Chapter 1

Marine Environmental Metabolomics

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Additional information is available at the end of the chapter

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Abstract

Marine environmental metabolomics studies the interactions of marine organisms with their environment using metabolomics to characterise these interactions. There are many advantages in using this method to study interactions between organisms and the environment and to assess the function and health of organisms at the molecular level. In fact, metabolomics is finding an increasing number of applications in the marine sciences. These range from understanding the response of organisms to abiotic pressure to researching the response of organisms to other biota. These interactions can be studied at different levels, from individuals to populations for more traditional eco-physiological or ecological studies. Marine organisms have developed a high diversity of chemical defences to avoid predators and parasites. This study therefore highlights the complexity of chemical interactions in the marine environment. The research methods include ^{1}H- and ^{13}C-NMR spectroscopy, mass spectrometry, analytical and preparative chromatography, and a multitude of bio-assays.

Keywords: metabolomics, marine, environmental, allomones, kairomones, pheromones

1. Introduction

Environmental metabolomics is the application of metabolomics for characterising the interactions of organisms with their environment [1]. Marine environmental metabolomics is the application of metabolomics to characterise the interactions of marine organisms with their environment [2].

As Greek scholars claimed, everything is born out of struggle and need. The pressing need of organisms is to adapt to the environment or adapt the environment to the interests of the species. The survival and progress of the different living entities represent a secret driving

force that harnesses metabolites to trigger sophisticated chemical interactions. Although the production of these bio-active substances requires an enormous effort from the organism in terms of energy, the adaptive advantage gained in return is as spectacular as it is necessary for survival.

The progressive degradation of the marine environment on the other hand (animal/plant pests, pollution, etc.) leads us to the need to protect it, and to do so, we have to understand it. Scientists are increasingly clear that our understanding of the marine environment is incomplete without a deeper understanding of its metabolomics. There is even talk of a new science, although its name is not clear. Some call it chemical ecology [3], others ecological bio-chemistry [4], and others consider it part of synecology or biocenotics. According to the principle of science itself "given enough time, only the necessary will survive" [3] so one can expect that the day will come when chemists and biologists will decide on the best name for it.

2. Terminology

Coactones or semiochemicals are the compounds released by an organism which evoke a reaction in another organism of a different or the same species. When these compounds act at a distance, they are called allelochemicals, and their interaction is known as allelopathy [5].

Interactions can be inter-specific or intra-specific, depending on whether they affect individuals of different or the same species, respectively. The chemical factors that affect organisms of different species can in turn be allomones or kairomones, while the chemical factors that affect individuals of the same species can be by auto-toxins, or pheromones [3].

Allomones are semiochemicals that favour the emitter, but not for the receiver. Examples in the marine environment include toxins, digestibility reducing factors, repellents, feeding deterrents, anti-fouling compounds, escape substances, suppressors—antibiotics and cytotoxins [6].

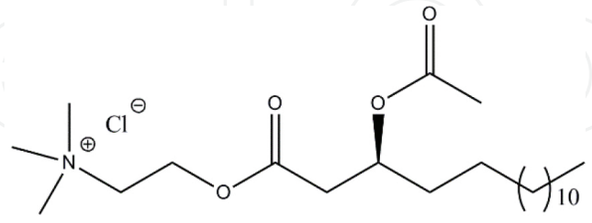
Kairomones are semiochemicals that favour the receiver. Examples from the marine environment include predator attractors or substances that predators use to locate their prey, adaptation inducers, like the spine-development factor in rotifers, warnings signals of danger or toxicity, which benefit the receiver, such as colourants that generate bright colours with characteristic designs on the most toxic animals, growth stimulators, etc. [6].

Pheromones are the semiochemicals released into the environment to influence behaviour or some biological function in the same species. These include sexual/social/warning/territorial marking/trace and communication pheromones [7]. One particular and highly important case of the latter refers to migration pheromones or tele-mediators [8].

3. Toxins

Animals that are mobile or have hard shells or spines are typically not defended by noxious or toxic chemicals. This is the case of the sea urchin or the spiny lobster. Contrarily, the spotted

trunkfish (*Lactophrys bicaudalis*) secretes a colourless toxin from glands on its skin when touched. Predators as large as nurse sharks can die as a result of eating a trunkfish. Methane chemical ionisation gas chromatography-mass spectrometry was used to study pahutoxin (**1**) and choline chloride esters of 16C (**2**), 17C (**3**) and 18C (**4**) fatty acids from Caribbean trunkfish (*Lactophrys triqueter*) toxin (**Figure 1**) [9].



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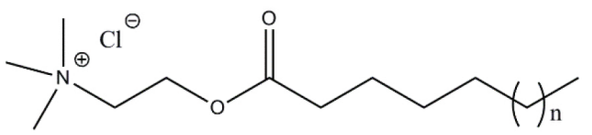

2, n=10
3, n=11
4, n=12

Figure 1. Caribbean trunkfish toxins.

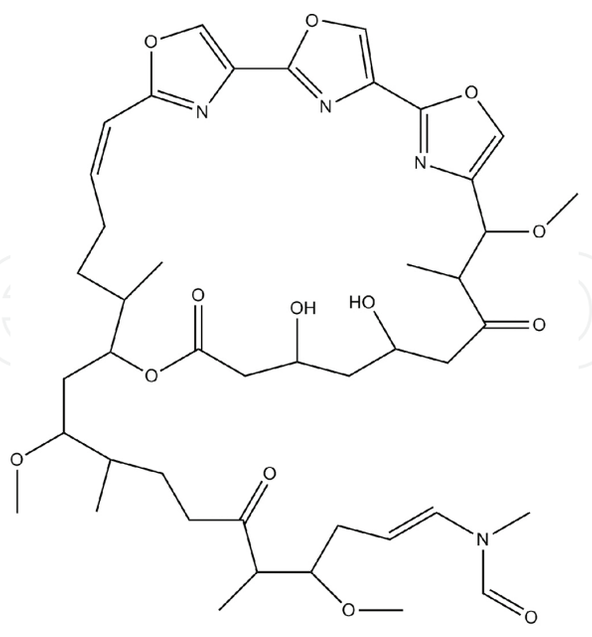

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Figure 2. Jaspisamide A, a nudibranch egg ribbon toxin.

On the other hand, marine snails are strongly protected. Nudibranchs, or sea slugs as they are also known, are an example that is commonly quoted of how organisms with powerful chemical defences have little need for a physical defence like a hard shell to protect them from predators. Nudibranchs usually obtain their chemical defences from the sponges, bryozoans and sea squirts that they eat, but cases have been reported in which these are produced by *de novo* bio-genesis [10]. Nudibranchs also put defensive compounds in their soft egg ribbons. One known example is the case of the jaspisamides A (**5**), B and C, chemical compounds that, while produced by the Okinawan sponge *Jaspis* sp., have been isolated from nudibranch egg masses (**Figure 2**) [11]. These trisoxazole macrolides are cytotoxic and antifungal metabolites initially isolated from the egg ribbons of the *Hexabranchus* nudibranch [12]. They possess a characteristic macrolide portion, comprising three contiguous oxazole units. Trisoxazole macrolides depolymerise F-actin and form a 1:1 complex with G-actin, thereby exhibiting potent toxicity towards eukaryotic cells [13].

4. Repellents/feeding deterrents

Sponges are an abundant group of coral-reef invertebrates that are very chemically rich. Recent studies have shown that many sponge chemicals effectively repel potential predators, and many of the distasteful compounds have now been isolated and structurally characterised [14]. Bioassays are usually run to locate sponges that accumulate repellents. As an experimental methodology, *Preference assays* offer a range of potential prey species to common predators. Species that are avoided by predators frequently have chemical defences. *Caging experiments* also cast light on the role played by predators in habitats, such as coral reefs, where they abound, in eliminating poorly defended prey. Ecologists first identify low preference prey species, before determining whether they are equipped with a chemical defence by adding chemical extracts taken from them to a food item that predators readily eat. This feeding experiment is simple: each fish (from a typical sample of 10–15 individuals) is offered a food pellet containing the extract and an identical food pellet without the extract. The numbers of control and treatment pellets eaten are recorded in tables and graphs and then compared to see whether the fish find the extract distasteful. The assay is then made more realistic from an ecological point of view by placing control and extract-treated foods on the reef where many different species of fish can feed on them.

Soft fleshy seaweeds found where herbivorous fish and invertebrates abound typically deter herbivory by producing distasteful secondary metabolites. *Halimeda* spp. are among the most common seaweeds on tropical reefs where herbivory is intense. These calcified seaweeds produce feeding deterrents. Halimedatrial (**6**) a structurally unprecedented diterpenoid trialdehyde was identified as the major secondary metabolite in six species of these calcareous reef-building alga (**Figure 3**) [15]. In laboratory bioassays, this metabolite is toxic to reef fish, significantly reducing feeding in herbivorous fishes, and has cytotoxic and anti-microbial activities. When plants from most of the *Halimeda* spp. on Guam suffer grinding or crushing damage, they immediately convert halimedatetraacetate, a less-deterrent secondary metabolite, into halimedatrial, a more powerful deterrent to feeding (**6**). The conversion process would

be triggered when fish bite or chew *Halimeda* plants. The process of rapid conversion is known as activation. Extracts from injured plants contained more halimedatrial and were more deterrent towards herbivorous fishes than extracts from control plants. Herbivore-activated defences are common in many families of terrestrial plants, but this was the first example of an activated defence in a marine plant [16].

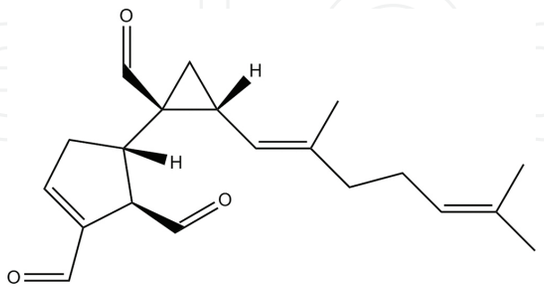

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Figure 3. Halimedatrial.

Gorgonians, a type of soft coral, are close relatives of hard corals, but they do not have a hard calcium carbonate skeleton. Their soft texture seems to make them a target for a range of reef predators, but the many novel compounds they produce act as an effective defence to protect them from these predators. Hence, for example, *Erythropodium caribaeorum* and *Verrucella umbraculum* produce several diterpenes B, **7** (**Figure 4**) [17–20].

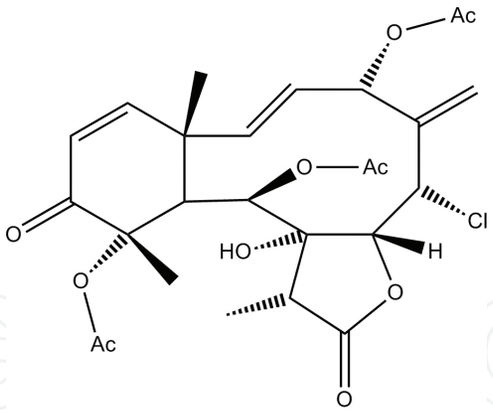

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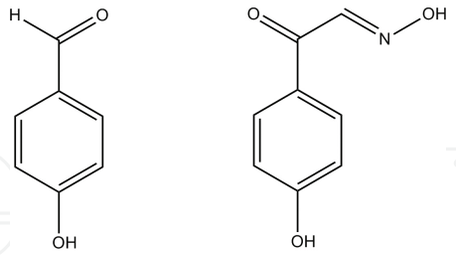
Figure 4. Erythrolide B.

Biomass screening of a new marine-derived strain of *Penicillium roqueforti*, as produced by liquid-state fermentation, led to the identification of the compound 4-hydroxy-benzaldehyde **8** (0.92%) [21]. This natural product is a feeding deterrent factor that restrains the greatest predator of the *Isodictya erinacea* sponge, the *Perknaster fuscus* starfish [22]. Although it is the first time that this has been described in fungi, this substance is structurally related to other previously known metabolites in these organisms that are involved in the shikimic acid

pathway, such as oxime-2-(4-hydroxyphenyl)-2-oxo acetaldehyde **9**, a metabolite previously isolated from *P. olsonii* (**Figure 5**) [23].



8 **9**

Figure 5. Allomones of *Penicillium roqueforti* (**8**) and *Penicillium olsonii* (**9**).

This seems to suggest that the shikimic acid pathway allows fungi to produce their allomones by *de novo* bio-genesis. After these and many other analogous discoveries [2], the idea began to take hold that these allomones are not produced by sponges, but by some symbiotic fungus that lives on them. One fact that supports this idea is that other fungal allomones have been identified among the components of others sponges [24], oysters [25] and algae [26, 27].

5. Anti-fouling compounds

Many sessile marine organisms are surprisingly clean given the abundance of algal spores and invertebrate larvae that could settle and grow on them. Some seaweeds and invertebrates produce compounds that deter or kill larvae and spores attempting to colonise them as a way of keeping clean. Zosteric acid **10** (**Figure 6**), isolated from the young shoots of a seagrass, is an example of a potent natural anti-fouling compound [28, 29].

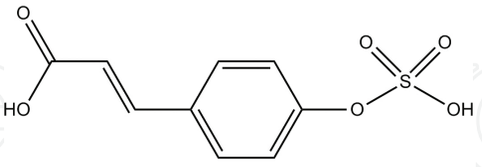


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Figure 6. Zosteric acid.

6. Kairomones

Saponins act as repellents in sea cucumbers, and many species produce these cytotoxic secondary metabolites. Despite the deterrent, they are still colonised by multiple symbiotic

organisms, including the Harlequin crab, *Lissocarcinus orbicularis*, which is one of the most widespread in the Indo-Pacific Ocean. The authors have identified the nature of the molecules secreted by sea cucumbers that attract symbionts for the first time. The kairomones recognised by the crabs are saponins—like holothurin A, **11** (**Figure 7**)—ensuring symbiosis. The success of this symbiosis is due to the ability that crabs showed during evolution to overcome the sea cucumber's chemical defences, with their repellents evolving into powerful attractants [30].

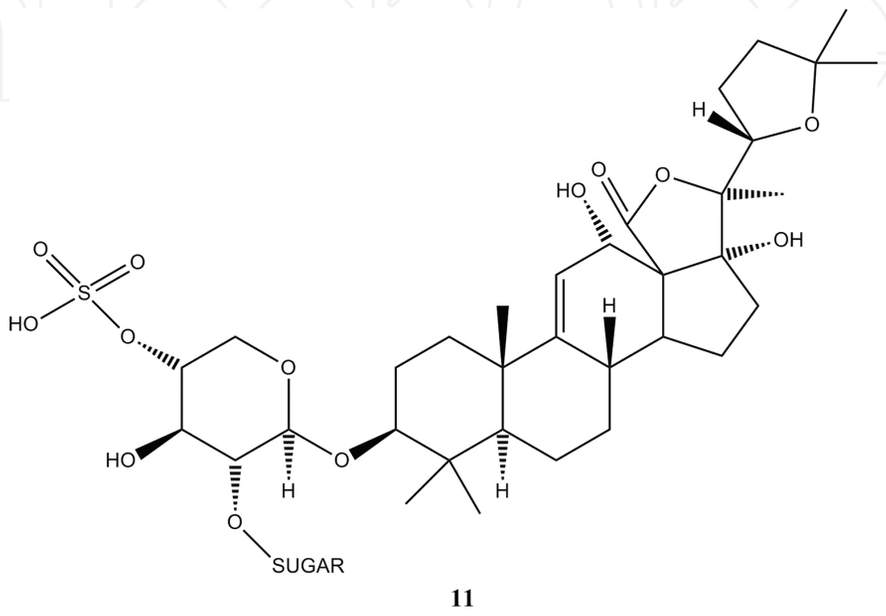

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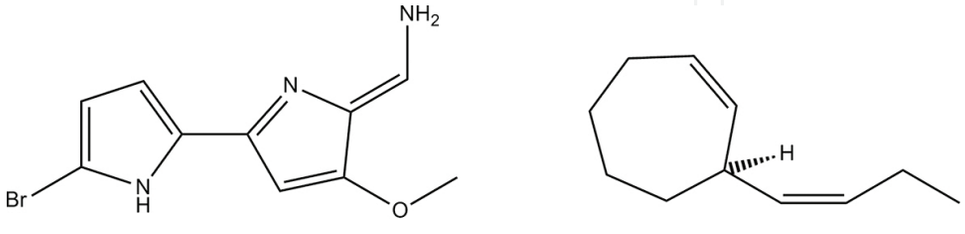
Figure 7. Holothurin A.

7. Pheromones

Pheromones are secreted or excreted chemicals that trigger a social response in members of the same species. In the marine environment, there are some well-known examples that affect behaviour or physiology: alarm pheromones, sex pheromones, etc. There are papers in the field of alarm pheromones reporting how the nudibranchs *Tambje* spp. use alarm pheromones,



12 **13**

Figure 8. Tambjamine B (**12**) and ectocarpene (**13**).

such as tambjamine B, **12** (**Figure 8**), to alert others to a threat [31]. Similarly, there are papers in the field of sex pheromones that report how male copepods can follow a three-dimensional pheromone trail left by a swimming female [32], and male gametes of many brown algae use a pheromone, such as ectocarpene, **13,** (**Figure 8**) to help find a female gamete for fertilisation, a phenomenon known as chemotaxis [33, 34], in fact there are even videos of this on internet.

8. Research methods

Isolating and identifying natural products requires the use of physio-chemical fractionation and purification techniques (**Figure 9**). These products can be explored once enough biological matter is obtained, either from organisms collected directly from their natural habitat or using bio-processes (fermentation, photo-bio-reaction) or marine aquaculture to grow them. The biomass obtained can be frozen, freeze-dried or chemically set in a dissolvent to conserve it.

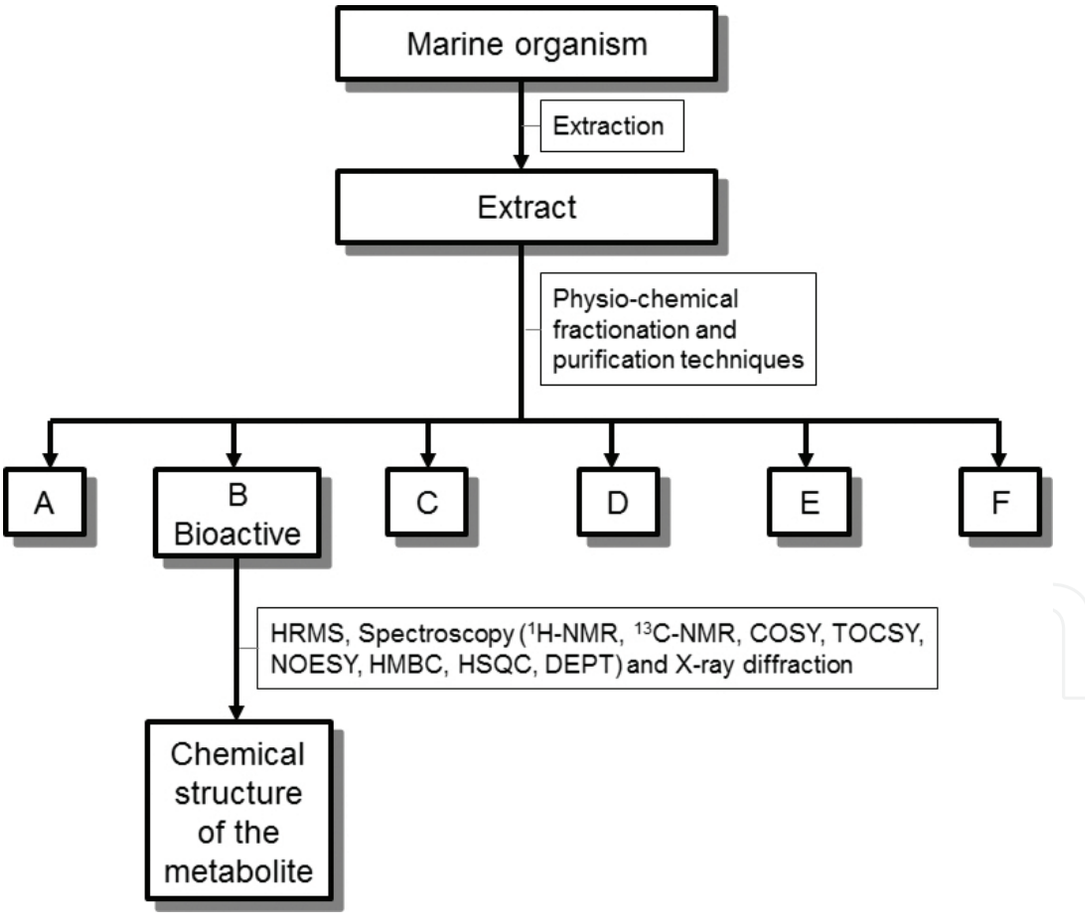


Figure 9. Methodology to identify bioactive substances.

The preliminary separation techniques used in laboratories are performed with adsorption chromatography, using gravity flow columns at low or medium pressure, or using liquid-

liquid partition methods [35]. The latter technique can be applied simply with the help of a decanting funnel, and using one of several variants, it can provide low, medium or high-polarity fractions. This was the case of the fractions obtained from the growth medium used for *P. roqueforti* fungus [36]. Throughout the process to separate the components, there is always the option of using a *Sephadex*-type molecular size exclusion chromatography, which separates families of chemical components of similar molecular size. There are several resins on the market under the common name of the technique (*Gel Filtration*). A very popular one is *LH-20* [37]. Other separation techniques can then be used, such as adsorption chromatography in flash-type columns, either in normal phase or in reverse phase, allowing us to work on a scale of grams, or the case of preparatory plates for thin-layer chromatography (HPTLC/TLC). Finally, the isolated component is crystallised to get pure crystals [38].

Structural elucidation using spectroscopic methods requires perfectly pure substances—crystalline, amorphous or oily. If crystals are successfully obtained, an X-ray diffraction study could then be performed. If not, if the purified component has a non-crystalline structure, then the literature recommends obtaining ^{1}H- and ^{13}C-nuclear magnetic resonance spectra and mass spectra. Once we have the spectra and these are studied, the researcher proposes the structure of the component. This process requires a meticulous revision of the structures previously described in the literature.

Because of the immense number of known products, it is much easier to resort to a powerful database. In these days, many databases on the subject are available to the scientific community online, from the more conventional *Chemical Abstracts Registry*, now part of *Scifinder*, to more specific ones like *MarinLit*® published by the University of Canterbury (New Zealand), which encompasses all the literature published on natural marine products. Mention must also be made of the antibase (*Chemical Concepts*), which deals solely with natural products isolated from micro-organisms and higher fungi.

Once the bibliographic background has been checked and the conclusion has been drawn that the component isolated is new, a more refined structural elucidation has to be carried out. A second high-resolution mass spectrum (HRMS) is required for this to determine the exact molecular formula of both the molecule and the fragments of it that form in the apparatus' injection block. With this information, the two-dimensional structure of the new metabolite isolated can be deduced [39].

Obtaining the three-dimensional structure of molecules requires high-resolution nuclear magnetic resonance techniques. Such spectra provide data on coupling between nuclei that are close together in space and their dihedral angles, using the coupling constants J_{HH}. Complex two-dimensional resonance experiments called COSY, TOCSY, NOESY, HSQC, HMBC, DEPT-90, DEPT-135, etc. provide all the other necessary information [40, 41].

Apart from the techniques indicated above, analytical methods provide important tools in the qualitative and quantitative analysis of substances allowing us to establish their identity and the precise quantity of each component of a given mixture [36]. Instrumental techniques include high-resolution liquid chromatography (HPLC or UHPLC) and gas chromatography

(GC). Once connected to modern mass spectrometry, these tools resolve countless analytical problems (UHPLC-MS/MS or GC-MS) [21].

9. Results and discussion

A new *Paecilomyces variotii* strain was isolated from the marine habitat. The fungal biomass necessary for the chemical study was successfully produced on a laboratory scale. Twenty-eight structural groups were identified from volatile compounds, a large part of which are lipid compounds involved in the fatty acid pathway, fragments from its catabolism, terpenoids and a metabolite from the shikimic acid pathway. Two other non-volatile compounds, olein and ergosterol peroxide, were also isolated and identified using spectroscopy [42].

The screening of the biomass of a new marine-derived strain of *Penicillium roqueforti*, produced by liquid-state fermentation, led to the identification of several volatile organic compounds active in the fatty acid pathway, together with fragments produced as a result of their catabolism, terpenoids, and metabolites from the shikimic acid route. In addition, three non-volatile organic compounds: 9(11)-dehydroergosterol peroxide, 4-hydroxy-benzaldehyde and D-mannitol were isolated and identified using spectroscopy. The results have shown that this fungal strain produces no mycotoxin in the culture conditions applied and thus is useful for industrial applications where high value-added biomolecules are generated [21].

A GC-MS chemical screening on the biomass of a marine protist of the *Schizochytrium* genus enables the authors [43] to identify 24 kinds of organic compounds belonging to the *n*-alkanes, 1-alkenes, 1-alkanols, free fatty acids, methyl and ethyl esters of saturated and unsaturated fatty acids, saturated and unsaturated glycerides, wax esters, sterols, mono-, sesqui- and tri-terpenes. Thus, this organism from the base of the food chain, which accumulates so many nutrients and does not produce toxins, has been proposed as a very interesting specimen for modern functional nutrition.

The chemical constituents of the fermentation broth of the marine-derived fungus *Penicillium roqueforti* were determined. Several volatile organic compounds involved in the fatty acid pathway were identified, along with a terpene and a cyclic dipeptide. Three kinds of non-volatile metabolites were also identified by spectroscopy: alkanes, fatty acids and 1-alkanols. The results showed that the fermented broth of this fungal strain does not produce mycotoxins in the growing conditions used, which is an important factor, given the importance of this species for nutraceuticals [36].

A recent proposal is to study *Spirulina* metabolites (a blue-green alga) as a laboratory practise for bio-organic/bio-chemistry students. They propose that students tackle the separation and analysis of metabolites of nutritional interest using simple thin-layer chromatography (TLC) plates [44].

10. Conclusions and future direction

There are an estimated 22,000 known marine metabolites. Their value as potential drugs for industry—cosmetic, nutraceutical and pharmaceutical—is well documented; in fact in recent years, companies have appeared such as the Spanish company *Pharmamar*, that are trying to sustainably exploit the issue.

However, only a few marine metabolites have been developed commercially. This is perhaps due to the fact that marine environmental metabolomics is scarcely 60 years old, apart from the fact that the major bio-technology and/or pharmaceutical companies have invested very few resources in this field. But irrespective of whether or not marine metabolites have an industrial application, an understanding of their three-dimensional chemical structures and the bio-genetic pathways that living creatures use to produce them is already of great value in the field of marine chemical ecology.

Marine organisms use chemistry for many different purposes. The obvious objectives are to form cellular structures, genetic expression (DNA) and primary metabolism, which guarantees their basic welfare. There is also a secondary metabolism, controlled by enzymes, which is used by organisms to produce, accumulate and disseminate active biological substances into the environment that are essential for the survival of both the organism itself and others of the same or a different species.

For some time, these metabolites were classed under the definition of marine natural products (MNPs), but this definition is defective as it ignores the ecological function or role that they have. That is why more precise words such as allomone, kairomone or pheromone are increasingly applied to them, as we have explained in this chapter.

However, the future is promising, as there is an increasing awareness of the need to study the marine environment in-depth. The proof of this is the creation of four faculties of marine sciences in Spain in recent decades, which means that there is now a bachelor's degree in marine sciences, along with a range of Masters and PhD courses that focus on the sea as their field of study. Subjects like "Chemistry of Marine Natural Products" have suddenly appeared on our syllabuses. At the same time, our students are presenting their degree/master projects or their doctoral theses on marine environmental metabolomics, all of which augers a promising future for this exciting field of science.

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