Opinion

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Coral aquaculture to support drug discovery

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Marine natural products (NP) are unanimously acknowledged as the 'blue gold' in the urgent quest for new pharmaceuticals. Although corals are among the marine organisms with the greatest diversity of secondary metabolites, growing evidence suggest that their symbiotic bacteria produce most of these bioactive metabolites. The *ex hospite* culture of coral symbiotic microbiota is extremely challenging and only limited examples of successful culture exist today. By contrast, *in toto* aquaculture of corals is a commonly applied technology to produce corals for aquaria. Here, we suggest that coral aquaculture could as well be a viable and economically feasible option to produce the biomass required to execute the first steps of the NP-based drug discovery pipeline.

Marine NP and drug discovery

New drug approval in the past decade has been comparatively lower than in previous years [1]. Simultaneously, the development of new drugs from bioactive NP (see Glossary) has been relatively slow [2]. In this urgent quest for new drugs, researchers and policy makers unanimously acknowledged that NP produced by marine organisms could be the way forward [3,4]. Unlike plants, which have been a traditional source of compounds for the treatment of several disorders, marine organisms possess primitive versions of human genetic systems and therefore hold particular promise for the development of new drugs [5].

Marine bioprospecting has been mainly focused on tropical coral reefs [6]. These highly diverse, complex, and fragile ecosystems are the main habitat of most coral species, a group of marine invertebrates that has been an important source of new molecules [7]. In the last decade, the discovery of NP from corals was higher than from sponges, the old-time target group on the quest for new marine NP; this trend suggests a shift in bioprospecting efforts towards a hitherto untapped biodiversity [6].

Currently, the search for new marine NP typically depends on the harvest of wild specimens. This is a major

Glossary

ADMET: acronym for the step of the preclinical trials in the drug discovery pipeline termed Adsorption, Distribution, Metabolism, Excretion, and Toxicity (Box 1).
Agar microdroplets: serially diluted bacterial suspensions are combined with preheated agarose and emulsified into microdroplets. Microdroplets are then cultivated in approximations of the original substrate in chromatography columns equipped with filters to prevent contamination of the media reservoir, allow the passing of free-living cells and retention of microdroplets within the column.
Bioactive natural products (NP): chemical compounds produced by a living organism that display a biological activity similar to a drug on another living organism.
Bioprospecting: the process of discovery of new NP from biological resources.
Captive breeding: the process of breeding animals in controlled environments, also known as *ex situ* aquaculture for aquatic organisms.
Double encapsulation method: novel method to culture microorganisms by encapsulating them within agar spheres, which are then encased in a polymeric membrane (permeable to nutrients and cues from the environment) and incubated in a simulated or natural environment.
Heterologous expression of gene clusters: technique that involves expressing a foreign set of genes and proteins in a cell that does not normally express them.
Hollow-fiber membrane chamber: 48–96 hollow-fibre polyvinylidene fluoride membranes (0.1 μm pore size) connected with injection and sampling devices using syringes to cultivate serially diluted bacterial samples *in situ*.
Holobiont: refers to the coral host and the associated community of microorganisms including endosymbiotic dinoflagellates (popularly known as zooxanthellae), bacteria, archaea, viruses, fungi, and endolithic algae.
Ichip: a central chip made of plastic polyoxymethylene containing 384 miniature chambers (Φ = 1 mm) where bacteria serially diluted in liquid agar-based medium to approximately one bacterium per chamber get captured, stuck between 0.03 μm membranes, and this is maintained by screwing two additional 384 matching well plates. Chips are then immersed in their natural environment for cultivation.
Immunofluorescent viability screening: technique that uses the specificity of fluorescent-labeled antibodies to their antigen and highlights viable microbes.
Mariculture: production of marine organisms in the ocean using natural seawater. It is also known as *in situ* aquaculture.
Micropropagation: the process of multiplying stock material to produce a large number of progeny using tissue explants derived from a single polyp or colonial corals.
Soft corals: marine cnidarians from the order Alcyonacea (suborders Alcyoniina, Calcaxonina, and Protoalcyonaria) that do not produce calcium carbonate skeletons but contain sclerites (spiny skeletal elements) instead.
Stony corals: marine cnidarians from the order Scleractinia that generate a hard skeleton of calcium carbonate in the form of aragonite.

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limitation for the development of new marine drugs [3,5] and commonly entails two major bottlenecks: sustainability and replicability. Sustainability issues are associated with the large amounts of biomass that are usually required for drug discovery. In the past, extensive collection of reef organisms for drug development purposes negatively affected reef communities as a whole and disrupted ecological processes of ecosystems that were already at risk [8]. Replicability is constrained as a result of environmental variability and community level changes to the chemical ecology of the target organisms [9]. Individuals of the same species sampled in different areas, or time frames, may not display the same chemical composition and therefore may not guarantee the supply of the target metabolite (a pitfall commonly termed as 'loss of the source').

Aquaculture of marine invertebrates may overcome these two bottlenecks as animal biomass can be continuously produced using homogenous environmental conditions, a realization that has prompted much researches on aquaculture for drug production. These efforts have primarily focused on sponges, which proved extremely difficult to grow under controlled conditions [10]. Concurrently, a wide array of effective technologies for the culture of corals has been developed over the last decades. Originally developed for the production of corals for aquaria and reef restoration purposes, these technologies may provide a useful baseline for biotechnological production of coral NP. Here, we show that corals have great potential as sources of new NP and that coral aquaculture could be a feasible route for the supply of these NP for drug development.

Bioactivity in corals

Corals are popularly classified as stony or soft (these classifications do not include gorgonians, a related Cnidarian taxon popularly known as sea fans or horny corals). The number of species described in the order Scleractinian (stony corals; Figure 1A) is approximately 1500, whereas about 3300 species have been described within the order Alcyonacea (soft corals; Figure 1B) (Appletans, W. *et al.* (2012) World Register of Marine Species, http://www.marinespecies.org). Nearly 3000 new NP have been isolated from corals in the past two decades, from which several promising leads for drug discovery were identified [11]. However, NP discovery has been uneven among the two coral types because only about 3% were yielded from stony corals [12]. It is possible that the larger chemical diversity currently known from soft corals is associated with biased bioprospecting efforts: screening efforts have included approximately 30 species of stony versus approximately 300 of soft corals [12]. Intriguingly, the percentage of bioactive bacterial isolates associated with stony corals is higher than with soft corals [13].

Coral tissues, skeleton, and mucus layer (Figure 2) contain dense and diverse populations of bacteria and archaea (100–1000-fold more than in seawater) that confer various benefits to their host, such as support to coral nutrition and, most importantly, protection against pathogens. The bioactivity displayed by these symbiotic microorganisms is diverse and has provided promising leads for drug discovery (e.g., cytotoxicity, antibiotic activity, and activity against human cancer cell lines) [11]. These findings are in line with the growing awareness that

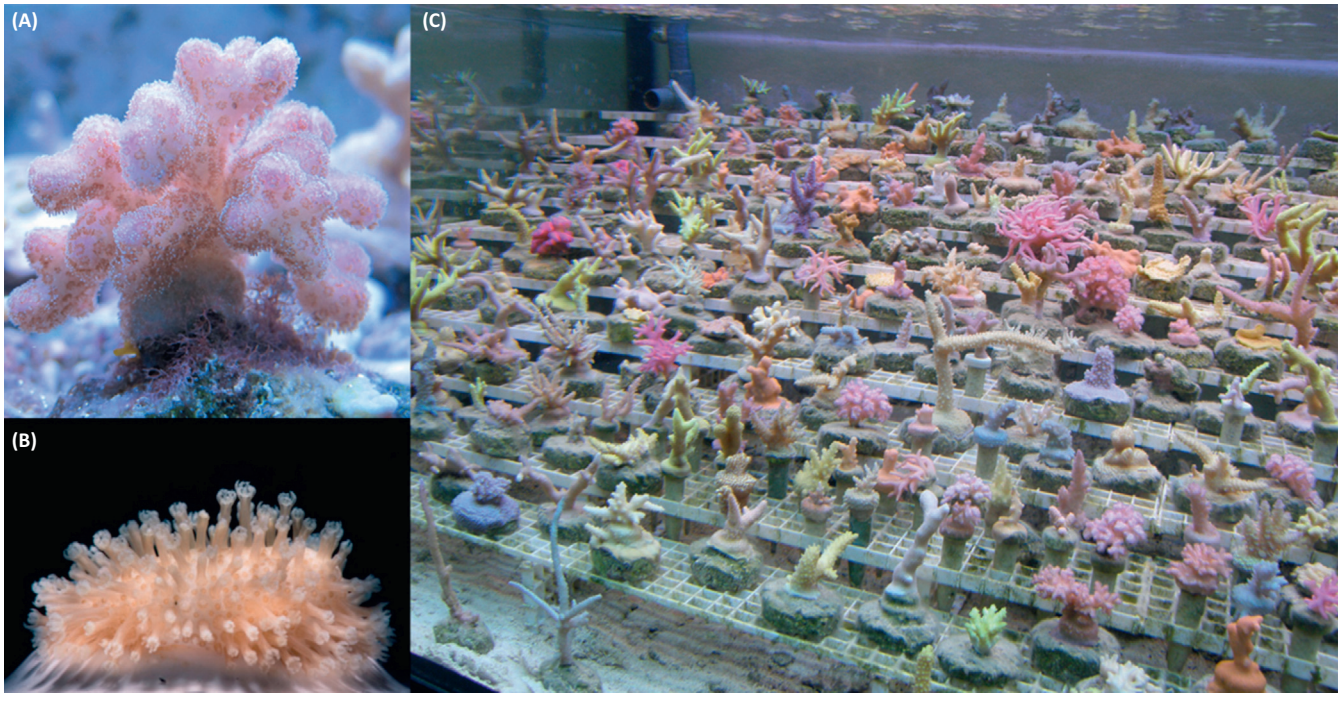

Figure 1. **(A)** Stony coral *Stylophora pistillata* and **(B)** soft coral *Sarcophyton glaucum*. Stony corals generate a hard skeleton whereas soft corals have soft tissues containing spiny skeletal elements. **(C)** Culture of stony corals in a grow-out tank for *ex situ* coral aquaculture is also shown (copyright: Maternidade do Coral Lda., Portugal).

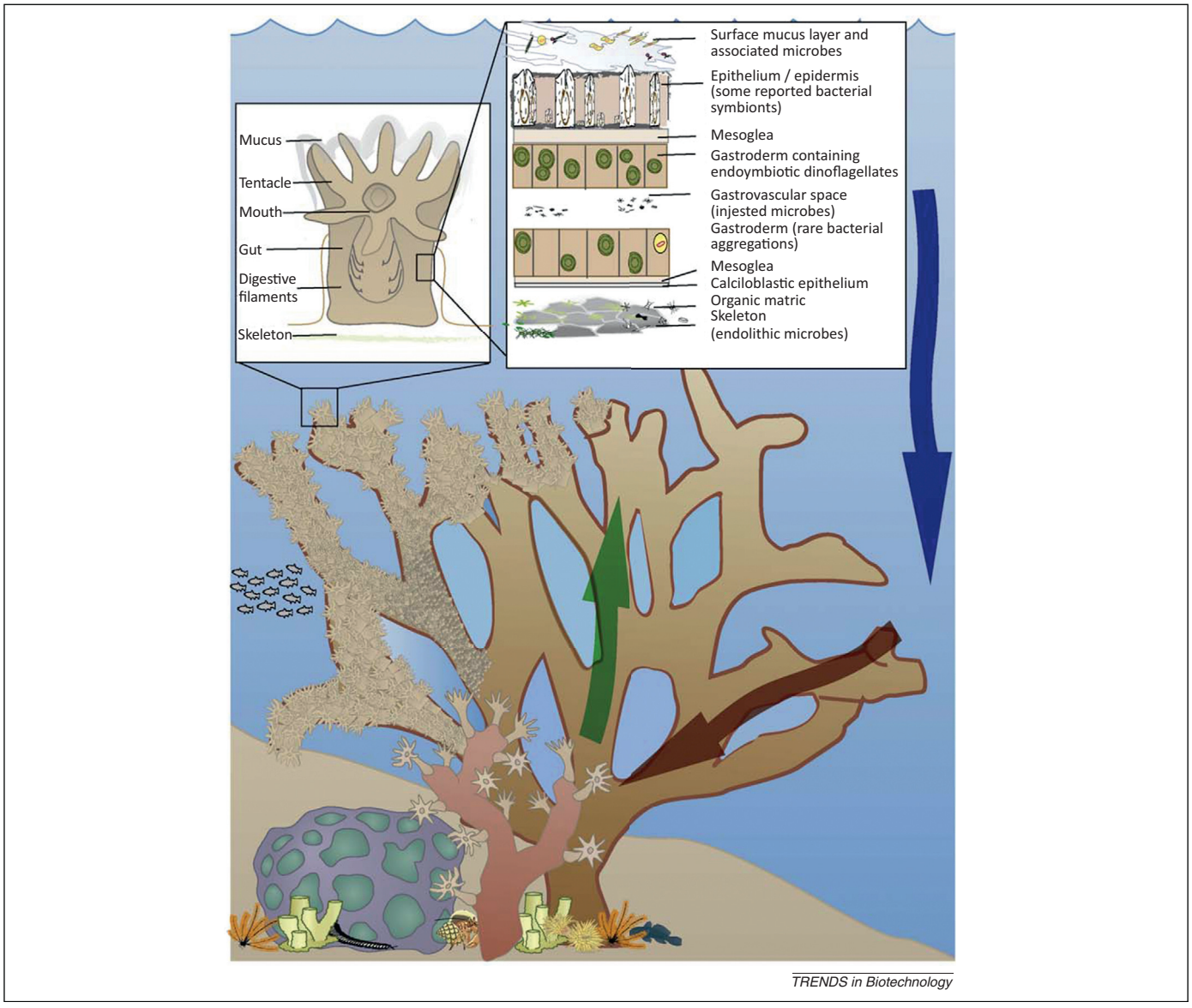

Figure 2. The complexity of the coral reef structure results in many microhabitats. The host structure provides various microhabitats for microbial colonization within the coral colony, coral polyps, and coral tissues. Each microbial compartment on the reef is influenced by physical and biological environmental conditions that vary in time and space. Environmental variability through the water column (dark blue arrow) is related to reef depth and reefal position. Biological variability along the branch axis (brown arrow) is related to environmental (light and water flow) and biological factors (colony openness, endosymbiotic dinoflagellate density, respiration, and photosynthesis). Variability along the branch apical to the basal axes (green arrow) is related to the variable 3D structure of host, polyp, density, and niche environments for microbial colonization and biofilm formation. Reproduced, with permission, from [14].

NP discovered in several marine invertebrates are actually produced by their symbiotic microbiota [1]. Nevertheless, it is our opinion that the potential of stony corals and their microbial consortia has been neglected. We suggest that NP research should be expanded towards stony corals, with a particular focus on their unique microbiota.

Culturing coral microbiota for drug discovery

The complexity and variability of the marine environment, along with the range of microhabitats that corals are composed of (Figure 2), have been major drivers promoting the diversification of coral microbial symbionts and their functional pathways [14]. This functional diversity offers great potential for drug discovery [15]. However, limited amounts of biomass of individual microbial strains can generally be isolated from corals because of the relatively small quantities of tissue and mucus that can be collected and the relatively small proportion of the microbial biomass that these individual strains may represent. As a result, there is usually not enough biomass available per strain to perform the drug discovery process. Additional biomass is usually produced through traditional culture methods, with pure cultures being isolated by serial plating on solid medium or serial dilutions in liquid medium and later screened for NP [16]. The use of such culture-dependent methods limits the true potential for the discovery of new drugs. The main reasons for this constraint is that only 0.001–1% of total microbial diversity has been successfully cultured and the fraction that is successfully isolated may not be representative of the environment it was isolated from [16]. These shortfalls may be due to: (i) opportunistic microbes that out-compete slow-growing microorganisms, (ii) the inability to reproduce environmental or nutritional conditions using traditional culture

media, (iii) insufficient time allowed for the growth of isolates, and (iv) the suppression of complex networks of cell-to-cell interactions (e.g., signaling and exchange of metabolites) [16,17].

Recent advances in culture methods (e.g., using diverse culture media, conditions, and incubation periods) have increased the cultivable fraction of the total microbial diversity [18]. Novel methods typically attempt to simulate natural environmental conditions and range from community culture to *in situ* culture techniques [18]. *In situ* culture techniques can be grouped into two broad types: encapsulation (agar microdroplets [19] and double encapsulation [20,21] methods) and diffusion chambers (e.g., Ichip [22], hollow-fibre membrane chamber [23]). Such *in situ* techniques are among the most promising approaches that may help to increase the proportion of culturable microbes as they allow interactions from cell-to-cell and between isolates and their native environment, and have been successfully used to isolate numerous unknown microorganisms [20,22]. However, despite recent advances in the cultivation of microorganisms, most of the microbial diversity remains untapped. Culture-independent methods, although not without caveats, have helped researchers filling this knowledge gap by: (i) improving the cultivation process (e.g., by immunofluorescent viability screening) [18], (ii) heterologous expression of gene clusters, including silent gene clusters (i.e., gene clusters that are not expressed in the organism from which they originate) of new bioactive pathways from environmental DNA (eDNA) cloned in appropriate vectors and hosts [24–26], or (iii) providing information on particular functions or proteins by screening entire metagenomic sequences obtained from eDNA [15].

Considering the high adaptability of coral symbionts to their environment [14], and the techniques now available to the drug discovery process [15,18], it is our opinion that coral aquaculture could be performed under a variety of conditions so as to trigger targeted responses in the microbial community. Ultimately, this approach may be used to increase the biomass of target metabolites and the consequent chances of successfully isolating promising NP.

Coral aquaculture

Current coral culture methods include mariculture (*in situ*) and captive breeding (*ex situ*; Figure 1C) [27]. Micropropagation through tissue explants is also a versatile *ex situ* approach [28]. Although *ex situ* practices occur worldwide, because a proximate source of natural seawater is not mandatory [29–31], *in situ* coral aquaculture has been observed in coastal habitats of tropical regions, such as the Caribbean [32], Red Sea [33], Western Indian Ocean [34], and Indo-Pacific [35]. Both *in situ* and *ex situ* culture methods have advantages and disadvantages [36]. *In situ* aquaculture represents a low cost and simple methodology that entirely relies on the environment to supply essential ingredients for coral growth, such as light and nutrients. The ability to manipulate culture conditions *in situ* is fairly limited and the cultures are vulnerable to environmental stresses such as predation, thermal anomalies, and eutrophication. *Ex situ* coral culture, although more expensive, enables the manipulation of abiotic and biotic factors that maximize coral production and *ex situ* grown corals show better recovery after fragmentation [37]. It is important to note that although *ex situ* aquaculture allows the control of environmental conditions, it may change the holobiont composition and cause either the loss of important microorganisms present in nature [38], or involuntarily provide the conditions for growing microorganisms previously undiscovered from field collections.

Although coral aquaculture is an established technology that allows the production of monoclonal organisms [37,39] it has never been applied for drug discovery research from NP isolated from hard corals. This is probably related to the low tissue to skeleton ration in these corals. Soft tissue accounts for less than 5% of the hard coral wet weight [40], which implies that substantial amounts of hard coral have to be obtained to perform the first steps in the drug discovery process. Hence, high-throughput screening for NP on stony corals will require a substantial initial investment in aquaculture in comparison to soft-bodied animals such as soft corals and sponges, for which initial screening for NP can easily be done using small amounts of biological materials obtained through wild harvest.

To reduce these initial investments in aquaculture, we suggest that research on coral culture should focus on methods to increase the amount of soft tissue around the coral skeleton. For example, feeding with zooplankton increases the overall growth of hard corals, and specifically stimulates tissue growth, protein synthesis, and fat storage [41]. Selective breeding could also be used to optimize coral aquaculture through the selection of genotypes that combine high biomass productivity and high NP synthesis. Such practices, however, would only be feasible through *ex situ* culture because the corals are kept in an enclosed system and fully monitored by researchers. Possibilities to induce NP formation prior to harvesting should be investigated as well.

Because our current understanding of the stimuli that cause the coral holobiont to synthesize certain NP is still very limited, we suggest that the manipulation of environmental factors could be used to maximize metabolite production (Box 2). As an example, light conditions can be manipulated to maximize growth rates and metabolite production of the soft coral *Sinularia flexibilis* [42]. Key abiotic factors known to affect coral growth, such as nutrient availability and water flow, may also influence metabolite production. Further, biotic factors, such as interspecific competition, predation, and co-culture conditions should also be tested because these interactions may also be chemically mediated [43].

Replicability of NP production could be achieved through maintaining stable, nonvarying environmental conditions in the culture system, which are paramount to maintain a stable community of bioactive metabolite-producing symbiotic microorganisms in the invertebrate host. The ability to optimize and maintain conditions for NP production in cultured corals is best achieved in controllable *ex situ* culture systems. Therefore, we suggest that the potential of *ex situ* coral culture to supply NP for drug discovery is unquestionably favored compared to mariculture. Ultimately, the identification and manipulation of environmental stressors triggering mucus production or bacterial growth [44,45]

Box 1. Theoretical drug discovery and development pipeline

0. **Prediscovery**. Researchers gain in-depth knowledge on the disease to be treated (e.g., how genes are altered, how they affect proteins and, ultimately, how the disease affects the patient).
1. **Drug Discovery**
   - 1.1. *Basic research*: target identification and validation.
   - 1.2. *Lead discovery*: search for a molecule that can act on the selected target and change the course of the disease being addressed; the lead compound can be identified from nature, created in the laboratory (*de novo*), or synthetized by genetically engineered organisms.
2. **Preclinical Trials**. Researchers run a series of *in vitro* and *in vivo* tests with an optimized compound to clarify how the candidate drug works and its safety profile. The different amount of metabolite biomass for each step of these trials and its estimated costs are detailed in Table I. These trials include the following steps:
   - 2.1. *Primary screening of crude extracts on validated pharmaceutical targets*
   - 2.2. *Isolation of the active molecules through bioassay-guided fractionation and biochemical characterization of the active molecules*
   - 2.3. *Hit-to-lead development including optimization of the physico-chemical properties of the active molecule*
   - 2.4. *ADMET*
   - 2.5. *In vivo animal tests, which comprise tests of both toxicity and efficacy*
3. **Clinical Trials**
   - 3.1. *Phase I*: the drug is first used in humans (in a small sample of 20–40 patients) to test whether the drug is safe.
   - 3.2. *Phase II*: the effectiveness of the drug is evaluated in humans carrying the disease (100–500 patients) to determine if the drug is working by the mechanism hypothesized and if it does improve the condition of the patients.
   - 3.3. *Phase III*: the drug is tested in a higher number of patients (1000–5000) to evaluate the statistical significance of recorded results on the drug's safety and efficacy, as well as the benefit–risk trade-off.
4. **Launch**
   - 4.1. *Submission to launch*: an application is issued to the legal authority in the country of operation, requesting the approval to market the drug as a new medicine.
   - 4.2. *Large scale manufacturing and launching*: after approval, the new medicine undergoes large-scale manufacturing. The researchers continue to monitor its effects, as 'Phase IV' trials may be required to evaluate long-term safety on patients.

Table I. Drug discovery costs for two bioactive compound concentrations

			Scenario 1 (1000 mg/kg)				Scenario 2 (10 mg/kg)			
Development step	**Screening**	**Isolation**	**Hit to lead**	**ADMET**	**Animal testing**	**Total cost**	**Hit to lead**	**ADMET**	**Animal testing**	**Total cost**
Amount of pure compound needed (mg)	–	–	50	50	2000	–	50	50	2000	–
Amount of coral needed (kg)	0.5	50	0.05	0.05	2	–	5	5	200	–
Costs per product for production of biomaterials (€)	58	5750	6	6	230	6049	575	575	23 000	29 958
Costs per product for product development (€)	10	10 000	50 000	50 000	60 000	170 010	50 000	50 000	60 000	170 010
Percentage of total costs represented by coral culture at each development step	85.19	36.51	0.01	0.01	0.38	3.44	1.14	1.14	27.71	14.98

may also lead to new practices, such as the collection of the coral mucus and its microbial metabolites without destroying the coral host, that is, the nonlethal harvest.

Economic feasibility

To assess the economic feasibility of coral aquaculture for drug discovery, we performed a scenario analysis using anticancer research as an example. A useful case study in this respect has been published on the production of flexibilide, the main bioactive compound in the soft coral *Sinularia*. This compound is present in the coral tissue at a concentration of 0.5 g/kg coral wet weight. One kg of flexibilide can be produced for €5 million [46], which constitutes only 0.5% of the average sales value of an anticancer drug (~€1 billion/kg) [47]. If a similar product concentration in the tissue is assumed for montiporyne A, the most potent bioactive compound in the tissue of the stony coral *Montipora* sp. [48], a comparable calculation can be made for the feasibility of stony coral culture. The specific growth rate of *Montipora* sp. is 0.9%/day [49]. Production costs for a coral growing at this rate can be estimated at €115/kg [37]. Because in *Montipora* sp. the soft tissue only accounts for approximately 5% of the total wet weight [40], the costs to produce biomass for 1 kg of pure metabolite were calculated as €115/kg × 20 g wet weight/g tissue × 2000 g tissue/g compound, resulting in a total cost of €4.6 million to produce biomass for 1 kg of montiporyne A, which accounts for 0.46% of its potential sales value. However, it must be noted that information on metabolite concentrations in stony corals is very scarce. The only relevant paper we found reported a concentration of aplysinopsin in *Tubastrea aurea* of 1.20 mg/g of wet weight [50]. Future studies on NP in stony corals should attempt to include such data. In addition, the specific variation of the ratio between total wet weight (including the skeleton) and tissue wet weight in stony corals is largely variable (5% for *Montipora verrucosa*, 0.2% for *Pocillopora damicornis*, and 0.5% for *Porites lobata* [40]).

Notwithstanding these favorable figures, the current practice among pharmaceutical companies is to start clinical development of natural drug leads only after successful development of a chemical synthesis route for the natural molecule [10]. Therefore, we limited our further analysis to the application of coral aquaculture to produce metabolite for preclinical drug development (Box 1).

We calculated the costs for culture-based preclinical trials of one successful anticancer drug lead obtained from a coral (see Table I in Box 1). Hereby, it was taken into consideration that the completion of each consecutive development step requires increasing amounts of metabolite

biomass per species tested. It was also considered that the amounts of materials needed for the step in the drug discovery pipeline usually known as ADMET (Adsorption, Distribution, Metabolism, Excretion, and Toxicity) and animal testing depend on both the potency of the compound and its abundance in the biological materials. Concentrations reported for bioactive compounds in invertebrates vary largely. The concentration of bioactive compounds in sponges ranges from 300 μg/kg of wet biomass to an exceptional 10 g/kg of wet biomass [51], but no such information is available for corals. Low concentration metabolites will only be detected by bioactivity assays if they are extremely potent. By contrast, if that is the case, lower quantities of these compounds will be needed for preclinical development.

The average amounts of pure compounds needed for hit-to-lead development, ADMET, and *in vivo* testing were fixed at 50, 50, and 2000 mg, respectively (see Table I in Box 1). Two scenarios regarding the product concentration in tissues were considered: (i) high concentration: 1 g/kg; and (ii) low concentration: 10 mg/kg. These scenarios also simulate the situation for highly potent metabolites in low concentrations. The culture of soft and stony corals requires similar technology, and specific growth rates for both coral types are in the same range [37,46]. Therefore, production costs were estimated at approximately €115/kg for both coral types [37]. Costs for coral aquaculture (actual costs and percentage of total development costs) were calculated for each of the five development steps and varied between 0.01 and 27.71% of the costs involved in each step, apart from screening and isolation (see Table I in Box 1). The estimated costs of coral aquaculture for metabolite production represent 3.44 and 14.98% of the total cost of the drug discovery process for a high and low concentrated compound, respectively. Again, it should be noted that the soft tissue of stony corals may only account for 0.2–5% of the total wet weight (in the case study shown in Table I in Box 1, we use 1%). Hence, the low concentration scenario in Table I in Box 1 may represent a high concentration scenario for stony corals.

Concluding remarks

One of the bottlenecks affecting marine drug discovery is the availability of sufficient material for complete biological and chemical evaluation and eventual production [5,52]. We suggest that coral aquaculture offers a strong potential for both the discovery of highly diverse metabolites and the production of large amounts of bacterial biomass. Although coral aquaculture techniques are already available, it is our opinion that they should be optimized for bioprospecting, genotype selection, and metabolite production (Box 2). Furthermore, the calculated costs for metabolite biomass production through coral aquaculture are relatively low, representing only between 3.5 and 15.0% of the total preclinical development costs (see Table I in Box 1). We therefore conclude that the 'supply issue' commonly reported for marine drug discovery may not apply to the drug development process of bioactive NP originating from corals. Although this underlines the untapped potential that coral aquaculture represents for drug discovery, there are yet outstanding questions (summarized in Box 2) that remain to be answered in order to advance this field.

Box 2. Outstanding questions

- **Who produces the metabolite?** Although it is increasingly recognized that metabolites are produced by symbiotic bacteria and not by the invertebrate host, accurate information for most of the marine natural products from invertebrates currently known is still missing.
- **What drives metabolite production?** The specific environmental factors that trigger metabolite production by the invertebrate host or its endosymbiotic microorganisms are still poorly understood.
- **How do we maximize coral tissue growth and metabolite production?** Although optimized methods to maximize coral tissue growth and metabolite production are needed, little work has been done to establish such culture protocols because most previous culture efforts were aimed at providing corals for the ornamental industry. Future studies should include selective breeding.

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