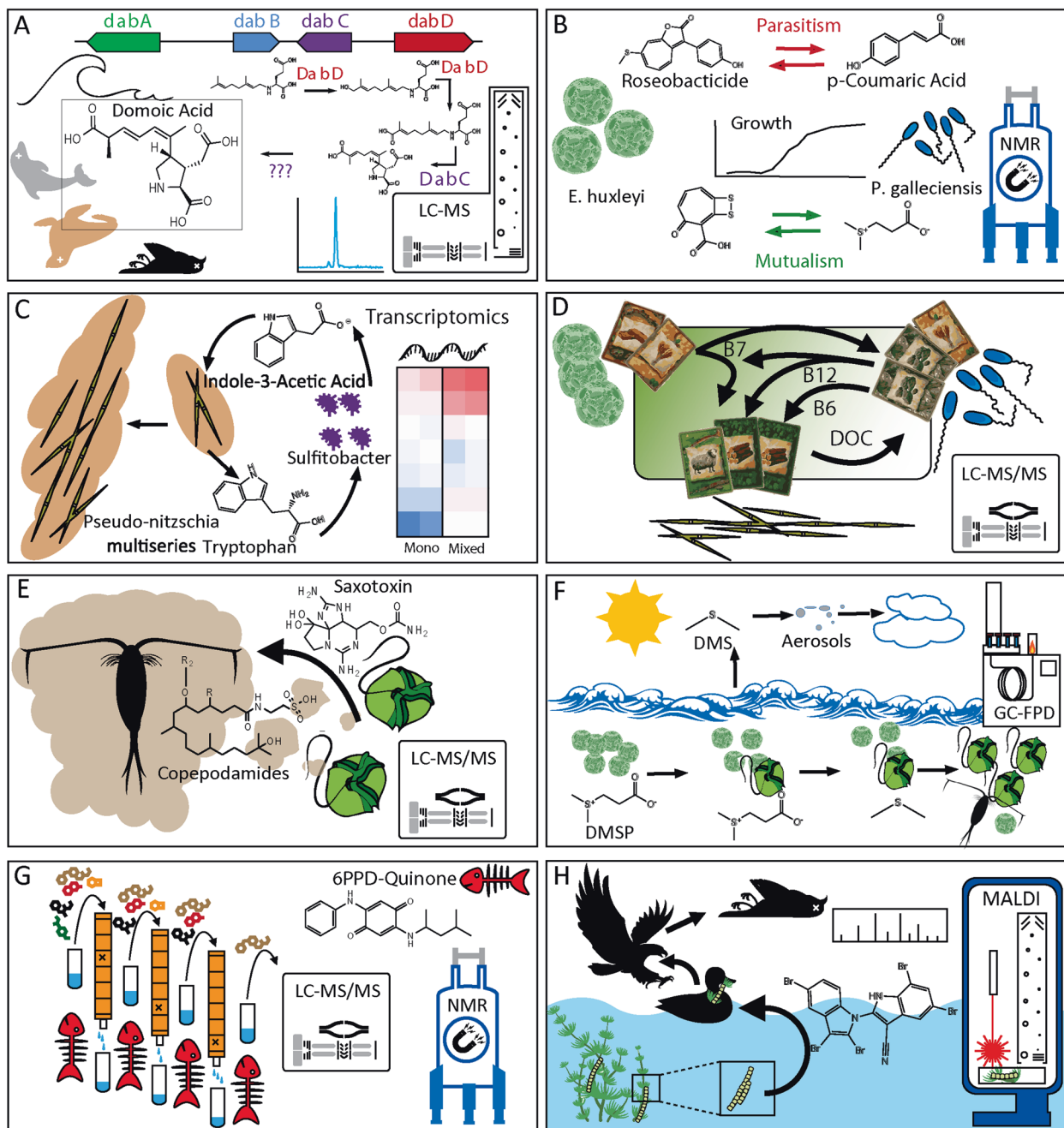
Fig. 3 Success stories of deciphering chemical mediators in in aquatic chemical ecology. Domoic acid's biosynthetic pathway was characterized with transcriptomics, biochemistry and NMR (**a**), p-coumaric acid and roseobacticide are exchanged through a parasitic interaction between a phytoplankton and a bacteria characterized through dose-response growth experiments and NMR (**b**), and metabolomics and transcriptomics were used to characterize the exchange of indole-3-acetic acid and tryptophan between a diatom and symbiotic bacteria (**c**). Many microorganisms have not retained the ability to biosynthesize cobalamin (Vitamin B$_{12}$) and rely on other microorganisms to produce it in exchange for other resources (**d**). Copepodamides were the first molecules discovered to mediate "chemical warfare" between zooplankton and their prey (**e**), and DMS has been known to modulate cloud formation and climate and a new role has been discovered to modulate algae-grazer interactions through GC-FPD (**f**), 6PPD from tire runoff into water sources and its ecological effect was characterized UPLC-HRMS/MS and NMR (**g**), and AP-MALDI-MSI was used to study cyanobacteria colonies on plant leaves to detect aetokthonotoxin (**h**).

metabolically to p-coumaric acid that is not from an *E. huxleyi* source, but rather derived from another non-haptophyte algae or from runoff of plant-derived dissolved organic matter [63, 64].

Algae-bacteria interactions in the phycosphere are facilitated by proximity between cells which often takes the form of bacterial colonization of host algal cells. The flavobacterium *Croceibacter atlanticus* inhibits diatom growth when it attaches to the diatom,

possibly in order to increase colonizable surface area of the diatom [65]. This interaction exemplifies antagonism of bacteria upon the microalgae. Here, microscopy, flow cytometry and genomics work can be augmented by introducing MS-based metabolomics work to characterize which molecules are exchanged between the flavobacterium and the diatoms leading to the growth inhibition.

An example of the use of omics techniques to characterize symbiosis is that *Sulfitobacter's* production of indole-3-acetic acid (IAA) increases *Pseudo-nitzschia's* cell division rate (Fig. 3c) [66]. Metabolomics and transcriptomics were used to characterize this signaling molecule's role, linking two of the ocean's microorganisms. Furthermore, this *Sulfitobacter* responds with an increased growth rate only when co-cultured with *Pseudo-nitzschia*, likely by taking up tryptophan produced by the diatom, establishing a symbiotic relationship promoting growth in both organisms through the exchange of small signaling molecules. Environmental concentrations of IAA are sufficient to induce an ecologically relevant response as observed in culture. The study identified bacteria as the IAA producer by identifying transcripts of genes involved in IAA biosynthesis in both the laboratory and the environment, and through measurement of IAA in axenic culture experiments. However, in the environment, it is possible that some IAA sources from terrestrial runoff of plant-derived DOM affects the aquatic microbial community as IAA is a common plant hormone and bacterial natural product [67].

The small molecule cobalamin (Vitamin B_{12}) is critical in regulating microbial communities. For example, diatoms scavenge cobalamin from the environment rather than make it themselves, following principles of the "Black Queen Hypothesis" of gene-loss reductive evolution in organisms, creating microbial dependences (Fig. 3d) [68, 69]. The term is an analogy to the situation in the game "Hearts", when a player strategizes to not to take the queen of spade cards [68]. Cobalamin availability controls which phytoplankton can grow well where. It is needed as a cofactor for enzymes, and some plankton have evolved enzymes (metE) that function without a cobalamin cofactor, though less efficiently. The model diatom *Thalassiosira pseudonana* was used to study how diatoms respond metabolically to cobalamin limitation for the first time through both targeted and non-targeted metabolomic methods [70]. Cobalamin-limitation to diatom *Thalassiosira pseudonana*, which does not contain the metE gene in its genome, leading to a requisite cobalamin requirement (auxotrophy), revealed a differential response in production of several metabolites. Key findings include less dimethylsulfoniopropionate (DMSP) and glycine betaine under cobalamin limitation which normally are used to balance osmotic pressure, and that the diatoms enzymatically transform hydroxy-cobalamin into ado-cobalamin using an enzyme conserved among diatoms. These findings demonstrate the value of applying metabolomics to ecologically relevant processes like vitamin limitation to shed light on physiological responses.

Bacterial presence can also help dinoflagellates with iron acquisition through siderophore production: *Marinobacter* produces the weak siderophore vibrioferrin and co-occurs with dinoflagellate *Gymnodinium*, which may benefit from iron-chelating siderophore presence in iron-depleted environments [71–73]. Vibrioferrin is one of only a few marine siderophores with a characterized structure [74–77].

Small molecules mediate microalgae-grazer interactions
Interactions between harmful algae and grazers are exemplified by the interactions between dinoflagellate *Alexandrium catenella* and copepod *Acartia tonsa* [78]. While copepods graze on toxic *A. catenella* to gain energy, they suffer negative outcomes to their reproductive abilities.

An important step in the discovery of chemical cues that mediate the interaction between zooplankton and their prey was the discovery of taurolipid "copepodamides" (Fig. 3e) [79]. Copepodamides are produced by copepods and induce a 20-time increase in dinoflagellate *Alexandrium's* saxitoxin toxin production. Different species of copepods combine an amide and a taurine to 8 unique isoprenoid fatty acids to form this set of signaling molecule lipids that can be detected both in lab culture experiments and in the environment [79]. These novel molecules

also induce bioluminescence in *Lingulodinium* dinoflagellates which confers a competitive advantage in deterring predation [80]. In addition to affecting dinoflagellate toxin production, copepodamide presence affects diatoms as: increased domoic acid toxin production from *Pseudo-nitzschia* and a decrease in colony size in *Skeletonema* [81]. Environmental concentrations of these molecules are relevant to cause restructuring of ecosystem dynamics and mediate predator-prey interactions in nature. Ecological warfare between copepods and diatoms were further underscored by the discovery that copepods that feed on blooming diatoms have a low hatching success rate due to a set of three 10-carbon aldehyde molecules that stop embryonic development [82].

Dimethyl sulfide (DMS) is an important source of sulfur-containing aerosol compounds (sulfate, methane sulfonate, sulfuric acid) which cause water vapor condensation and thereby promote cloud formation (Fig. 3f) [83]. DMS therefore holds a critical role in the natural climate feedback loop. In addition to its role in regulating climate, DMS, produced by some species of blooming plankton, has been found to regulate algae-grazer interactions [84]. The enzyme DMSP lyase was studied in bloom-forming coccolithophore *Emeliania huxleyi* and diatom *Thalassiosira pseudonana* and surprisingly found that it enhances grazing rates and promotes growth in grazers like *Oxyrrhis marina*, revealing the key ecological impact of the "eat-me" signaling molecule DMS.

Multi-omics empowers ecosystem-level systems biology studies
Studies that integrate multiple 'omics techniques may yield a more complete systems biology level understanding of any organism [85]. For example, the Ingalls group integrated transcriptomics and metabolomics to show that taxonomy and metabolomics can be linked. The results enabled them to link organisms with different metabolites they produce and consume with respect to light oscillations. One finding was that the *Crocosphaera* cyanobacteria produced the osmolyte trehalose at the end of the light period to store energy for the night. Integrated metabarcoding and metabolome data was utilized for the study of the effects of copepod feeding on planktonic interactions in aquatic environments, allowing for the conclusion that copepod feeding preferences are related to the metabolic stage of the prey rather than prey abundance [86].

Capturing human impacts as a fundamental part of aquatic ecosystems
While metabolomics tools enable us to capture a broad range of metabolites, they are also well suited to detect and annotate a wide range of anthropogenic molecules. For example, aquatic ecosystems contain numerous xenobiotics that have become a fundamental part of their chemotypes [87].

Human-caused pollution to urban rivers was studied by combining bacterial metagenomic amplicon sequencing with GC-MS to characterize differences between clean and contaminated parts of an urban river, to understand how the organisms' metabolomes shift when exposed to pollution [88]. In polluted waters that contained fecal bacteria, there were higher levels of sugar alcohols and short-chain fatty acids, which can enhance biofilm formation.

The chemical impact of humans on aquatic ecosystems is exhibited by the recent characterization of a salmon-killing xenobiotic, causing "urban runoff mortality syndrome" [89]. Although it is not a natural product or related to microbial turnover, anthropogenic contaminants represent a paradigm shift to the field of chemical ecology. The toxic molecule *N*-(1,3-dimethylbutyl)-*N*'-phenyl-p-phenylenediamine (6PPD) from tire tread rubber from roadway runoff is abundant in many freshwater creeks that are the home of U.S. Pacific Northwest Coho Salmon. Abiotic transformation processes are involved to produce 6PPD-

quinone, found to be abundant in the aquatic ecosystem and toxic to Coho Salmon. The structure of this compound was characterized using UHPLC-MS/MS and NMR following extensive bio-activity guided fractionation steps (Fig. 3g). This example of humans unintentionally interfering with the salmon life cycle is one of many stories of how human waste runoff into aquatic ecosystems affects native organisms.

Another more indirect human impact on aquatic ecosystems is the increased abundance of the compound aetokthonotoxin and related die-offs of bald eagle populations in the south of the US [90]. The discovery of "The Eagle Killer" molecule aetokthonotoxin typifies how small molecules, facilitated through anthropogenic impacts and produced at the base of the food web can affect the whole food chain. The *Aetokthonos* cyanobacteria colonizes a freshwater invasive plant *Hydrilla* and produces a molecule aetokthonotoxin which bioaccumulates up the food web to top predator bald eagles, causing neurological disease, and ultimately the death of eagles (Fig. 3h). As aetokthonotoxin contains multiple bromine atoms, it has been speculated that its biosynthesis benefits from increased bromide salt concentrations related to anthropogenic sources. The identification of aetokthonotoxin and its linkage to the decline of bald eagle populations was a milestone for ecotoxicologists, as the novel toxin can now be monitored and better regulated. For example, monitoring of bromide levels in reservoirs will aid in bald eagle conservation. The discovery of this unprecedented natural product was facilitated by atmospheric-pressure matrix assisted laser desorption/ionization MS imaging (AP-MALDI-MSI) which was used to study the cyanobacterial colonies on the plant's leaves to detect small molecules produced by the bacteria in situ, and to identify the molecular formula of the novel molecule.

Finally, anthropogenic impacts significantly alter aquatic environments, for example through ocean warming and ocean acidification, which decreases calcification and causes bleaching events in corals [91, 92]. Nutrient loading in aquatic environments has been shown to increase biomass and calcification and carbon fixation rates of coral *Acropora pulchra* [93]. Multi-omics studies using transcriptomics, proteomics, and metabolomics have been used to characterize the metabolic exchange and regulation of the coral-microalgal symbiotic relationships. Some of the key findings include new insights into heat stress influencing protein folding, antioxidant biosynthetic gene expression, and catabolism of lipid stores in some symbioses [94–96]. As a result of detrimental anthropogenic impacts on the natural environment, humans used metabolomics as a method to aid in the conservation of endangered freshwater mussels, critical in their role as ecosystem engineers [97, 98]. To understand the higher mortality in mussels relocated to alternative environments or to captivity for management purposes, mussels were interrogated with non-targeted GC-MS and LC-MS [97]. It was found that relocation of mussels from the native environment changed amino acid and nucleotide metabolism, likely reflecting a stress response due to the change in habitat.

EMERGING METABOLOMICS TOOLS FOR AQUATIC CHEMICAL ECOLOGY
New tools enable large-scale metabolomics data analysis and compound annotation
With the constant improvement of mass spectrometers' sensitivity, scan speed and accessibility, thousands to millions of individual mass spectra are typically obtained per metabolomics study. Similar to sequencing-based omics studies, data analysis has thus become a major bottleneck and has reached a point that is far beyond manual data interpretation. Hence, scalable software solutions are indispensable to extract conclusions from data in metabolomics experiments. Fortunately, both mass spectrometer vendors and open-source developers have created numerous

software tools for raw file processing, feature extraction, feature annotation, and downstream statistical analysis. Multiple software platforms, with graphical user interface (GUI), web-based apps, or scripting packages provide streamlined workflows that include quantification and enhanced detection of chromatographic features as well as annotation of MS data [99–106]. While each platform has unique capabilities and serves different user preferences, the interconnection of different platforms enables the best customizable data analysis solution, especially at the interface of feature detection, annotation and statistical analysis.

Feature-based Molecular Networking (FBMN) through the GNPS environment, for example, allows for the seamless import of processed raw mass spectrometry data, as well as export to further annotation and statistical analysis from a wide range of vendor and open source software tools [107–109]. FBMN builds upon molecular networking, which connects related molecules by their MS/MS spectral similarities [35]. FBMN can also make use of ion mobility separation information in addition to its algorithm to characterize chromatographic features and separate isomers with similar MS/MS spectra. The FBMN concept and workflow allows for improved annotation of mass spectra in combination with the ability for semi-quantitative or relative quantitative analysis, enhancing the user's downstream research and potential for new compound discovery and quantification from understudied organisms such as from aquatic fungi and aquatic macroalgae [110].

Since "unknown" features in a non-targeted experiment typically represent the majority, additional tools are needed to bridge this large gap of unidentified data. In addition to matching experimental MS/MS spectra against spectral libraries, other tools predict MS/MS fragments from structure data and match those predicted MS/MS fragments against the experimental spectra. Such approaches are typically called in silico spectrum annotation. As structural databases are significantly larger than spectral libraries (typically 10-100 fold), in silico annotation tolls have much larger coverage, and allow for annotation of features at varying confidence and information levels (e.g. molecular formula, chemical class, or planar structures) [111, 112]. Recent advances include the use of Network Annotation Propagation (NAP), which was developed to improve the accuracy of existing in silico spectrum annotation tools by adding molecular networking to re-rank in silico annotations between connected nodes to generate consensus annotations within the networks, which typically improves the confidence in the annotations [111]. In recent years, several tools have been developed to improve in silico spectrum annotation further. SIRIUS is a computational tool that analyzes isotope patterns and fragmentation trees, which are related to the fragment peaks of a molecule to identify molecular formulas [113]. SIRIUS is often used in combination with CSI:Finger ID, which allows the matching of fragmentation trees of experimental spectra with fragmentation trees from structural databases. ZODIAC, improves the prediction of molecular formulas from SIRIUS by also taking into account similar fragments and losses between from other derivatives from spectral networks [114]. CANOPUS (class assignment and ontology prediction using MS) is another computational tool within the SIRIUS software suite, that allows chemical class level prediction through fragmentation trees [109]. An application of this tool on a crude extract of a cyanobacteria *Rivularia sp*. demonstrated these predictions by superclass, class and subclass: CANOPUS identified the major compound to be a cyclic peptide that was not annotated or identified by other computational methods. NMR was used to confirm the structural predictions and characterize the group of rivulariapeptolide, allowing for a greater understanding of aquatic natural products from microorganisms [115]. The easy-to-use GUI and integration of these tools improves the user experience for in silico spectrum annotation. Another way to predict unknown molecules is to improve the size of the library: "Suspect IDs" have

been generated by using molecular networking and analog searches, in which unknown MS/MS spectra are matched against the GNPS MS/MS spectral library. Spectra that show high spectral similarity, but different precursor masses with a delta matching common modifications, were considered to be related compounds, which were then included in a new "suspect library" available on GNPS [116]. This generates new library spectra out of known library spectra, making the library of standards more comprehensive.

Data sharing and computational advances allow the reutilization of existing metabolomics data

The increasing willingness of researchers to share their data in public repositories and the communities' efforts to organize the data and their meta-data allows for global repository-scale analyses through new software tools such as the MS Search Tool (MASST) [100, 117]. Akin to BLAST which is commonly used to compare biological sequence information against public databases, MASST allows querying a single MS/MS spectrum, for example from a known compound, against all public MS/MS data. The output will tell in which dataset the spectrum or in a broader sense the compound of interest is present. This information can then be used to hypothesize about the occurrence or origin of certain compounds, even without the need for full identification.

MASST has been used for example for the repository scale spectrum search of the potent neurotoxin domoic acid. Surprisingly, the results of this analysis indicated that domoic acid was present in data sets from coral reefs in Hawaii, an environment that is typically not known for the presence of *Pseudo-nitzschia*, the microalgae that produces domoic acid. In another study, MASST was used for the contextualization of metabolites and xenobiotics, detected in coastal environments [118].

Besides spectrum matching of single spectra against entire MS repositories through MASST, there are more tools emerging to perform multivariate analysis, such as Principal Coordinate Analysis (PCoA) of whole repositories [107]. An important aspect for repository scale analysis is the use and organization of controlled metadata. Through the ReDu template in GNPS for example, the user can provide a metadata sheet with controlled vocabulary in addition to their raw metabolomics data, which allows the seamless integration of their data into a global analysis, such as PCoA [119]. With the rise of LC-MS/MS centric meta-analysis tools and repositories, there has been significant effort from the imaging MS (IMS) community to centralize data storage which can then be leveraged for high-confidence annotation of ion features [120]. With the rise of new instrument platforms such as for single cell proteomics or μm-range lateral resolved MALDI imaging platforms, new and effective strategies are needed for the high demands of data analysis, clean-up and annotation [121, 122]. Data from the METASPACE imaging mass spectrometry repository has also been used to filter out background features from single-cell MALDI imaging data with a software tool called SpaceM [123]. Such tools further integrate other spatial data, such as fluorescent microscopy images, or single cell transcriptomics/genomics data. Such data integration approaches are fundamental to increase for example spatial resolution (through image fusion) or compare transcriptional to translational regulation [121, 122]. In the microbial ecology context, multi-omics data layers, such as combined metabolomics and transcriptomics/genomics data can further be used to identify biosynthesis genes or the producing organisms [100, 124, 125]. Connecting molecules to organisms is therefore not an easy to tackle problem, despite it being of high interest to many scientific fields [15, 23].

Bioinformatic approaches to integrate metabolomes with community composition

The CoNet tool can be used to detect associations between genes, metabolites, and taxa and also hosts a command-line usage that allows for interactions between these variables to be easily visualized in Cytoscape [126]. This computational tool does co-occurrence analysis and network interference analysis to predict relationships between measurements of genes, metabolites, and taxa. It also can take metadata as an input to look for associations between environmental variables and other metadata variables with these biological measurements. CoNet was applied to determine which microorganisms (based on 16 S rRNA gene data) in Arctic soil are co-correlated and anti-correlated with pH and identify microorganisms that cross-feed in a complex environmental microbiological sample [126]. Another recent tool to connect metabolites with microorganisms is "mmvec" which uses neural networks to determine how likely a metabolite is to be linked with microorganisms [125]. In order to generate databases of these associations and to verify computationally predicted associations, characterizing metabolites of laboratory-cultured phytoplankton species and natural phytoplankton communities is paramount [127].

Advances in functional annotation of metabolites at scale

While the identity of most metabolites, especially in complex environmental systems, are still unknown, even less information is available about the biological function and activities that these compounds might have. Thus, in addition to tackling the bottleneck of metabolite annotation, novel experimental strategies are needed to assign metabolite function at scale. The idea of pairing functional assays with high-throughput metabolomics, often referred to as functional metabolomics, is hence very compelling [128, 129]. While bioactivity-guided assays have been applied and refined in the natural product field for decades, the pairing to high-throughput metabolomics approaches is not straightforward [130–132]. Major bottlenecks are the scalability of bio-assays related to the fraction number per metabolomics sample, unambiguous assignment of activities to individual compounds as well as their structural annotation. Recent advances in the combination of bio-activity and non-targeted metabolomics workflows include the integration of high-throughput phenotyping and metabolomics as well as molecular networking approaches with bioactivity assays for the assignment of antibacterial and antiviral assays [133–135], as well as molecular interactions such as metabolite-metal and metabolite-protein binding [115, 136, 137]. Similar, bioactivity-guided LC-MS approaches are widely used in the environmental chemistry community, often termed effect-directed analysis [138, 139]. For example, connecting toxicity with non-targeted chemical profiling and downstream de novo structure elucidation led to the above mentioned discovery of a salmon mortality-inducing compound from rubber wear-off from car tires that enter aquatic systems through storm water run-off [89].

CONCLUSIONS AND FUTURE DIRECTIONS

Non-targeted metabolomics tools offer great potential to provide molecular insights into complex ecosystems and expand biological conclusions from multi-omics studies. The top four key advantages and emerging solutions that we see for this field are: (1) improved metabolite annotation and democratization of spectral libraries and data, (2) improved functional metabolite assignments (i.e. toxicity) (3) integration of multi-omics datasets, and (4) contextualization and re-use of data. Yet, key challenges that remain are: (1) Lack of medium-scope studies to bridge the knowledge gap between quantitative targeted and non-targeted qualitative environmental studies, (2) ultra-complexity of environmental samples, (3) and annotation of remaining unknown metabolites.

Inherent limitations of current metabolomics approaches are that targeted studies lose the scope of the thousands of unstudied molecules [140, 141], whereas, non-targeted studies face the

challenge of limited library sizes that most often only allow the annotation of less than ten percent of the total number of features. The improvement of quantitative capabilities for non-targeted studies will pave the way forward to bridging this knowledge gap. While the challenge of spectral annotation through spectral libraries remains, it is constantly improved by the increase in spectral library coverage, in silico spectral annotation tools and compound suspect identifications [109, 113, 116].

Despite these potential advances, challenges that an unknown proportion of these spectra are from abiotic backgrounds, and another fraction are from the detection of isotopes that are not unique compounds will remain. Other challenges arise from the inherent ultra-complex nature of environmental samples: One study aimed to separate isomers in DOM with high performance liquid chromatography found that the ultra-complex nature of the mixture was a central barrier and that the complexity is an order of magnitude higher than what was previously expected [142]. Furthermore, highly hydrophilic biodegradable dissolved molecules are not well detected with typical methods of solid phase extraction to concentrate DOM prior to mass spectrometry, so many of these species are likely overlooked during DOM analysis [143]. New experimental methodology is still needed to improve the chemical extraction and characterization of environmental metabolomics samples, and to provide standardized and repro-ducible computational frameworks for the community to share, re-utilize, and contextualize data [144, 145]. In addition to limitations in aquatic metabolomics, a recent review of machine learning strategies for integrating multi-omic marine datasets identified some challenges identified with data integration to be the variability and noise of data, limited metadata, inaccessible computational tools, and high cost [146].

Connecting chemotypes with genotypes
Along with advances in metagenomics and increased accessibility of high-quality Metagenome-Assembled Genome (MAG), impor-tant future developments in metabolomics tools for aquatic microbial ecology may include the better integration of MAGs and genome mining strategies with metabolomic data. New tools and infrastructures like NPOmix and The Paired Omics Data Platform pave the way to connect thereby molecules to their biosynthesis genes [124–126, 147]. It is estimated that two-thirds of the work on a multi-omic projects goes to data processing and integration, highlighting how the current data analysis approaches are a time and energy consuming processes, with much room for improve-ment [148].

Metabolomics methods for global change assessment
We anticipate that metabolomics tools will play a central role in assessing the impact of climate change on aquatic systems. It has been predicted that climate change induced rising ocean temperatures favor larger and more frequent harmful cyanobac-terial blooms [149], and understanding and perhaps leveraging chemical cues that influence harmful algal blooms [150], is of great importance. Metabolomics tools may play an integral part to identify such metabolites in multi-stressor experiments. An important aspect hereby well be the scaling from single chemical entities and interactions to chemotype-wide ecology of molecules. A fully structurally resolved inventory of all metabolites / DOM pool, would be the basis to build up models for DOM persistence (intrinsic and emergent) along with ecological/environmental parameters [15, 151, 152]. Multi-dimensional DOM fractionation and LC-MS/MS analysis, combined with bioactivity screening approaches, such as cytological profiling high content screening methods or antimicrobial assays, could link DOM chemical composition to biological functions. When done at high resolution and scale, identifying new natural products and their biological properties can be done directly from environmental samples

[153]. Such developments will not only drastically improve our understanding of the DOM black box, but also unleash its tremendous molecular complexity as a potential source of structural and pharmaceutical/biotechnological novelty.

Waiting for the "AlphaFold-Moment" in metabolomics
Machine learning, and in particular, advanced deep learning tools like AlphaFold, and large language models (LLMs), are transform-ing the field of biological research [154]. AlphaFold, developed by DeepMind, has made it possible to accurately predict the 3D structures of proteins, which has historically been a fundamental challenge in structural biology [155]. This breakthrough in machine learning has the potential to accelerate the discovery of new drugs and therapies, as well as help us better understand the underlying mechanisms of many diseases. Meanwhile, LLMs, like OpenAI's GPT-4, are being used to analyze large amounts of biomedical literature, making it possible to quickly identify new hypotheses and connections between metabolites and organisms [156]. This can lead to new insights into the relationships between genes, proteins, metabolites and microbial community dynamics, offering completely new scales to describe and understand ecosystem function. A similar breakthrough in the field of proteomics occurred when the SEQUEST software was developed to match tandem mass spectra from peptides to database sequences [157].

Besides advanced AI tools such as ChatGTP, many machine learning approaches are already well established in the metabo-lomics community. Especially in the realm of supervised multi-variate statistics, machine learning classification tools such as random forest and XG-Boost are commonly used [42, 158]. At the same time, some of the above mentioned in silico spectrum annotation tools make use of deep neural networks to learning MS/MS fragmentation, compound class prediction, or de novo structure elucidation and are rapidly improving [110, 114, 159]. However, high confidence compound annotation through the prediction of MS/MS spectra from structure libraries is still an unsolved problem, and the field is still waiting for an "AlphaFold Moment" in mass spectrometry-based metabolomics.

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ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Daniel Petras.

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