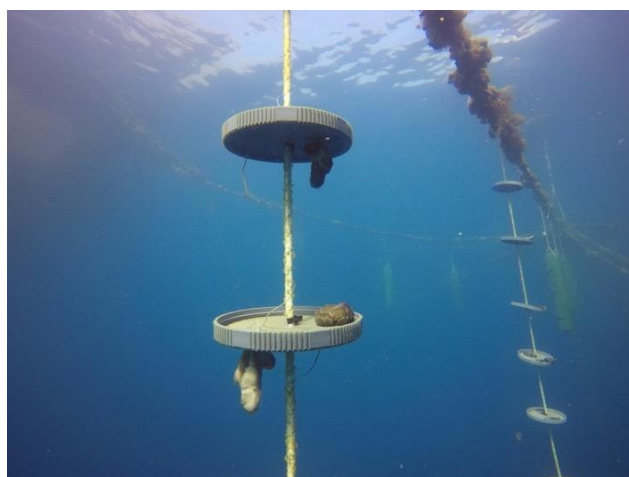TECHNICAL UNIVERSITY OF CRETE

SCHOOL OF CHEMICAL AND ENVIRONMENTAL ENGINEERING

Improvement of water quality and bioproduction of high added-value products from the integrated cultivation of marine sponges in fish aquaculture systems

Βελτίωση ποιότητας υδάτων και βιοπαραγωγή φυσικών προϊόντων υψηλής προστιθέμενης αξίας από τη συγκαλλιέργεια θαλάσσιων σπόγγων σε ιχθυοκαλλιεργητικές μονάδες

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"Και τα παρτάλια οι σκέψεις μας, πειρατική σημαία.

Όλα στραβά γινήκανε, και όλα είναι ωραία."

– Quote from «Σιμούν», a song by Thanasis Papakonstantinou –

Despoina Varamogianni-Mamatsi

Table of Contents

Contents

List of Figures

[13]

[14]

[16]

List of Tables

Abstract

Mariculture, typically supported by marine cage systems, constitutes a significant economic sector for several countries across the world and plays an increasing role in fish supply. During the last decades, a pronounced activity has been reported in the Mediterranean Sea, with Greece being one of the major producers of seabream and seabass finfish worldwide. With the gradual increase of global population, and consequently, the demands for fish and fish-related products, mariculture systems are expected to face an even more unparalleled growth. To enhance productivity, fish farmers currently use larger densities of feed and chemicals that are necessary to ensure the health and growth within the culture. A significant portion of these administered substances remains freely dissolved in the water column, or can either end up as particles in marine sediments, leading to sound threats for aquatic and human life. Unconsumed fish feeds, accompanied by massive excretions of fish faeces and other metabolic waste products are the main cause for nutrient enrichment of water and sediments near fish farms. On the other hand, aggressive administration of therapeutants, such as antibiotics, parasiticides, anesthetics, disinfectants and hormones, or antifouling booster biocides that are used to prevent fouling in submerged production structures, can potentially lead to high organic loadings, chemical pollution and bioaccumulation of contaminants in fish stocks or other marine life. Other pollutants that may occur in fish farm environments include heavy metals and polycyclic aromatic hydrocarbons (PAHs), discharged from offshore and coastal anthropogenic activities. As a result, nutrient and organic pollution can provoke a series of adverse effects, including water deterioration, hypoxia, biological pollution, eutrophication and habitat destruction, with severe economic losses for fish farmers.

To alleviate aquaculture pollution through eco-sustainable and socially acceptable manners, integrated multi-trophic aquaculture (IMTA) systems emerge as feasible solution. Such systems combine fish aquaculture with rearing of secondary extractive species, in a way that nutrients and fish wastes are recycled to promote growth of the co-cultured organisms, while the latter present an additional monetary benefit to the enterprise, through their own economic value. Over the years, many organisms have been documented as promising IMTA candidates. Among them, marine sponges stand as excellent candidates, in light of their innate filter-feeding properties and their capability to retain a variety of organic waterborne substances, from particulate to dissolved forms. Besides their high bioremediation capacity for an array of biological and organic pollutants, their biomass is considered a "gold mine", given their applicability in various biotechnological fields, from bath sponges to bioactive compounds resources. However promising, only few existing studies have conceived the "sponge-driven bioremediation/bioproduction" concept in aquacultures worldwide, with Greece being practically inactive. Considering the thriving aquaculture sector of Greece and the vast diversity of sponges

existing in Aegean Sea, it is essential to gain a better view of the integration potential of native sponge species in fish farms.

The aim of this dissertation is to (a) assess the bioremediation capacity of Mediterranean sponges through controlled laboratory experiments involving typical aquaculture biological and chemical pollutants and to (b) explore the valorization potential of candidate sponges through the targeted analysis of known metabolites and bioactivity screening. The cleanup experiments were employed for the four ubiquitous Mediterranean species *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*, which were distinguished for their high natural abundance, variability in body form and satisfactory performance under rearing conditions. The *in vitro* capability to mitigate aquaculture-related biological pollution was assessed for three phytoplanktonic cells, in an attempt to simulate sponges' response to eutrophic algal blooms. The tested microalgal substrates were exhibiting different size/motility characteristics and belonged to genera of *Nannochloropsis* sp. (~3.2 μm, nonmotile), *Isochrysis* sp. (~3.8 μm, motile), and *Phaeodactylum* (~21.7 μm, nonmotile). Sponge explants were exposed for 7 h to microalgae-enriched seawater under different experimental setups. First, it was shown that all four candidates were capable of retaining their cleanup capacity across a span of four or five successive days. When exposed to varying cell concentrations approximating the gradient from oligotrophic to highly eutrophic systems, sponges maintained their optimal filtering activity. The same argument was partially true when exposed to different illumination conditions. Different feeding preferences were observed among sponge species for microalgal substrates with distinct size and motility traits. Overall, the study sponges exhibited a wide range of retention efficiencies for the different phytoplankton cells, with the highest average values found for the species *A. oroides* (70%) and *S. foetidus* (44%).

The same candidate sponges were further investigated for their ability to remove typical aquaculture-related dissolved organic pollutants from seawater. This series of *in vitro* experiments involved the exposure to (i) individual chemicals belonging to antibiotics (i.e., oxytetracycline), antifouling biocides (i.e., diuron and Irgarol 1051) and polycyclic aromatic hydrocarbons (i.e., 2,6-dimethylnapththalene, phenanthrene), as well as (ii) complex organic mixtures, involving filtrates of fish feed and excreta. All sponges were capable of uptaking the various organic substances, by exhibiting a pronounced preference for lipophilic pollutants. To further support this argument, a strong positive correlation was revealed between sponge's cleanup capacity and substrate hydrophobicity. Among the examined sponges, *A. oroides* demonstrated the greatest filtering performance across an array of dissolved organic substances, with the highest rates reported for the highly lipophilic pollutants. At a later stage, we intended to shed light on the processes dictating dissolved organic matter (DOM) removal by sponges. In all studied species, active pumping was found to play a prominent role in the assimilation of dissolved pollutants. This was explained by the much faster rates exhibited from this mechanism

compared to the values derived for passive adsorption of pollutants onto dead sponges' surface. Finally, the uptaken pollutants were shown to be strongly retained by sponges and they were hardly released back to seawater as a result of desorption or sponge excretory mechanisms.

The final criterion to assess the suitability of the selected sponge species as components of integrated aquaculture, was the valorization potential of their cultivated biomass. For this reason, the two best-performing species in terms of bioremediation capacity, namely *A. oroides* and *S. foetidus*, were selected and further analyzed for targeted known biomolecules and bioactivities. This assessment was performed in specimens subjected to rearing in direct proximity to a fish aquaculture for more than a year, as well as to conspecifics from adjacent sponge populations, to estimate the effect of farming on the natural bioproduction potential. An array of natural products was identified in the extracts of both farmed and wild sponges, with the majority of them belonging to alkaloids, benzenoids, indoles, lipids and polyketides. Metabolomic analysis revealed also species-specific chemical patterns, with *A. oroides* and *S. foetidus* extracts dominated by alkaloids and lipids, respectively. More importantly, farmed and wild explants of each species demonstrated similar chemical fingerprints, with the majority of the metabolites showing modest differences in their content and on a sponge mass-normalized basis. Furthermore, farmed sponge extracts presented similar or slightly lower antibacterial activity against methicillin-resistant *Staphylococcus aureus*, compared to the extracts resulting from wild sponges. Anticancer assays against human colorectal carcinoma cells (HCT-116) revealed marginally active extracts from both wild and farmed *S. foetidus* populations.

As shown, sponges, along with other marine organisms, harbor a wealth of bioactive compounds with potential biotechnological applications in drug, cosmetics and food industry. In this context, a comprehensive overview of the most popular bioassays used in natural products biodiscovery is provided, along with their properties and practical considerations for their selection. A particular focus is given on the exploration of antimicrobial, antibiofilm, cytotoxic, antiviral, antioxidant and anti-ageing potential of the associated extracts. Furthermore, quality control procedures and flowlines involved in bioactivity-guided identification and purification of compounds are introduced. The review concludes with an application-oriented study focusing on drug discovery, dietary supplements, and cosmetics, the industrial pipelines most commonly supplemented with marine-derived natural products. Safety and regulatory issues that are critical to the transition of substances to a higher stage of development are also presented, along with an outlook on trends and future developments.

Altogether, sponge farming can be regarded as a promising source of high added-value natural products, in addition to an effective cleanup technology for different types of aquaculture pollution. Based upon the bioremediation/bioproduction potential of the different sponge species investigated

throughout this dissertation, both *A. oroides* and *S. foetidus* are proposed as the best candidates for future large-scale farming adjacent to fish farms.

Περίληψη

Η θαλάσσια καλλιέργεια, υποστηριζόμενη συνήθως από συστήματα θαλάσσιων κλωβών, αποτελεί σημαντική οικονομική δραστηριότητα για πολλές χώρες σε όλο τον κόσμο και διαδραματίζει αυξανόμενο ρόλο στον εφοδιασμό ψαριών. Τις τελευταίες δεκαετίες, έχει αναφερθεί έντονη δραστηριότητα στη Μεσόγειο Θάλασσα, με την Ελλάδα να είναι ένας από τους σημαντικότερους παραγωγούς τσιπούρας και λαβρακιού παγκοσμίως. Με τη σταδιακή αύξηση του παγκόσμιου πληθυσμού, και κατά συνέπεια, των διατροφικών απαιτήσεων σε ψάρια και προϊόντων ιχθύων, τα συστήματα θαλάσσιας καλλιέργειας αναμένονται να παρουσιάσουν ακόμη μεγαλύτερη ανάπτυξη. Για την ενίσχυση της παραγωγικότητας, οι ιχθυοκαλλιεργητές χρησιμοποιούν επί του παρόντος μεγαλύτερες ποσότητες ιχθυοτροφών και χημικών ουσιών που είναι απαραίτητες για τη διασφάλιση της υγείας και την ανάπτυξη εντός της καλλιέργειας. Ένα σημαντικό μέρος αυτών των χορηγούμενων ουσιών παραμένει διαλυμένο στη στήλη του νερού ή μπορεί είτε να καταλήξει ως σωματίδια σε θαλάσσια ιζήματα, εγκυμονώντας σημαντικούς κινδύνους για την θαλάσσια και την ανθρώπινη ζωή. Οι μη καταναλωθείσες ιχθυοτροφές σε συνδυασμό με τις μαζικές εκκρίσεις περιττωμάτων και άλλων μεταβολικών αποβλήτων των ψαριών, είναι η κύρια αιτία για τον εμπλουτισμό των υδάτων και των ιζημάτων με θρεπτικά συστατικά κοντά σε ιχθυοτροφεία. Από την άλλη πλευρά, η χορήγηση θεραπευτικών ουσιών, όπως αντιβιοτικά, παρασιτοκτόνα, αναισθητικά, απολυμαντικά και ορμόνες, καθώς και η χρήση υφαλοχρωμάτων ενισχυμένων με βιοκτόνα που χρησιμοποιούνται για την πρόληψη της βιοεπικάθισης στις βυθισμένες δομές παραγωγής, μπορεί δυνητικά να οδηγήσει σε υψηλά οργανικά φορτία, χημική ρύπανση και βιοσυσσώρευση ρύπων στα εκτρεφόμενα ψάρια ή άλλους θαλάσσιους οργανισμούς που ενδιαιτούν στην ευρύτερη περιοχή. Άλλες χημικές εισροές περιλαμβάνουν βαρέα μέταλλα και πολυκυκλικούς αρωματικούς υδρογονάνθρακες (ΠΑΥ) που προέρχονται από ανθρωπογενείς δραστηριότητες στην ξηρά και τη θάλασσα. Συνεπακόλουθα, η ρύπανση από θρεπτικά και οργανικά συστατικά δύναται να προκαλέσει μια σειρά από δυσμενείς περιβαλλοντικές επιπτώσεις, όπως υποβάθμιση του νερού, υποξία, βιολογική ρύπανση, ευτροφισμό και καταστροφή των οικοτόπων, με σοβαρές οικονομικές απώλειες για τους ιχθυοκαλλιεργητές.

Για να περιοριστεί η ρύπανση σε μία υδατοκαλλιέργεια μέσω οικολογικά βιώσιμων και κοινωνικά αποδεκτών τρόπων, τα ολοκληρωμένα συστήματα πολυτροφικής υδατοκαλλιέργειας (IMTA) αναδεικνύονται ως εφικτή λύση. Τέτοια συστήματα συνδυάζουν την ιχθυοκαλλιέργεια με την εκτροφή δευτερευόντων ειδών, τα οποία καταναλώνουν τα θρεπτικά συστατικά και τα απόβλητα των ψαριών επιτυγχάνοντας συνεχή ανάπτυξη και παράγοντας βιομάζα με σημαντική οικονομική

αξία. Κατά την πάροδο των χρόνων, αρκετοί οργανισμοί έχουν αναδειχθεί ως πολλά υποσχόμενοι υποψήφιοι για συστήματα ολοκληρωμένης υδατοκαλλιέργειας. Μεταξύ αυτών, οι θαλάσσιοι σπόγγοι επιφυλάσσουν μεγάλο δυναμικό υπό το πρίσμα των έμφυτων ιδιοτήτων τους ως θαλάσσια φίλτρα και της ικανότητάς τους να συγκρατούν μια ποικιλία οργανικών υδατογενών ουσιών, σωματιδιακής ή διαλυμένης μορφής. Πέρα από το υψηλό δυναμικό βιοαποκατάστασης ως προς μία πληθώρα βιολογικών και οργανικών ρύπων, η βιομάζα των σπόγγων θεωρείται «ορυχείο χρυσού», δεδομένου των εφαρμογών της σε διάφορους βιοτεχνολογικούς τομείς, ως σφουγγάρια μπάνιου αλλά και πηγές βιοδραστικών ενώσεων. Αν και ελπιδοφόρα, μόνο λίγες υπάρχουσες μελέτες έχουν διερευνήσει την ιδέα της βιοαποκατάστασης/βιοπαραγωγής από την συγκαλλιέργεια σπόγγων σε ιχθυοκαλλιέργειες παγκοσμίως, ενώ αντίστοιχες μελέτες εντός Ελλάδας είναι πρακτικά ανύπαρκτες. Λαμβάνοντας υπόψη τον ακμάζοντα τομέα της υδατοκαλλιέργειας στον ελλαδικό χώρο και την πλούσια ποικιλία σε σπόγγους που υπάρχει στο Αιγαίο Πέλαγος, είναι απαραίτητο να διερευνηθεί η δυνατότητα ενσωμάτωσης των γηγενών ειδών σπόγγων σε ιχθυοτροφικές μονάδες.

Στόχος αυτής της διατριβής είναι (α) να αξιολογήσει την ικανότητα βιοαποκατάστασης των μεσογειακών σπόγγων μέσω ελεγχόμενων εργαστηριακών πειραμάτων που περιλαμβάνουν χαρακτηριστικούς βιολογικούς και χημικούς ρύπους των ιχθυοκαλλιεργειών και (β) να διερευνήσει το δυναμικό αξιοποίησης της παραγόμενης βιομάζας των σπόγγων μέσω της στοχευμένης ανάλυσης γνωστών μεταβολιτών και του ελέγχου της βιοδραστικότητας των εκχυλισμάτων τους. Τα πειράματα καθαρισμού πραγματοποιήθηκαν για τέσσερις ευρέως διαδεδομένους μεσογειακούς σπόγγους *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* και *Sarcotragus foetidus*, οι οποίοι επιλέχθηκαν βάσει της υψηλής φυσικής τους αφθονίας, των μορφολογικών χαρακτηριστικών τους και της ικανοποιητικής ανάπτυξή τους σε πειραματική σπογγοκαλλιέργεια. Η ικανότητα εξυγίανσης βιολογικά ρυπασμένων υδάτων ιχθυοκαλλιέργειας αξιολογήθηκε εργαστηριακά ως προς τρεις φυτοπλαγκτονικούς οργανισμούς, σε μια προσπάθεια προσομοίωσης της απόκρισης των σπόγγων σε ευτροφικά περιβάλλοντα με άνθιση φυτοπλαγκτού (algal blooms). Τα εξεταζόμενα μικροφύκη παρουσίαζαν διαφορετικά χαρακτηριστικά μεγέθους/κινητικότητας και ανήκαν στα γένη *Nannochloropsis* sp. (~3.2 μm, μη κινητικό), *Isochrysis* sp. (~3.8 μm, κινητικό) και *Phaeodactylum* (~21.7 μm, μη κινητικό). Μοσχεύματα σπόγγων εκτέθηκαν για 7 ώρες σε θαλασσινό νερό εμπλουτισμένο με μικροφύκη υπό διαφορετικές πειραματικές συνθήκες. Αρχικά, αποδείχθηκε ότι και οι τέσσερις υποψήφιοι σπόγγοι ήταν σε θέση να διατηρήσουν την ικανότητα καθαρισμού τους σε διάστημα τεσσάρων ή πέντε διαδοχικών ημερών. Υπό έκθεση σε μία βαθμίδα κυτταρικών συγκεντρώσεων που προσομοίαζαν ολιγοτροφικά έως και εξαιρετικά ευτροφικά συστήματα, οι σπόγγοι διατήρησαν τη βέλτιστη ικανότητα φιλτραρίσματος

τους. Το ίδιο παρατηρήθηκε εν μέρει και κατά την πραγματοποίηση πειραμάτων υπό διαφορετικές συνθήκες φωτισμού. Διαφορετικές προτιμήσεις πρόσληψης παρατηρήθηκαν μεταξύ των εξεταζόμενων σπόγγων για μικροφύκη με διακριτό μέγεθος και κινητικά χαρακτηριστικά. Συνολικά, οι μελετώμενοι σπόγγοι εμφάνισαν ένα ευρύ φάσμα αποτελεσματικοτήτων συγκράτησης των διαφορετικών κυττάρων φυτοπλαγκτού που εξετάστηκαν, με τις υψηλότερες μέσες τιμές να σημειώνονται για τα είδη *A. oroides* (70%) και *S. foetidus* (44%).

Οι ίδιοι υποψήφιοι σπόγγοι διερευνήθηκαν περαιτέρω για την ικανότητά τους να αφαιρούν από το νερό διαλυμένους οργανικούς ρύπους που απαντώνται σε περιβάλλοντα υδατοκαλλιέργειας. Αυτή η σειρά εργαστηριακών πειραμάτων περιλάμβανε την έκθεση σε (α) μεμονωμένες χημικές ουσίες που ανήκουν στα αντιβιοτικά (π.χ. οξυτετρακυκλίνη), αντιρρυπαντικά επιχρίσματα (π.χ. diuron και Irgarol 1051) και πολυκυκλικούς αρωματικούς υδρογονάνθρακες (π.χ. 2,6-διμεθυλναφθαλένιο και φαινανθρένιο), αλλά και (β) πολύπλοκα οργανικά μείγματα, που περιλάμβαναν διηθήματα ιχθυοτροφών και περιττωμάτων ψαριών. Όλοι οι σπόγγοι ήταν ικανοί να προσλαμβάνουν τις διάφορες οργανικές ουσίες, επιδεικνύοντας έντονη προτίμηση στους λιπόφιλους ρύπους. Πιο συγκεκριμένα, παρατηρήθηκε μια ισχυρή θετική συσχέτιση μεταξύ της καθαριστικής δράσης των σπόγγων και της υδροφοβικότητας των υποστρωμάτων. Μεταξύ των σπόγγων που μελετήθηκαν, το *A. oroides* επέδειξε την υψηλότερη απόδοση κατακράτησης ως προς τις διαφορετικές διαλυτές οργανικές ουσίες που εξετάστηκαν, με τα υψηλότερα ποσοστά να παρατηρούνται πάντα για τους περισσότερο λιπόφιλους ρύπους. Σε μεταγενέστερο στάδιο, διερευνήσαμε τις διεργασίες που διέπουν την απομάκρυνση της διαλυμένης οργανικής ύλης (DOM) από τους σπόγγους. Σε όλα τα είδη που μελετήθηκαν, η διαδικασία ενεργητικού φιλτραρίσματος των σπόγγων βρέθηκε να παίζει κυρίαρχο ρόλο στην αφομοίωση των διαλυμένων ρύπων. Κάτι τέτοιο δικαιολογήθηκε από τους αυξανόμενους ρυθμούς που παρατηρήθηκαν μέσω αυτού του μηχανισμού σε σχέση με τις τιμές που προέκυψαν από την παθητική προσρόφηση των ρύπων στην επιφάνεια των νεκρών σπόγγων. Τέλος, επιβεβαιώθηκε ότι οι προσλαμβανόμενοι ρύποι συγκρατούνται σταθερά από τους σπόγγους και μετά βίας επαναπελευθερώνονται πίσω στο θαλασσινό νερό μέσω της εκρόφησης ή των μηχανισμών απέκκρισης των σπόγγων.

Το τελικό κριτήριο για την αξιολόγηση της εφαρμοσιμότητας των γηγενών σπόγγων σε ολοκληρωμένα συστήματα υδατοκαλλιέργειας, ήταν το δυναμικό αξιοποίησης της καλλιεργούμενης βιομάζας τους. Για το λόγο αυτό, τα είδη με τις καλύτερες επιδόσεις ως προς το δυναμικό βιοαποκατάστασης, αναλύθηκαν περαιτέρω για στοχευμένα γνωστά βιομόρια και βιοδραστικότητες. Η συγκεκριμένη διαδικασία υλοποιήθηκε για σπόγγους που προέρχονταν από καλλιέργειες σε γειτνίαση με ιχθυοκλωβούς, αλλά και από παρακείμενους φυσικούς πληθυσμούς, για να εκτιμηθεί κατά πόσο οι συνθήκες καλλιέργειας επηρεάζουν τη βιοσυνθετική ικανότητά

τους. Στα εκχυλίσματα που μελετήθηκαν ανιχνεύθηκε μια ευρεία γκάμα φυσικών προϊόντων, τα οποία ανήκαν στα αλκαλοειδή, βενζενοειδή, ινδόλες, λιπίδια και πολυκετίδια. Επίσης, η μεταβολομική ανάλυση αποκάλυψε χαρακτηριστικά χημικά μοτίβα ειδικά για κάθε είδος σπόγγου, με τα εκχυλίσματα των *A. oroides* και *S. foetidus* να κυριαρχούνται από αλκαλοειδή και λιπίδια, αντίστοιχα. Ωστόσο, το πιο σημαντικό εύρημα ήταν ότι τα καλλιεργούμενα και φυσικά μοσχεύματα από καθένα είδος σπόγγου παρουσίαζαν παρόμοια χημικά αποτυπώματα, με την πλειονότητα των μεταβολιτών να εμφανίζουν μικρές διαφορές ως προς τη σχετική σύστασή τους και ως προς τις απόλυτες συγκεντρώσεις τους. Επιπλέον, τα εκχυλίσματα καλλιεργημένων σπόγγων παρουσίασαν παρόμοια ή ελαφρώς χαμηλότερη αντιβακτηριακή δράση έναντι του *Staphylococcus aureus* που είναι ανθεκτικός στη μεθικιλλίνη, σε σύγκριση με τα εκχυλίσματα που προέρχονται από φυσικούς πληθυσμούς σπόγγων. Οι βιολογικές δοκιμασίες ως προς ανθρώπινα κύτταρα ορθοκολικού καρκινώματος (HCT-116) αποκάλυψαν οριακά δραστικά εκχυλίσματα τόσο από φυσικούς όσο και από καλλιεργημένους πληθυσμούς του είδους *S. foetidus*.

Όπως φαίνεται, οι θαλάσσιοι σπόγγοι, μαζί με άλλους θαλάσσιους οργανισμούς, φιλοξενούν μία μεγάλη ποικιλία βιοδραστικών ενώσεων με δυνητικές βιοτεχνολογικές εφαρμογές στη βιομηχανία φαρμάκων, καλλυντικών και τροφίμων. Στο πλαίσιο αυτό, πραγματοποιήθηκε μια ολοκληρωμένη επισκόπηση των πιο δημοφιλών βιοδοκιμών που χρησιμοποιούνται κατά τη διαδικασία βιοανακάλυψης φυσικών προϊόντων, μαζί με τις ιδιότητές τους και διάφορες άλλες πρακτικές λεπτομέρειες σχετικά με την επιλογή και τη χρήση τους. Ιδιαίτερη έμφαση δόθηκε στις βιοδοκιμές που συνήθως εφαρμόζονται για τον έλεγχο των αντιμικροβιακών, αντιβιοφίλμ, κυτταροτοξικών, αντιικών, αντιοξειδωτικών και αντιγηραντικών ιδιοτήτων των εκχυλισμάτων. Επιπλέον, παρουσιάστηκαν οι διεργασίες ποιοτικού ελέγχου καθώς και η ροή εργασιών που ακολουθείται κατά την ταυτοποίηση και τη σταδιακή κλασμάτωση/απομόνωση των βιοδραστικών ενώσεων καθοδηγούμενης από βιοδοκιμές. Η ανασκόπηση ολοκληρώθηκε με την παρουσίαση μίας πρακτικής μελέτης στοχευμένη στην ανακάλυψη φαρμάκων, συμπληρωμάτων διατροφής και καλλυντικών, που αποτελούν τις βιομηχανικές οδούς που συχνά προμηθεύονται με φυσικά προϊόντα θαλάσσιας προέλευσης. Επίσης, επισημάνθηκαν διάφορα ζητήματα ασφάλειας και κανονισμών που είναι κρίσιμα για τη μετάβαση των υπό εξέταση ουσιών μεταξύ των διαφορετικών σταδίων ανάπτυξης και των διαφορετικών φάσεων των κλινικών μελετών, μαζί με τις προοπτικές, τις γενικότερες τάσεις και τις μελλοντικές εξελίξεις που αφορούν το πεδίο των βιοδοκιμών.

Εν κατακλείδι, η σπογγοκαλλιέργεια μπορεί να θεωρηθεί ως αποτελεσματική τεχνολογία αποκατάστασης ρυπασμένων μονάδων υδατοκαλλιέργειας, αλλά και μία πολλά υποσχόμενη πηγή φυσικών προϊόντων υψηλής προστιθέμενης αξίας. Με βάση το δυναμικό

βιοαποκατάστασης/βιοπαραγωγής των διαφόρων ειδών σπόγγων που μελετήθηκαν καθ' όλη τη διατριβή, τόσο το *A. oroides* όσο και το *S. foetidus* προτείνονται ως οι καλύτεροι υποψήφιοι για μελλοντική συγκαλλιέργεια σε ιχθυοκαλλιεργητικές μονάδες.

Chapter 1. Introduction
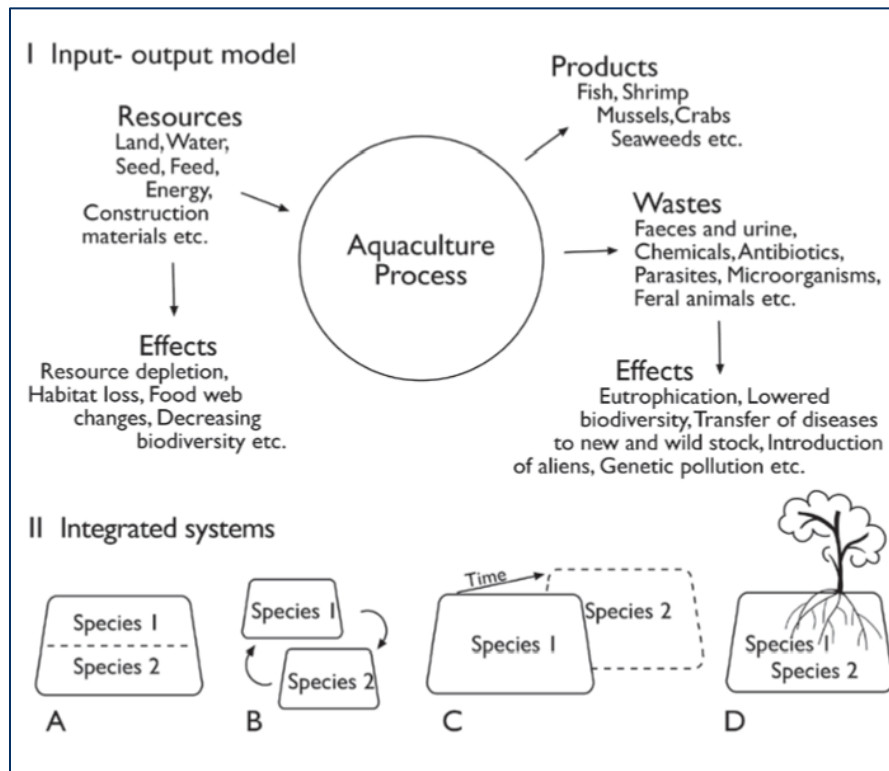
Figure 1.1. I. Diagram illustrating the use of resources by aquaculture, generation of wastes, and direct/indirect environmental effects; II. Main models of integrated aquaculture in marine or brackish waters (Soto, 2009).

1.1. Aquaculture

1.1.1. Global Aquaculture Industry: Current State and Trends

The term of aquaculture broadly refers to the cultivation of aquatic organisms involving human intervention in the rearing process to enhance production in several operations, such as reproduction, storage, feeding, and protection against predators (Araujo et al., 2022; Weissenberger, J., 2017). Over the past 40 years, aquaculture sector has experienced rapid growth as a means to meet global demands for seafood and protein. It is currently considered as the fastest growing food production sector worldwide, with an average annual growth rate estimated as high as 6.7% over the past three decades (FAO, 2022; Froehlich et al., 2017; Nadarajah and Flaaten, 2017). In 2021, total aquaculture production reached a peak value of 126 million tonnes live weight, with an estimated farm gate value of USD 296.5 billion. Out of these numbers, farmed finfish represented the greatest proportion (47.1%), while a further 27.9%, 14.6% and 9.4% was attributed to production of seaweeds, mollusks, and crustaceans, respectively (Mair et al., 2023).

For decades, finfish production accounts for the largest share of world aquaculture (**Figure 1.2**). According to predictions of Food and Agriculture Organization (FAO) of the United Nations, there is going to be an even higher demand for fish and fish-related products in the upcoming years, considering that urban population will have increased by more than two-thirds, reaching 10 billion people by 2050 (FAO, 2018a).
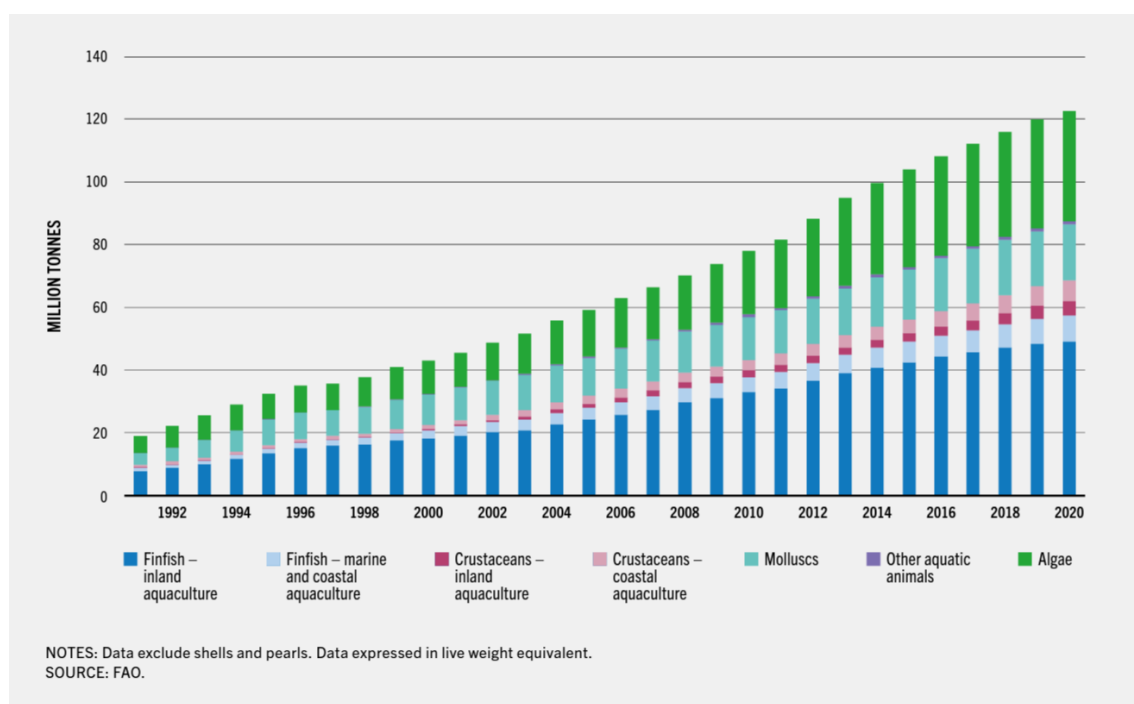
Figure 1.2. Evolution of global aquaculture production over the period of 1991-2020 (FAO, 2022).

1.1.2. Types of Aquacultures

1.1.2.1. Inland aquaculture

Depending on the environment in which cultivation takes place, global aquaculture activities can be broadly classified into two main types; inland aquaculture and mariculture. In general, inland practices use freshwater, but in some cases, farmers exploit saline water in inland areas (e.g., Egypt) or inland saline-alkali water (e.g., China) (FAO, 2014). Consequently, inland fish production may be carried out in lakes, rivers, ponds, artificial ponds, reservoirs, tanks and other environments (**Figure 1.3**) (Araujo et al., 2022; FAO, 2018a; Weissenberger, J., 2017).

Figure 1.3. Inland aquaculture systems.

1.1.2.2. Mariculture

In contrast, marine aquaculture, or commonly known as mariculture, is the cultivation of fish, or other marine organisms, for food and other human-benefit purposes, that takes place in the marine environment. This type of aquaculture can be practiced either;

Figure 1.4. Typical installation of a mariculture system consisting of marine cages and pens.

1) entirely in the sea for the whole production cycle, which concerns species dependent on wild seeds from the sea, or;

2) during the grow-out phase of the production cycle. In this case, rearing of the species is priorly carried out in land-based

hatcheries or freshwater, followed by transfer of juvenile fish to sea-based infrastructures, that are fed until harvest (e.g., Atlantic salmon) (Afewerki et al., 2023; FAO, 2022).

The most widely used technology in modern sea-based fish farms is the "marine cage culture". In such systems, production is conducted by rearing finfish within enclosed structures, comprised from cages and pens, which are placed near the surface of seawater bodies (**Figure 1.4**). Currently, cage cultivation systems provide the vast majority of fish products across the world (Wang et al., 2020). Countries such as Norway and Chile, that are endowed with large areas of fjords protected from rough sea, along with China, dominate world mariculture of finfish species particularly with sea cages (FAO, 2022).

Global marine fish production can take place in completely or partially human-made structures in areas close to the sea, onshore or in intertidal zones, such as coastal ponds and gated lagoons (e.g., coastal aquaculture). Alternatively, practices can be expanded into offshore sites, comprising the open-sea aquaculture. Because countries usually combine production from open-sea and coastal aquaculture for data reporting to FAO, the specific two terms are often mixed. This is particularly the case for finfish produced from both coastal ponds and cages in the sea, especially in East and Southeast Asia, where these two systems are widely employed (FAO, 2018a, 2020; Salomon and Markus, 2018).

Coastal aquaculture plays an important role in livelihoods, employment and economic development among coastal communities of many developing countries, particularly in Asia and Latin America (FAO, 2022). Remarkably, several developing countries in East and Southeast Asia rely more on coastal aquaculture for farmed finfish production than mariculture in the open-sea, especially in regions that are exposed to typhoons every year, including China, the Philippines and Vietnam (FAO, 2020). Contrarily, finfish production in America, Europe and Oceania mostly relies on open-sea mariculture (FAO, 2022).
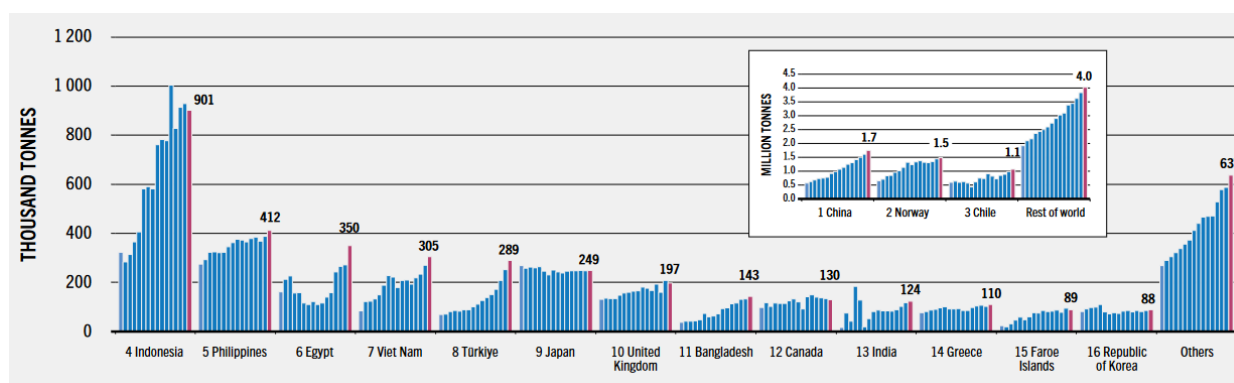
Figure 1.5. Global marine aquaculture production of finfish by major producers in 2020 (FAO, 2022).

Based on the data reported by FAO in 2020, finfish mariculture (reported as open-sea and coastal aquaculture) is practiced across the world, with production concentrated in specific countries (**Figure 1.5**). Among these, China is by far the largest producer, resulting in 1.7 million tonnes, followed by Norway with similar production values. Other major producers include Chile, Indonesia, Philippines, Egypt, Vietnam, Turkey, Japan, United Kingdom, Bangladesh, Canada, India and Greece, with a finfish production of over 5.1 million tonnes in total. Overall, mariculture practices reached a total fish production of 8.3 million tonnes (36.2 billion USD), with coastal aquaculture accounting for only 37.4% (FAO, 2022; Wang et al., 2020).

Table 1.1. World production of major mariculture finfish species (FAO, 2022).

	2000	2005	2010	2015	2020	Percentage of total, 2020
	(thousand tonnes, live weight)					
Finfish in marine aquaculture						
Atlantic salmon, *Salmo salar*	895.7	1 266.6	1 433.8	2 380.2	2 719.6	32.6
Milkfish, *Chanos chanos*	429.7	542.9	750.5	1 012.3	1 167.8	14
Mullets nei, Mugilidae	92.4	173.7	102.7	129.2	291.2	3.5
Gilthead seabream, *Sparus aurata*	87.3	110.8	142.3	168.8	282.1	3.4
Large yellow croaker, *Larimichthys croceus*	0.0	60.9	83.3	142.4	254.1	3
European seabass, *Dicentrarchus labrax*	60.7	90.9	118.0	149.1	243.9	2.9
Groupers nei, *Epinephelus* spp.	7.6	57.1	77.2	149.2	226.2	2.7
Coho(=Silver) salmon, *Oncorhynchus kisutch*	108.6	115.1	124.8	140.7	221.8	2.7
Rainbow trout, *Oncorhynchus mykiss*	155.3	202.0	287.7	204.1	220.1	2.6
Japanese seabass, *Lateolabrax japonicus*	0.6	79.6	104.8	120.6	196.9	2.4
Pompano, *Trachinotus ovatus*	0.0	0.0	80.0	110.0	160.0	1.9
Japanese amberjack, *Seriola quinqueradiata*	136.8	159.7	138.9	140.3	137.1	1.6
Nile tilapia, *Oreochromis niloticus*	1.6	5.3	20.3	49.8	107.4	1.3
Barramundi(=Giant seaperch), *Lates calcarifer*	18.1	27.0	52.7	68.7	105.8	1.3
Red drum, *Sciaenops ocellatus*	2.1	42.4	53.0	71.3	84.3	1
Subtotal of 15 major species	*1 996.6*	*2 933.9*	*3 569.9*	*5 036.7*	*6 418.2*	77

According to the latest FAO data, finfish mariculture is currently focused on the production of 15 species, with salmonoids, and especially Atlantic salmon (*Salmo salar*), being the main cultivated fish worldwide. The production of the specific species approximated the 2.7 million tonnes in 2020, accounting for 32.6% of total marine finfish aquaculture. Together with the other 14 dominating species, such as milkfish (**Table 1.1**), they explained the 75.6% of all finfish species produced by open-sea and coastal aquaculture globally (FAO, 2022).

1.1.3. World distribution of aquaculture

The criteria for selecting an aquaculture production system differ significantly across regions, countries and the cultivated species. Inland aquaculture of finfish production is dominated by developing countries such as China, India and Indonesia, yet some middle-income countries offer additional contributions (FAO, 2022, 2020). Meanwhile, a small number of countries belonging to the Organization for Economic Cooperation and Development (OECD), including Norway, Chile, Japan, the United Kingdom, Canada and Greece, are active players in the mariculture of finfish species, with a particular focus on coldwater salmonids (FAO, 2020). African production is mainly driven by inland practices given the lack of infrastructure, technical expertise and investment to support marine culturing (Carballeira Braña et al., 2021).

Table 1.2. Types of aquaculture production by region and by farmed species in 2020 (FAO, 2022).

	Africa	America	Asia	Europe	Oceania	World	Share in world total (%)
			(tonnes, live weight)				
1. Finfish	1 857 209	1 179 727	45 526 599	551 802	5 124	49 120 461	90.2
2. Crustaceans	2	72 541	4 401 336	3 145	177	4 477 201	8.2
3. Molluscs	…	…	192 671	…	…	192 671	0.4
4. Other aquatic animals	…	370	593 161	176	…	593 707	1.1
5. Algae	150	1 321	62 670	349	…	64 490	0.1
Inland aquaculture	**1 857 361**	**1 253 959**	**50 776 437**	**555 472**	**5 301**	**54 448 530**	**100**
1. Finfish	379 322	1 240 969	4 502 888	2 121 867	95 587	8 340 633	12.2
2. Crustaceans	7 617	1 193 549	5 549 811	418	8 420	6 759 815	9.9
3. Molluscs	5 994	688 077	16 158 709	578 712	116 363	17 547 855	25.8
4. Other aquatic animals	60	…	459 185	6 495	2 844	468 584	0.7
5. Algae	103 941	23 994	34 853 646	21 443	10 065	35 013 089	51.4
Marine aquaculture	**496 934**	**3 146 589**	**61 524 239**	**2 728 935**	**233 279**	**68 129 976**	**100**
1. Finfish	2 236 531	2 420 696	50 029 487	2 673 669	100 711	57 461 094	46.9
2. Crustaceans	7 619	1 266 090	9 951 147	3 563	8 597	11 237 016	9.2

3. Molluscs	5 994	688 077	16 351 380	578 712	116 363	17 740 526	14.5
4. Other aquatic animals	60	370	1 052 346	6 671	2 844	1 062 291	0.9
5. Algae	104 091	25 315	34 916 316	21 792	10 065	35 077 579	28.6
Total aquaculture	**2 354 295**	**4 400 548**	**112 300 676**	**3 284 407**	**238 580**	**122 578 506**	**100**

NOTES: …= no production or production data unavailable. Data exclude production of shells and pearls. Data may not match with totals due to rounding.

Although global fish aquaculture is currently dominated by inland practices, comprising 85.4% of the total production worldwide (FAO, 2022), marine farming has been viewed as a promising outlook for sustainable food supply (Gentry et al., 2017). This is mainly driven by the high impacts derived from land-based practices, which may involve resource intensity, pollution, disease occurrence and habitat destruction. Nowadays, momentum is heading towards marine environment as a farming resource given the fewer spatial conflicts, resources demands and the faster currents which can mitigate aquaculture-related pressures (Froehlich et al., 2017; Gentry et al., 2017; Holmer, 2010). Intriguingly, marine fish farming already plays an increasingly important role in food security, employment and economic development of several countries (**Table 1.2**).

1.1.4. Aquaculture in European Union

The European Union's (EU) Blue Growth Strategy identifies aquaculture as one of the sectors with the highest growth and job creation potential. Currently, the EU aquaculture sector provides jobs for 69,000 persons in 15,000 enterprises, including ten thousand owners and family members engaged in small family driven businesses (Nicheva et al., 2022).

Although EU aquaculture is dominated by mussels production, fish mariculture constitutes a significant economic sector across EU countries, by holding the biggest share in the total aquaculture value. Over the last decade, the specific activity reported a noteworthy 18% rise due to the increased production of high value finfish species, such as trout, salmon, seabass and bluefin tuna. This trend was further accompanied by the notorious price increase of some major species, such as gilthead seabream, that entered the market recently with booming production (**Figure 1.6**) (European Commission and EUMOFA., 2022). Furthermore, the aquaculture of turbot, halibut (*Hippoglossus hippoglossus*) and, more recently, sole (*Solea solea*) has given a boost to flatfish production, although combined production of these species was limited (Hough, 2022).

Based on the farming activity data of 23 European countries, rainbow trout (*Oncorhynchus mykis*) was the most important farmed species within EU in 2021, accounting for approximately 14% of total aquaculture production (**Figure 1.6**). With regard to rainbow trout, approximately 54% of its live weight produced during 2021 came from the combined output of France, Italy and Denmark. Greece

was proven to be a major producer of seabass and seabream, representing 69% of gilthead seabream and 53% of European seabass produced at EU level (Eurostat, 2023). Ireland was responsible for about 96% of the 13,700 tonnes of farmed salmon produced in EU. However, these values were far below of those reported for salmon farming in Norway (i.e., 1.5 million tonnes in 2021) (Eurostat, 2023).
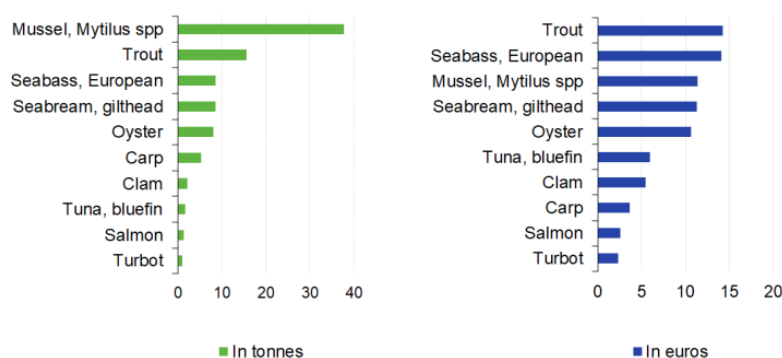
Figure 1.6. Main species of aquaculture production in European Union (%, EU, 2021) (Eurostat, 2023).

1.1.5. Aquaculture in the Mediterranean Sea

In general, aquaculture plays a major role in economic growth and food security across Mediterranean countries, while preventing overexploitation of wild stocks (FAO, 2018b). Considering that sea-fishing in the Mediterranean Sea is generally supported by small-scale vessels of low capacity, it is no surprising that aquaculture activity plays a relatively prominent role in the respective fisheries industry of Malta (accounting for 87.7 % of total fisheries production in tonnes live weight), Cyprus (85.1 %), Greece (71.0 %) and Italy (50.0 %) (Eurostat, 2023).

Interestingly, aquaculture is an activity with a long history in the Mediterranean region. It is speculated to be initiated even from ancient times, in places such as Egypt, as well as across Roman empire, where sea bass, sea bream, mullets and oysters were cultivated or simply kept alive off the Italian coast. Originally, Mediterranean aquaculture bloomed in coastal lagoons in the light of their particular ecological conditions that allowed the management of finfish and oyster populations. This set the foundations for the development of the modern Mediterranean aquaculture, characterized by the coexistence of diverse production systems that use a wide range of production techniques; from coastal lagoon management to highly intensive raceways or cage fish farming (Basurco, 2000).

The diversified character of Mediterranean coastal aquaculture is based on geographical differences (e.g., coastal lagoon, islands) together with a range of historical and socio-economic factors. The technology applied has evolved rapidly, both in modifying existing facilities (e.g., water recirculation for land-based installations) and in developing new projects (e.g., offshore cage technology). Cages are by far the most popular production technique, established in lagoons, sheltered

[35]

bays or semi-exposed and offshore conditions. The majority of farms uses cages, followed by land-based intensive raceways or tanks and semi-intensive production in earth ponds (Basurco, 2000; Rosa et al., 2012).

Generally, Mediterranean marine aquaculture is dominated by finfish, which comprised 83% of the total production in 2019, followed by mollusks (16%). Fish farming activity is highly pronounced in countries such as Egypt, by accounting for 31% of the total fish quantity in 2019, followed by Turkey (29%), Greece (14%) and Italy (12%) (Carvalho, N. and Guillen, J., 2021).

Although the majority of Mediterranean fish produced in 2019 resulted from capture fisheries (e.g., 57%), aquaculture still has an increasing role in fish supply. In fact, marine finfish production demonstrated the highest rise in the specific region (+15% compared to 2010), a fact entirely attributed to aquaculture practices (+71% compared to 2010), given the 6% decrease of capture fisheries in 2019 (Carvalho, N. and Guillen, J., 2021).

As shown in **Figure 1.8**, gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) are the most commonly farmed species in the Mediterranean Sea, the production of which approximated the 464,000 tonnes, with an estimated value of USD 2.24 billion in 2019. Remarkably, more than 95% of the world's seabream and seabass production currently comes from aquaculture, of which 97% originates from Mediterranean countries. Turkey and Greece are reported to be the main producers of these species, while Spain, France, Italy, Greece and Turkey are the main consumers. Initially, the specific

Figure 1.7. The main farmed species in the Mediterranean Sea; a) gilthead seabream (*Sparus aurata*) and b) European seabass (*Dicentrarchus labrax*). Source: FAO.

aquaculture activity was characterized by slow growth rates due to difficulties in producing large quantities of good quality fry. However, improvements at hatcheries in the late 1980s, such as reliable seed production techniques, formulation of specialized feeds and the application of intensive production systems (e.g., cages) led to the increased supply of juveniles, and since 2009, the production of seabass and seabream has increased by 115% and 95%, respectively. This trend was mainly attributed to Turkish aquaculture, which increased production of both species by 217% (Basurco, 2000; Carvalho, N. and Guillen, J., 2021).

Other marine finfish with pronounced farming activity involve mullets (*Mugil cephalus*), which are characteristic species of the Egyptian aquaculture. Contrarily, bluefin tuna accounted for less than 1% of farmed fish quantity, yet, holding an increased share in the total production value (i.e., 6%) (Carvalho, N. and Guillen, J., 2021). Furthermore, the emergence of meagre, red porgy (*Pagrus pagrus*) and other seabreams has been recorded, albeit in minor volumes (Hough, 2022).

(a)

(b)
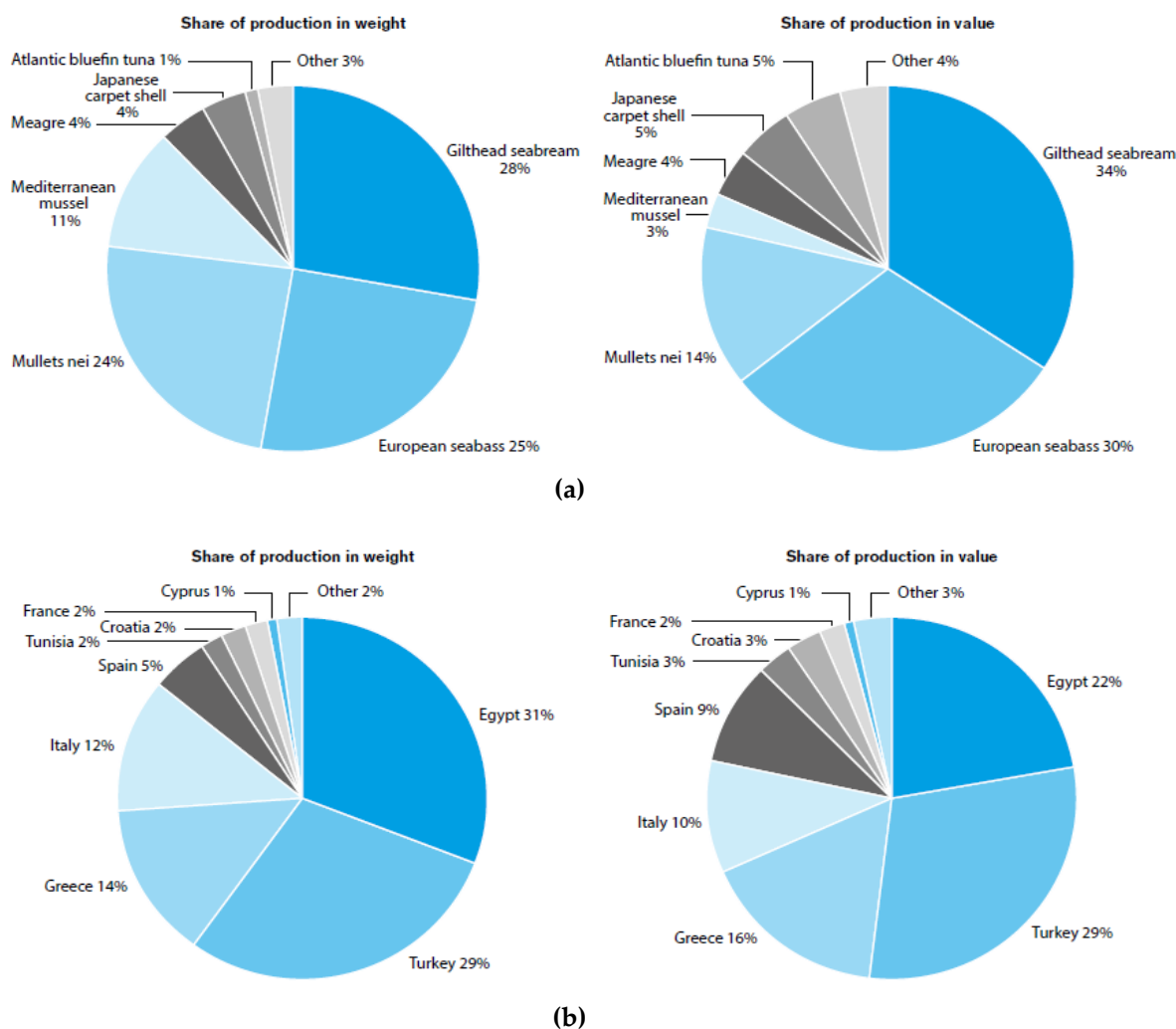
Figure 1.8. Aquaculture production in the Mediterranean Sea by a) species and b) country (Carvalho, N. and Guillen, J., 2021).

1.2. Intensification of Fish Mariculture Systems

In order to fulfill fish-related demands for an ever-increasing population, mariculture practices faced unparalleled growth and became a much more intensive industry, with larger farms, higher densities, and new technologies applied to the production (Phu et al., 2022; Rosa et al., 2020). Intensification of aquaculture was largely made possible by the use of fish cages. Marine cage culture is an open system that allows water to flow freely and interact strongly with the environment, allowing the release of produced fish wastes directly into seawater. Unlike the cultivation of other species, fish

cage cultivation requires feeding with additional concentrations of nutrients and chemicals to promote fish growth and help prevent disease, while fish metabolic processes may also yield excessive pollutants. Therefore, cage cultivation represents the main source of waste effluent discharge among all mariculture systems (Wang et al., 2020). Consequently, aquaculture intensification has been associated with a series of environmental concerns and it has been shown to induce negative effects on the surrounding ecosystems (Phu et al., 2022; Rosa et al., 2020).

1.2.1. Fish Mariculture Effluents: Composition, Waste-Load, and Environmental Impacts

Through recurring input of feeds and chemicals that are necessary to ensure the health and growth of fish, mariculture systems generate considerable amounts of effluent that can have undesirable impacts on the environment (Wang et al., 2020). Such negative impacts include nutrients pollution, derived from uneaten feed and fish metabolic waste, as well as chemical pollution resulting from various substances used in the production process (e.g., antifouling biocides, aquaculture medicinal products, polycyclic aromatic hydrocarbons). Both types of pollution can potentially lead to a series of adverse effects, with sound consequences for marine life. Other concerns are associated with the spread of farmed fish genes, parasites, and diseases to wild populations and habitat destruction. Furthermore, mariculture discharges might cause undesirable socio-economic issues in coastal waters and to the adjacent area. Landscape visual impact and odor, derived from marine litter, can potentially provoke conflicts between stakeholders, such as tourist industries, nature conservation organizations and water quality boards (Carballeira Braña et al., 2021; Gökalp et al., 2021; Phu et al., 2022; Rosa et al., 2020). The types of wastes contained within the mariculture effluents, as well as their environmental impact on aquatic and terrestrial life, are discussed below.
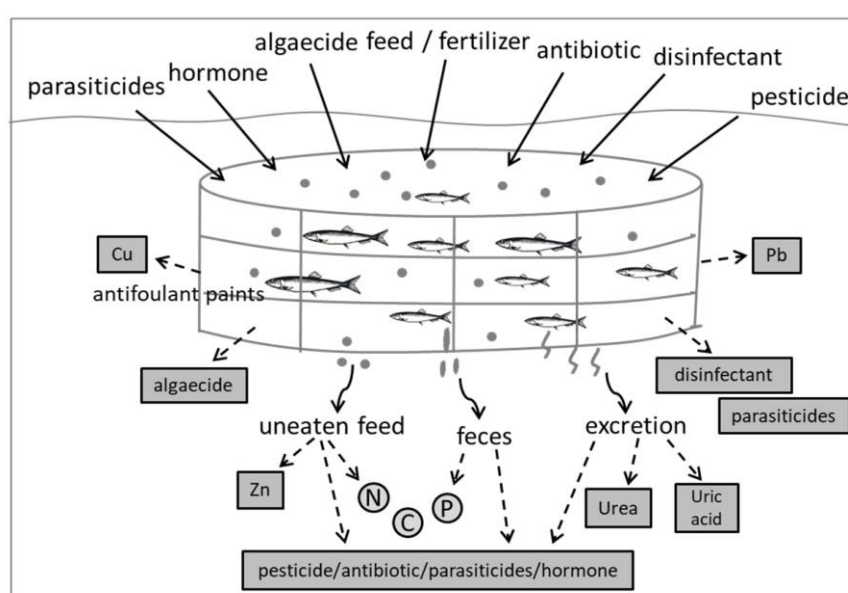
Figure 1.9. Schematic diagram of mariculture effluent discharges (Wang et al., 2020).

[38]

1.2.1.1. Nutrient Pollution

1.2.1.1.1. Fish Feeds

Fish maintained under intensive culture conditions are totally dependent on the external provision of a nutritionally "complete" diet throughout their culture cycle. Traditionally, "complete" diets have taken the form of a dry or moist pelleted feed consisting of a combination of different feed ingredients, the overall nutrient profile of which, as near as possible, approximates to the nutrient requirements of the fish species in question under conditions of "maximal" growth. Alternatively, complete diets may consist of a single food item of high nutrient value (i.e., trash fish, live food organisms), or a combination of both (Tacon, A.G.J., 1986). Currently, the major components of pellet fish feeds are agricultural products, fish meal and fish oil (Olsen, 2011).

Feeds in intensive mariculture systems are introduced into the cages twice or even continuously throughout the daylight hours, for a period of at least 14 months until the cultivated fish reach minimum commercial size (Pelekanakis et al., 2023; Rico et al., 2019; Rosa et al., 2020; Wang et al., 2020). However, up to 50% of this supplied feed is reported to be lost to the surrounding water and sediment (Carballeira Braña et al., 2021), with the majority of its content (i.e., approximately 80%) being biodegradable (Qi et al., 2019).

1.2.1.1.2. Metabolic waste products

Production densities in intensive aquaculture systems are so high that can reach 200 kg of fish per cubic meter of water (Rosa et al., 2020). Consequently, aquaculture practices are accompanied by significant release of species-related metabolic wastes. Such effluents may contain ingested but undigested food (faeces), or food ingested and eliminated as excretion (e.g., urea, uric acid) during production (Wang et al., 2020).

1.2.1.1.3. Waste Load and Environmental Impact

All the abovementioned types of pollutants include high contents of protein, carbohydrates, lipid, nitrogen, and phosphorous, which when released in the receiving waters, they can either transport in the water column as dissolved forms or settle on the seabed and around the fish farm as particulate matter (Qi et al., 2019; Wang et al., 2020). Excess nutrient enrichment can impact the adjacent area (near-field) and beyond (far-field) (Wang et al., 2020).

Nitrogen, needed for protein synthesis, and phosphorus (P), needed for DNA, RNA, as well as energy transfer, are both required to support aquatic plant growth, and constitute the key limiting nutrients in most aquatic and terrestrial ecosystems (Conley et al., 2009). Abundant loads of uneaten feed pellets, detritus and metabolic waste products can potentially serve as source of excess nutrients, leading to the proliferation of primary producers (e.g., phytoplankton) that may trigger micro- and

[39]

macroalgal blooms (Carballeira Braña et al., 2021; Wang et al., 2020). Excessive growth of phytoplankton reduces the transparency of the water column, affecting the photosynthesis of aquatic plants and impairing the balance between primary and secondary productivity (Wang et al., 2020). Particularly in deficiently managed areas, or in low hydrodynamic sites where water mixing is limited, this nutrient loading can lead to eutrophication, potentially altering not only biomass and community structure but also fish stocks. It can further cause oxygen depletion (e.g., hypoxia) in the water column, with consequent deterioration of water quality (Carballeira Braña et al., 2021; Rosa et al., 2020).
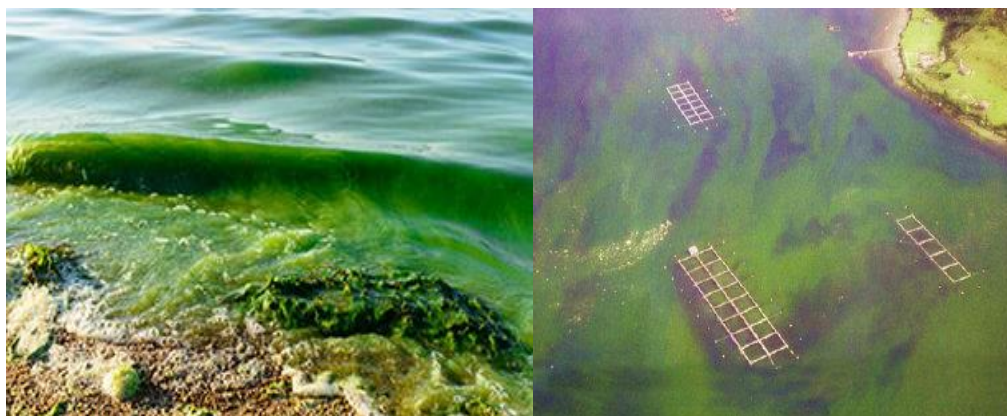
Figure 1.10. Harmful algae blooms affecting coastal sites (left) and salmon cages in Southern Chile (right) (adapted from *https://www.niehs.nih.gov/health/topics/agents/algal-blooms/index.cfm* and *https://seos-project.eu/marinepollution/marinepollution-c03-p04.html*, respectively).

Furthermore, ammonia that is excreted directly by the fish and produced through the decomposition of nitrogen-based uneaten food is toxic to marine animals, especially at high pH levels. In particular, unionized ammonia is even more harmful than ammonium ions and it reduces fish fertility, while increasing susceptibility to diseases. Ammonia is rapidly taken up by primary producers because of their higher preference for reduced forms of nitrogen or oxidized by *Nitrobacteraceae* bacteria into nitrite, which is toxic to aquatic animals. Nitrification continues until nitrite is oxidized into nitrate, which is less toxic than its previous forms. At low levels of oxygen as found in sediments, nitrate is reduced into dinitrogen gas, then sulfate-reducing bacteria transfer electrons to sulfur forms (sulfate, thiosulfate, and elemental sulfur) releasing toxic sulfides (Carballeira Braña et al., 2021).

The vast majority of phosphorus contained in fish feed (i.e., 80%) has been found to be lost to surrounding waters, with a large portion ending up in the sediment. Phosphorous introduced from aquaculture pollution can be more mobilizable (more easily released to the overlying water and posteriorly taken up by primary producers) than its typical form in the environment, hindering recovery and increasing the potential to cause eutrophication (Carballeira Braña et al., 2021).

1.2.1.2. Chemical and Organic-Matter Pollution

The most significant environmental concerns associated with intensive mariculture are those dealing with high loads of organic matter (Wang et al., 2020). Besides nutrients, mariculture-derived effluents such as metabolic byproducts and uneaten feed are highly enriched in organic compounds (Kim et al., 2022). In the water column, these substances could be either in the dissolved form or attached to suspended particles, which can subsequently settle to, and deposit upon, seabed sediments (Wang et al., 2020). Organic matter contributes to nutrient pollution, which further leads to all the prementioned threats for the marine ecosystem (e.g., hypoxia, eutrophication, water quality deterioration, habitat destruction) (Kim et al., 2022; Wang et al., 2020).

Organic pollution derived from fish mariculture is further enhanced by the use of chemicals, which are introduced to fish farms to enhance productivity and growth. Since the majority of mariculture practices rely on open-sea cages for fish cultivation, any chemical that may be introduced within the culture is discharged into the open water (dissolved organic matter; DOM) and sediments (particulate organic matter; POM) (Tornero and Hanke, 2016). These organic substances include therapeutants, which are the most commonly administered chemicals to prevent and treat disease outbreaks, hormones to enhance productivity and disinfectants to maintain hygiene throughout the production cycle. Antifouling agents, along with booster biocides, are also applied on aquaculture structures to avoid the clogging of meshes (Burridge et al., 2010; Carballeira Braña et al., 2021; Guardiola et al., 2012; Wang et al., 2020; Weston, 2000). Other contaminants, such as persistent organic pollutants (POPs, i.e., polychlorinated biphenyls (PCBs)), and polycyclic aromatic hydrocarbons (PAHs) are likely to be present in fish farm discharges (Justino et al., 2016; Nasher et al., 2013; Tornero and Hanke, 2016; Wang et al., 2010). Besides organic overloading, all these substrates can be metabolized and excreted, or accumulated in fish, with sound threats not only for aquatic, but also for human life (Justino et al., 2016).

1.2.1.2.1. Therapeutants

Chemotherapeutants widely used in fish aquaculture facilities include antibiotics to control bacterial infections, as well as algaecides and parasiticides to control algae and parasites (e.g., sea lice), respectively (Wang et al., 2010). Fish farmers are allowed to have access to a variety of properly authorized medicines to ensure animal health. The treatment protocols are tightly regulated in all jurisdictions, but they can vary among countries (Burridge et al., 2010; Tornero and Hanke, 2016). In Europe, the list of pharmaceuticals licensed for use in sea farming is limited to only 16 medicinal products (**Table 1.3**). Despite the strict regulations on the use of therapeutants in aquaculture practices, it appears that many other compounds are still legally available on the market and even if not fully licensed, they can be used on an off-label basis to overcome health threats (Tornero and Hanke, 2016).

Table 1.3. List of authorized veterinary medicines used in fish aquaculture among major producer countries of the European Economic Area (EEA) - AS, Atlantic salmon; CC, common carp; EE, European eel; ES, European seabass; FF, all finfish; GS, gilthead seabream; H, halibut; RT, rainbow trout; TB, turbot (Rico et al., 2019).

	Norway†	United Kingdom‡	Greece§	Spain¶	Italy††
Antibiotics					
Florfenicol	AS, H	AS, (RT)	GS, ES	RT	FF
Oxyetracycline		AS, RT	GS, ES	AS, RT, TB, GS, EE, ES, CC	FF
Chlortetracycline			GS, ES		FF
Amoxicillin		AS	GS, ES		RT
Flumequine			GS, ES	RT	FF
Sulfadiazine-trimethoprim	FF		GS, ES		FF
Oxolinic acid	AS,H, RT, TB		GS, ES		FF
Antifungals					
Bronopol	AS, RT	AS, RT		AS, RT	FF
Antiparasitics					
Azamethiphos	AS	AS			RT
Teflubenzuron	AS	AS			RT
Diflubenzuron	AS				RT
Emamectin benzoate	AS, RT	AS	GS, ES	AS, RT	FF
Deltamethrin	AS	AS, RT			FF
Cypermethrin	AS	AS			RT
Hydrogen Peroxide	AS	AS	GS, ES		FF
Formaldehyde			GS, ES	GS, TB	FF

Antibiotics

As aquaculture progressively grew into an intensive industry, more fish were concentrated in larger farms which caused an increase in bacterial disease occurrence (Culot et al., 2019). Antibiotics are used as chemotherapeutic agents to prevent or treat fish bacterial diseases, which are associated with stress conditions or poor seawater quality (e.g., high fish density, hypoxia) (Justino et al., 2016). According to FAO, an antibiotic is a "*drug of natural or synthetic origin that has the capacity to kill or to inhibit the growth of micro-organisms*" (Serrano, 2005). The major concern associated with their use is that the same properties causing the desired effects on target organisms are also the same properties causing adverse effects to indigenous non-target organisms (Rosa et al., 2020).

In general, the release of antibiotics around aquaculture sites can negatively impact the biodiversity of planktonic and benthic communities. Studies have shown that antibiotics can exert toxicity against algae and microcrustaceans, as well as non-target bacteria. Exposure of other marine biota to drug residues may also occur considering their potential bioaccumulation in the aquatic food chain (Boyd and McNevin, 2015; Grigorakis and Rigos, 2011). Furthermore, repeated exposure of bacteria to an antibiotic can lead to development of resistance, making the antibiotic less effective for its intended purpose (Boyd and McNevin, 2015).

Additionally, antibacterial therapy has been challenged by specialist groups, such as environmentalists and public health experts, on the basis that it may threaten human welfare. These concerns are mainly associated with the horizontal drug transmission via consumption/use of commercially-available aquatic products, where residual antibacterials are likely to be present (Rigos et al., 2010). In detail, they can induce a series of adverse effects;

a) Transmission of antibiotic resistance from aquaculture-related bacteria to bacteria responsible for human diseases, such as *Escherichia coli* (Boyd and McNevin, 2015; Carvalho et al., 2012; Rigos et al., 2010; Rosa et al., 2020).

b) Affection and disruption of normal intestinal flora (Okocha et al., 2018; Rigos et al., 2010).

c) Toxic effects, such as carcinogenesis, mutagenicity, immunopathological diseases, bone marrow depression and teratogenesis caused by systematic consumption of unlawful substances (e.g., chloramphenicol and nitrofurans) or legally used antibiotics (e.g., oxytetracycline) (Okocha et al., 2018).

d) Drug hypersensitivity reactions and emergence of allergic reactions (Grigorakis and Rigos, 2011; Rigos et al., 2010).

Typically, antibiotics are administered orally via feed for medication of farmed fish (i.e., medicated feed), but other means can be also used, such as direct injection and/or immersion in antibiotic bath solutions (Tornero and Hanke, 2016). In all cases, these substances, their metabolites and/or their degradation products eventually reach the surrounding environment and may cause adverse effects on wild organisms. However, medicated feed has been considered to be the most convenient way, given the lesser amounts required for drug treatment compared to the other practices (Ferreira et al., 2007).

Even so, it has been estimated that in intensive fish farming, approximately 70–80% of the antibiotic included in medicated feed enters the water of the culture system, with only a small portion being actually absorbed or metabolized by the organisms. This means that a considerable amount of the drug ends up in the environment through food surplus that is not consumed/ingested by fish, especially in the case of diseased fish which tend to have a reduced appetite. Medication can also pass into the environment via urinary, or digestive system with unabsorbed drug contained in faeces, unprocessed parent drug from renal and gill excretions or be eliminated as toxic metabolites following renal and faecal excretion. In any case, the antibiotic or its metabolites remain in the water until degraded by natural processes, diluted by mixing or until they are adsorbed onto sediments. Some antibiotics degrade rapidly, but most are persistent (Boyd and McNevin, 2015; Ferreira et al., 2007; Grigorakis and Rigos, 2011; Hektoen et al., 1995; Rosa et al., 2020). Therefore, antimicrobial agents may reach relatively high concentrations in aquatic ecosystems near aquaculture settings (Ferreira et al., 2007). Remarkably, sediments underneath fish farms have been reported to exert antibacterial activity due to drug enrichment (Samuelsen et al., 1992).

Oxytetracycline (OTC) and florfenicol are among the antibiotics with widespread use in global and European finfish aquaculture (Bondad-Reantaso et al., 2023; Rico et al., 2019), with OTC being the most persistent compound in seawater (half-life: 9-419 days; Weston, 2000). However, its beneficial characteristics, such as increased efficacy, low cost, high potency and broad-spectrum activity, make it probably the most employed antibiotic in global aquaculture (Ferreira et al., 2007; Tornero and Hanke, 2016). Administration of OTC is indicated for the treatment of fish bacterial diseases caused by *Aeromonas salmonicida* (furunculosis); *Aeromonas hydrophila* and *Aeromonas sobia* (aeromonosis); *Pseudomonas* (pseudomonosis); *Lactococcus garvieae* (lactococcosis) and *Vibrio anguillarum* (vibriosis). Target fish species typically subjected to OTC treatment involve fish of European or Mediterranean farms, such as salmonids (e.g., *Salmo* sp., *Oncorhynchus* sp., *Salvelinus alpinus*), turbot (*Psetta maxima*), sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), European eel (*Anguilla Anguilla*) and common carp (*Cyprinus carpio*) (Leal et al., 2019). Despite the use of antibiotics having been reduced drastically in recent years, following the introduction of vaccines and improved husbandry practices, antibacterial therapy still remains the last recourse to combat bacterial fish infections in aquaculture (Tornero and Hanke, 2016).

Parasiticides

The presence of internal and external parasites in cultured aquatic organisms is a common phenomenon (Boyd and McNevin, 2015). Parasitic diseases may not cause death to organisms, but can lead to an increase in production costs or a decrease in the product quality and eventually, rejection by markets due to non-conformity (Rosa et al., 2020). Thus, control of such infestation often relies on antiparasitic compounds.

A variety of treatments are available, but most practices employ parasiticides via in-feed and bath treatments. For instance, pyrethroids (deltamethrin, cypermethrin), hydrogen peroxide and organophosphates (azamethiphos) are applied via short bath treatments to kill ectoparasites. This treatment is used predominantly to combat infestation by sea lice (*Lepeophtheirus salmonis*), an ectoparasite which affects mainly salmonids (**Table 1.3**), by causing skin erosion, sub-epidermal hemorrhage and other secondary infections if not treated properly (Burridge et al., 2010). Other popular compounds, such as avermectins (emamectin benzoate) and benzoylurea insecticides (teflubenzuron, diflubenzuron) are included in commercial feeds (similarly to antibiotics) and administered for several days to kill a plethora of parasitic pests, including sea lice (**Table 1.3**) (Boyd and McNevin, 2015; Rico et al., 2019).

Despite being prescribed, and regardless of the method of administration, the portion of the parasiticidal compound not retained by the animals enters the culture system and spreads in the marine

environment, potentially causing harmful effects on the marine ecosystems adjacent to aquaculture facilities (Boyd and McNevin, 2015; Rosa et al., 2020). Environmental concerns related to antiparasitics include the possible effects to non-target invertebrate species in and around the fish farms, including principally microcrustaceans and decapods. Furthermore, some of the antiparasitics used in aquaculture are known to bind to particulate organic material and may be of concern to filter feeders, such as mussels or sediment dwelling organisms (Rico et al., 2019).

Antifungals

Fungal infections are particularly troublesome in fish hatcheries and a variety of compounds have been used to disinfect small fish before stocking them in grow-out systems (e.g., salmon) (Boyd and McNevin, 2015). Antifungals are usually applied in bath treatments, either in tanks, ponds or net-pen systems containing the eggs or small fish (Rico et al., 2019). Exposure is usually brief, but applied concentrations are relatively high (Boyd and McNevin, 2015). Bath treatments with fungicides are conducted by reducing the water volume and applying the chemical at the recommended concentration. In net-pen systems, the net depth is reduced and an impermeable barrier is installed to prevent chemical dispersal, so as to maintain chemical concentrations inside the net-pen for several minutes to 1 h (Rico et al., 2019).

Antifungal therapy in European fish aquaculture is mainly dominated by the use of bronopol (2-bromo-2-nitro-1,3-propanediol). This is the case of a wide-spectrum fungicide which acts against the cell walls of fungi with catalytic oxidation and production of active oxygen species, such as superoxide and peroxide. The bronopol application for egg disinfection has been reported in many other cultured fish species such as Atlantic salmon, cod, *Gadus morhua*, haddock, *Melanogrammus aeglefinus*, halibut, *Hippoglossus,* rainbow trout and bluefin sea bream (Jantrakajorn and Wongtavatchai, 2015; Rico et al., 2019). Although claimed to be eco-friendly, recent findings have indicated its potential to cause oxidative stress in aquatic life (Magara et al., 2021), while other popular antifungals, such as malachite green, have been accused for their carcinogenic and teratogenic properties (Oono et al., 2007).

1.2.1.2.2. Anesthetics

The usage of anesthetic substances is essential in fish farms since it allows the performance of painful procedures, ease handling and stress reduction during the execution of invasive activities (Bodur et al., 2018; Tsantilas et al., 2005). Anesthetic agents are routinely used to assist immobilization or sedimentation of brood animals during grading, tagging, transport, blood sampling, artificial breeding (stripping breeders) and vaccination (Bodur et al., 2018; Boyd and McNevin, 2015; Tornero and Hanke, 2016; Tsantilas et al., 2005). At the same time, it prevents fish from immuno-suppression, physical injury, or even death (e.g., heart attacks) during their handling, thus, preserving fish welfare

(Bodur et al., 2018; Coyle et al., 2004). Furthermore, anesthesia is necessary for invasive, physiological investigations on farmed fish, such as obtaining biopsies for DNA, which are essential for control and traceability of stocks (Bodur et al., 2018).

Compounds available for use are regulated in all jurisdictions (Burridge et al., 2010). There is a large range of substances and/or combination of them that are commonly used for sedation or anesthesia of fish such as MS-222 (tricaine metasulphonate), benzocaine, 2-phenoxyethanol, quinaldine, metomidate, clove oil and isoeugenol (Bodur et al., 2018; Brønstad, 2022; Tsantilas et al., 2005). Anesthetic substances are usually absorbed through the gills, after first being dissolved and added to the water in which the fish are (Tsantilas et al., 2005). However, the discharge of used anesthetic baths directly into natural seawaters (Boyd and McNevin, 2015; Tsantilas et al., 2005), has been a matter of environmental concern. When fish are exposed to an anesthetic, residues or metabolites of the substance remain in the flesh for a period of time until they are excreted or metabolized (Coyle et al., 2004). Although they are claimed to be used infrequently and in low doses (Burridge et al., 2010), there is limited information regarding the rate of degradation of most of these substances, or of their environmental toxicity (Boyd and McNevin, 2015). Quinaldine, MS-222, 2-phenoxyethanol and benzocaine are limited in their use because of health concerns for humans or fish, while repeated administration of clove oil has been reported to deleteriously affect corals (Boyer et al., 2009). Consequently, care should be taken with selection of anesthetics as they involve significant side-effects and these should be researched to inform the appropriate choice of drug (Brønstad, 2022).

1.2.1.2.3. Disinfectants

Disinfectants, or alternatively germicides, are "*agents that destroy infection-producing organisms*" (Russell Danner and Merrill, 2005). In global aquaculture, disinfectants are used to maintain hygiene throughout the production cycle and in some cases, to treat disease (Tornero and Hanke, 2016). For instance, in salmonids they are applied in order to prevent infectious salmon anaemia (ISA) and transfer of bacterial diseases from site to site (Burridge et al., 2010; Russell Danner and Merrill, 2005). Selection of disinfectants is performed according to their effectiveness against a list of endemic and state-regulated pathogens, which can vary in importance and prevalence on a global scale (Russell Danner and Merrill, 2005).

The list of commonly used disinfectant agents in marine and inland aquaculture, as well as, public and private fish hatcheries of USA is presented in **Table 1.4**. In Chile, the following products are identified as being applied in salmon aquaculture: Virkon®, iodine and detergents, chloramine-T, hypochlorite ($HClO_2$), chlorine dioxide (ClO_2), benzalkonium chloride, Superquats®, glutaraldehyde, formalin 40%, calcium oxide: CaO or quicklime, calcium hydroxide; $Ca(OH)_2$ or slake lime, sodium

carbonate: Na_2CO_3 or soda ash, Creolina®, synthetic phenols, halophenols and ethanol (95% and 70%) (Burridge et al., 2010). Formalin and iodophors are the most popular disinfectants used in European aquaculture (Tornero and Hanke, 2016).

Table 1.4. Efficacy and characteristics of commonly used disinfectants in USA (Russell Danner and Merrill, 2005).

Germicide	Use dilution	Level of disinfection	Active against					
			Bacteria	Lipophilic virus	Fungi	Hydrophilic viruses	M. tuberculosis	Bacterial 1 spore
Formaldehyde	2–3.2%	High	+	+	+	+	+	+
Hydrogen peroxide	3–25%	High	+	+	+	+	+	V
Chlorine	100–1,000 ppm Cl	High	+	+	+	+	+	V
Isopropyl alcohol	60–95%	Intermediate	+	+	+	V	+	−
Glucoprotamine	4%	Intermediate	+	+	+	+	+	−
Phenolic compounds	0.4–5% aqueous	Intermediate	+	+	+	V	+	−
Iodophors	30–50 ppm	Intermediate	+	+	+	+	V	−
Quaternary ammonium compounds	Active I 0.4–1.5% aqueous	Low	V	+	V	−	−	−

Note: + = yes; − = no; V = variable results. The efficacies of the disinfectants are based on exposure

Disinfectants are administered as a bath directly added to the water column and discharged outside the farm, but in most cases, they are released into the surrounding environment (Burridge et al., 2010; Wang et al., 2020). Although the discharge of disinfectants into the environment is regulated by both Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) in USA (Russell Danner and Merrill, 2005), it seems that there are no regulations regarding their use or quantities administered elsewhere (Burridge et al., 2010). Thus, chemical inputs from such substances can reach high levels and be cumbersome for aquatic life, especially in areas around wharves or in small sheltered coves. Furthermore, disinfectant formulations, such as the popular agent Virkon S® often contains surfactants (Russell Danner and Merrill, 2005), which are widely known endocrine disruptors affecting salmon, as well as other marine organisms (Burridge et al., 2010).

In general, most of the disinfectants used are quite soluble in water and should be regarded of low toxicity. However, lack of information regarding the nature of the actually used compounds and their associated quantities, make more difficult to assess risk to fish and to non-target organisms (Burridge et al., 2010).

1.2.1.2.4. Hormones

Hormones are used in fish farming to increase fish production, based on different techniques;

- Artificial reproduction, which sustains the production chain with constant yielding of seeds. By administrating exogenous hormones, fish farmers are able to induce/advance spawning or

[47]

delay/arrest fish maturation, depending on the present needs. Hormonal techniques for fish breeding are based on intramuscular or intraperitoneal injection in broodstocks, with the crude extract of the pituitary gland (PE) of mature fish (carp and salmon) and gonadotropin-releasing hormone (GnRH) of both mammalians (mGnRH) and salmon (sGnRH) being widely used.

- Sex reversal. This practice is used when different growth rate and/or weight gain are observed between the male and female, a phenomenon commonly occurred in teleost fish during puberty. In this case, hormonal treatment aims at the production of monosex population to increase growth rate or weight gain, by rearing individuals of the most profitable gender. Thereby, increased uniform lots and control of undesirable breeding are achieved.

In the case of increasing fish production based on sexual dimorphism, a variety of steroid hormones, either natural or synthetic, are registered in fish farms, with estrogens and androgens being the most popular. The administration of hormones for sex reversal treatment is conducted via immersion (bath treatments) or dietary supplementation (hormone incorporated in fish feed), as both methods reach a large number of fish. Hormones such as 17β-estradiol, estradiol valerate, 17α-methyl testosterone, 17α-methyl dihydrotestosterone and androgen are commonly used in immersion and diet techniques (Hoga et al., 2018).

Apart from the benefits hormone treatment can offer (e.g., maximum growth, elimination of early maturation in males and broodstock management), the associated chemicals must be handled carefully to ensure environmental, biological and food safety, since they can contaminate the environment and promote changes in the endocrine system inducing adverse reactions on the consumer health (e.g., carcinogenic effects). Hormonal residues have been reported in wild fish tissues in natural ecosystems, as well as in fishery products in the market (Hoga et al., 2018).

Hormones can be introduced into the environment by the incorrect or illegal discard of water containing residues of these compounds used to treat fish. The contamination may be originated by fish excretion, as well as from the medicated feed that remains unconsumed by fish. Although, fish liver metabolizes hormones into water soluble compounds, or inactive agents (which can be considered of low toxicity), these can turn back to free steroids by bacteria present in the environment. Nevertheless, it is estimated that more than 99% of administered hormones are metabolized and released within hours or days into the water (Hoga et al., 2018).

Countries all over the world have established different regulations on the use of hormones in aquaculture. EU prohibits the use of substances with hormonal action in food producing animals, including aquaculture. Other countries such as Canada, Australia, New Zealand, Argentina and the United States allow the use of natural steroid hormones, such as testosterone, progesterone and 17β-

estradiol, as well as the synthetics zeranol and trenbolone acetate. In those countries, only residues from synthetic compounds with maximum residue levels (MRLs) are controlled because they are considered to be the most potent endocrine compounds (Hoga et al., 2018).

1.2.1.2.5. Antifouling agents and booster biocides

Infrastructures used in aquaculture production systems invariably comprise a complex assortment of submerged components, involving cages, nets, floats and ropes. Each one of these structures provides potential surfaces for fouling attachment and growth. In particular, the cages, commonly made from polyamide or high-density polyethylene (PEHD), as well as their nets and supporting infrastructure, offer fouling organisms thousands of square meters of multifilament netting (Amara et al., 2018; Bazes et al., 2006). Along with the nutrients encountered in aquaculture wastes around cages (e.g., fish excretions, particles of uneaten feed), cage systems provide an excellent environment for the development of epibiota, and thus, fouling formation (Comas Morte, 2018).

Figure 1.11. Temporal organization of biofouling (Guardiola et al., 2012).

Marine biological fouling, usually termed marine biofouling, is a phenomenon visible on every surface that is submersed in seawater. Specifically, when man-made structures are placed in the aquatic environment are rapidly coated by a macromolecular film which favors colonization first by bacteria and unicellular organisms, followed by multicellular eukaryotes, such as macroalgae, barnacles and blue mussels (Bazes et al., 2006). Marine cage systems suffer significantly from the effects of biofouling, as they can result in severe economic consequences and a loss of profitability for the aquaculture industry. The most substantial problems associated with fouling formation, include;

     a)   Net occlusion. As fouling organisms settle and grow on nets, mesh openings occlude, resulting in restricted water and oxygen exchange.

     b)   Disease risk. Decreased levels of dissolved oxygen, driven by poor water exchanges, increase stress levels in fish, lower immunity and promote vulnerability to diseases.

[49]

c) Weight addition. The extra weight imposed by fouling development causes cage deformation and structural fatigue. Consequently, the maintenance and loss of equipment cause the increase of production costs for the industry (Amara et al., 2018; Comas Morte, 2018).

To prevent fouling formation when immersed in seawater, aquaculture infrastructures are priorly coated with antifouling paints which contain biocides. The latter are chemical substances that are capable of prohibiting or killing microorganisms responsible for biofouling. Generally, antifouling agents function by creating a toxic boundary layer at the surface of the paint, as the component biocides leach out to provide a constant threshold concentration in the water, therefore inhibiting the development of fouling communities (Amara et al., 2018; Comas Morte, 2018).

Figure 1.12. Biofouling of finfish cage netting: (A) water line fouling by the green algal genus *Enteromorpha* at a Scottish salmon farm, which has been developed on a copper-based coating that gives the red colour to the netting; (B) heavy fouling of a net immersed at a sea trout farm on the Danish east coast, dominated by mussels colonization (Braithwaite and McEvoy, 2004).

Although highly effective, many of these biocides, such as the triorganotin-based formulations (e.g., tributyltin (TBT)) have been accused in the past for their high toxicity against fish and shellfish, which led to their gradual elimination from the market (Amara et al., 2018; Comas Morte, 2018; Guardiola et al., 2012). Currently, new alternatives to antifouling paints involve copper-based substances (e.g., cuprous oxide (Cu_2O) and copper thiocyanate (CuSCN)), supplemented with booster biocides, such as organic or zinc-based, to control Cu resistant fouling organisms (Amara et al., 2018; Guardiola et al., 2012). The most widely used chemicals in aquaculture include more ecofriendly alternatives, such as the Irgarol 1051, Sea-Nine 211, diuron, chlorothalonil, and other metallic compounds like zinc pyrithione (ZnPT) and copper pyrithione (CuPT) (**Table 1.5**) (Amara et al., 2018; Konstantinou and Albanis, 2004).

Nevertheless, these alternatives to TBT may also exert toxicity and their release into the aquatic environment has been of particular concern during the recent years (Guardiola et al., 2012). More significantly, their extensive use has been associated with metal accumulation in cultured fish, and thereby, with lethal or sub-lethal effects and disruption of the immediate immune system (Nikolaou et al., 2014; Tornero and Hanke, 2016). Additionally, several studies have assessed the toxicity of booster biocides on non-target species, with the majority of these

[50]

chemicals being growth inhibitors of marine autotrophs, and influencing key species, such as sea grasses and corals. Therefore, there is an increasing interest in the impact of these compounds on the aquatic ecosystems (Guardiola et al., 2012).

Table 1.5. The main antifouling biocides currently used in aquaculture along with their physicochemical properties and their effects on aquatic life.

Antifouling Agent	Chemical structure and class	Mode of Action	Log(Kow)	Solubility (mg/L)	Persistence (in half-life time)	Environmental Impact	Reference
Copper	CuSCN; Cu_2O; Cu_2S (Organometallic salt)	Multi-site inhibitor (Metabolic processes)	Not Applicable	-	-	Immunotoxic to molluscs and teleosts, alteration of fertilization and early life stages of bivalves and corals	(Guardiola et al., 2012; Tornero and Hanke, 2016)
Irgarol 1051	$C_{11}H_{19}N_5S$ (s-Triazine)	Inhibitor of electron transfer process within photosystem-II	3.95	6.0-7.0 (slightly soluble)	100-350 days	Highly toxic to non-target photosynthetic marine organisms such as cyanobacteria, algae, macrophytes and symbiotic dinoflagellates in corals	(Guardiola et al., 2012; Loos, 2012; Martins et al., 2018; Tornero and Hanke, 2016)
Diuron	$C_9H_{10}Cl_2N_2O$ (Phenylurea)	Inhibitor of electron transfer process within photosystem-II	2.82	35-42 (relatively soluble)	33-365 days	Highly toxic to non-target photosynthetic marine organisms such as cyanobacteria, algae, macrophytes and symbiotic dinoflagellates in corals	(DeLorenzo and Fulton, 2012; Guardiola et al., 2012; Hannachi et al., 2022; Loos, 2012)
DCOIT (Sea-Nine 211®)	$C_{11}H_{17}Cl_2NOS$ (Isothiazolone)	Inhibitor of electron transfer process within photosystem-II	2.8, 4.5, 6.4	4.7 (in synthetic seawater) – 14 (unknown conditions)	<24 hours	Toxic effects on embryogenesis and larval growth of sea urchins, crustaceans, bivalve, and ascidians.	(Chen and Lam, 2017; Guardiola et al., 2012; Thomas and Brooks, 2010; Tornero and Hanke, 2016; Yebra et al., 2004)
Chorothalonil	$C_8Cl_4N_2$ (Organochlorine)	Inhibitor of mitochondrial electron transport	2.6-4.4	0.9	1.8-8 days	Highly toxic to fish and crustaceans, bioaccumulation in fish tissues.	(Guardiola et al., 2012; Martins et al., 2018; Thomas and Brooks, 2010; Van Scoy and Tjeerdema, 2014)

1.2.1.2.6. Other Contaminants

Other chemical pollutants encountered in aquaculture effluents involve heavy metals and organochlorine compounds. Metals such as zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), cobalt (Co), arsenic (As) and selenium (Se) may be incorporated in fish food formulations to fulfill complete mineral requirements of farmed fish. Others, such as cadmium (Cd), mercury (Hg), nickel (Ni) and lead (Pb), along with highly lipophilic compounds, including POPs (e.g., PCBs) and PAHs can be present as unintended additives/contaminants in fish feeds, or can end up in fish farm environments via aquaculture operations (i.e. use of engine boats by aquaculture personnel to reach fish cages) or offshore anthropogenic activities (e.g., maritime vehicle activity, oil industries) (Grigorakis and Rigos, 2011; Nasher et al., 2013; Sather et al., 2006; Tornero and Hanke, 2016). Environmental and health concerns about these contaminants are mainly associated with their bioaccumulation in fatty tissues of aquatic animals and biomagnification along the food chain, leading to health hazards for marine life and human beings (Justino et al., 2016; Retnam et al., 2013). The adverse implications caused by human exposure to these toxic entities are diverse and mainly include neurotoxic, immunological, mutagenic and carcinogenic effects (Grigorakis and Rigos, 2011; Justino et al., 2016; Retnam et al., 2013).

1.3. Bioremediation of Aquaculture Wastes

As the aquaculture industry intensively develops, its environmental impact inevitably increases. Over the years, various methods have been developed to treat aquaculture wastewater, which can be broadly classified into physical, chemical and biological processes (Crab et al., 2007). Among them, biological practices and, particularly, bioremediation techniques have gained widespread interest for sea cage operations due to their sustainability and their feasibility to be conducted on-site (Chávez-Crooker and Obreque-Contreras, 2010; Tom et al., 2021). According to FAO, bioremediation refers to the use of living organisms to remove contaminants, pollutants, or unwanted substances from soil or water (Zaid et al., 2003). Of those techniques, Integrated Multi-trophic Aquaculture (IMTA) strategies have emerged as key solutions for aquaculture sustainability, since they can minimize environmental concerns while delivering economic benefits, promoting the ecological approach to aquaculture and being socially acceptable (Chávez-Crooker and Obreque-Contreras, 2010; Chopin et al., 2008; Rosa et al., 2020).

1.3.1. Integrated Multi-trophic Aquaculture (IMTA) Systems

According to FAO, integrated multitrophic aquaculture is "*a practice in which by-products from one species are recycled to become inputs for another*" (FAO, 2014). More specifically, it is the farming, in

proximity, of aquaculture species from different trophic levels, and with complementary ecosystem functions, in a way that:

a) allows one species' uneaten feed and wastes, nutrients and by-products to be recaptured and converted into fertilizer, feed and energy for the other crops

b) exploits the synergistic interactions between species (Chopin et al., 2012).

In such systems, farmers combine the aquaculture of a main, feed-dependent species (e.g., fish; fed aquaculture), with rearing of secondary species, that utilize fed-aquaculture wastes (e.g., organic, inorganic material) for their own growth (extractive aquaculture). Extractive species typically involved in IMTA systems can be;

a) primary producers, such as seaweeds or other aquatic vegetation, that transform inorganic nutrients into organic biomass, or;

b) secondary producers, that use organic material from the water column or seabed as food. Suspension or filter-feeders are those IMTA components that sieve organic matter from the water column, and include shellfish and sponges. On the other hand, worms, sea urchins and sea cucumbers are typical deposit feeders that feed on organic material on or within the sediment.

Besides their role as natural biofilters, the co-cultivated extractive species have their own commercial value; their biomass can be harvested and turned into several value-added products, acceptable to consumers, by increasing the overall aquaculture value (Barrington et al., 2009; Chopin et al., 2008; Marine Institute, 2020; Rosa et al., 2020).
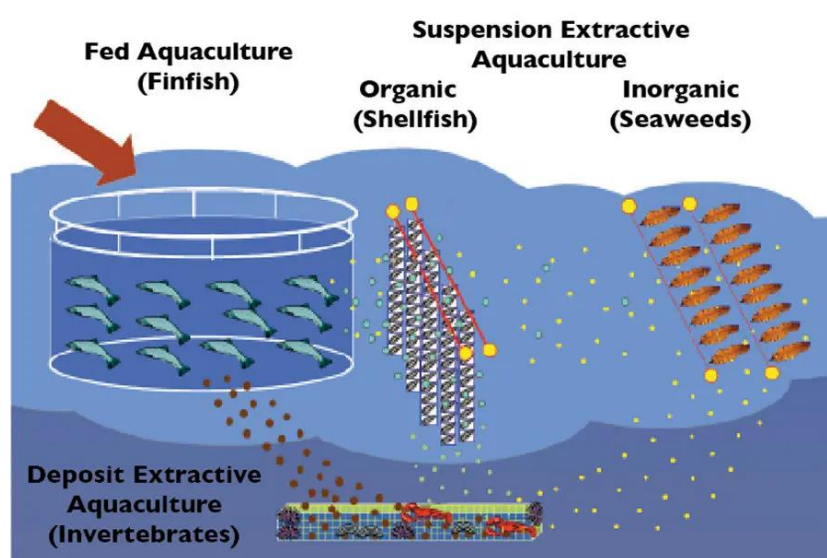
Figure 1.13. Conceptual diagram of an integrated multi-trophic aquaculture (IMTA) operation including the combination of fed aquaculture (e.g., finfish) with extractive species cultivation (e.g., primary or secondary producers) (Chopin et al., 2010).

[54]

In a nutshell, the overall aim of IMTA is to ecologically engineer food systems for environmental sustainability (e.g., delivery of bioremediation services for improved ecosystem welfare), economic stability (e.g., improved output, lower costs, product diversification, risk reduction and job creation in coastal and rural communities) and societal acceptability (e.g., better management practices, improved regulatory governance and appreciation of differentiated and safe products) (Chopin et al., 2012).

The inception of modern integrated mariculture traces back to 1972, when Ryther and colleagues adopted this scenario to address eutrophication issues arose from increased aquaculture nutrient release (Ryther et al., 1972). Over the last decades, the integrated aquaculture concept has been used to somehow mitigate the excessive nutrient/organic loading generated by intensive aquacultures in countries such as Australia, Canada, Chile, China, France, Japan, New Zealand, Scotland Thailand, and USA (Granada et al., 2016; Neori et al., 2004). Nowadays, it serves as a response to the global demand for seafood while assisting the sustainable expansion of aquaculture in coastal and marine ecosystems (Rosa et al., 2020).

1.3.2. Marine organisms with potential for IMTA production

Over the years, various organisms have been co-cultivated in the proximity of marine fish farms to be investigated as potential IMTA candidates. The integration of seaweeds is probably the most popular one, due to their high biomass buildup and productivity, low-cost demands for their cultivation, as well as their diverse commercial incorporation in animal feeds, fertilizers, and biofuels (Granada et al., 2016; Neori et al., 2004; Rosa et al., 2020). However, as photosynthetic organisms, biofiltration in seaweeds is activated when nutrients (N, P, C) are dissolved in the environment (Granada et al., 2016).

A valid alternative for nutrient and organic bioremediation is the use of marine animals. The reduction of excess nutrients, as well as microbial pollution and suspended solids within aquaculture can be achieved by the use of appropriately selected marine invertebrates. The bioremediation capacity of these organisms has recently attracted much interest. For instance, a variety of deposit or detritus feeders have been considered as potential IMTA components, such as crustaceans, sea cucumbers and polychaetes. In addition, suspension-feeding macroinvertebrates can be similarly adapted given their efficiency in filtering large volumes of water and retaining suspended particles, required for their food demands. Besides biological particles (e.g., microalgae, bacteria) the specific animals have also been proved to remediate inorganic substances, such as heavy metals and nutrients, as well as dissolved organic matter, including hydrocarbons and persistent organic pollutants. Among the proposed suspension feeders, mollusks and bivalves (e.g., mussels and oysters) have attracted most of the research effort as candidates for IMTA applications. Marine sponges, on the other hand, have been notably

understudied to this end. Although the latter constitute capable biofilters and the idea of integrated sponge/fish aquaculture has been discussed intensively during the past few years, their incorporation in such systems is still pretty new and unexplored (Granada et al., 2016; Rosa et al., 2020).

1.3.3. Marine sponges as promising candidates for integrated aquaculture

1.3.3.1. Sponge Physiology

The phylum Porifera, commonly known as sponges, comprises the earliest extant branch of multicellular animals on Earth, which diverged from the animal kingdom and evolved since early times (more than 500 million years ago) (Borisenko et al., 2019; Ereskovsky, 2010; Immanuel et al., 2015). The poriferan phylum is characterized by a notable diversity, with more than 9500 valid species (de Voogd et al., 2024) inhabiting benthic communities of oceans and seas, as well as freshwater environments, such as ponds, lakes, and streams (Gardères et al., 2016; Manconi and Pronzato, 2016). Sponges can vary in size and morphology; they can be massive and spherical, thin and encrusting, tall and tubular, along with many variations on these forms (Leys and Hill, 2012). Based on histological, skeletal and developmental characteristics, the phylum Porifera is generally divided into four classes; Calcarea, Demospongiae, Hexactinellida and Homoscleromorpha. In summary, Calcarea sponges are distinguished for their calcareous (calcite) skeleton, while Hexactinellida involves syncytial glass sponges, whose skeleton consists of silicone dioxide. The taxa Homoscleromorpha comprises sponge species possessing a basement membrane and metazoan-like cell junctions, whereas Demospongiae are the "common", most diversified taxa in terms of species (85% of all sponge species) and habitats (Borisenko et al., 2019; Manconi and Pronzato, 2015; Renard et al., 2013).

Sponges constitute morphologically simple, sessile organisms that lack a nervous, digestive or circulating system (Borisenko et al., 2019; Immanuel et al., 2015). In order to fulfill their food and oxygen demands, as well as other functions involving growth, reproduction and waste removal, sponges rely on actively induced, constant water flow through their bodies (Immanuel et al., 2015; Leys and Meech, 2011). Consequently, they are regarded as singular biological filters, with the entire body architecture being arranged around their aquiferous system. This comprises a complex network of canals and chambers harboring specialized cells that function in transport of water, while efficiently retaining a large variety of waterborne organic substances. Similarly to other filter-feeding organisms, sponges' diet includes dissolved organic matter, suspended particles, phytoplankton (e.g., diatoms), picoplankton such as bacteria, or fungi, as well as viruses present in the marine environment (Gardères et al., 2016; Gökalp et al., 2021; Hadas et al., 2006; Kowalke, 2000; Manconi and Pronzato, 2015).

A typical poriferan body structure involves an external layer of flat cells (pinacocytes), pierced with small inhalant apertures (ostia; formed by porocytes) and larger exhalant apertures (oscula),

comprising the pinacocyte epithelium (pinacoderm). This isolates the sponge internal structure (mesohyl) from the external environment. The mesohyl includes an extracellular matrix with the consistency of jelly, collagen fibrils or fibers (spongin), skeletal structures with mineral deposits (spicules), and cells. The water flows via numerous ostia into branching channels, which end in choanocyte chambers generating the water flow. From these chambers, water and wastes are discharged via outflowing channels into one or several osculae (Kowalke, 2000; Manconi and Pronzato, 2015; Renard et al., 2013).

Organic matter enters the sponge via diffusion, epithelial pinocytosis, and phagocytosis, all facilitated by water pumping at transport rates ranging from 0.002 to 0.84 cm_{water}^3 cm_{sponge}^{-3} s^{-1} (Maldonado et al., 2012; Simpson, 1984). Depending on the feed particle size, uptake is performed at three different levels;

a) The maximum size of filterable particles is determined by the diameter of ostia, which is typically around 50 μm. Large particles (>50 μm) that are not able to enter the ostia are engulfed on the sponge surface by epithelial pinacocytes (pinocytosis).

b) Smaller particles (<50 μm) capable of entering the ostia are taken up by pinacocytes lining the progressively narrower canal walls. The smallest particles (<5 μm size) are filtered by the bases of the choanocytes, which format the filtering chambers.
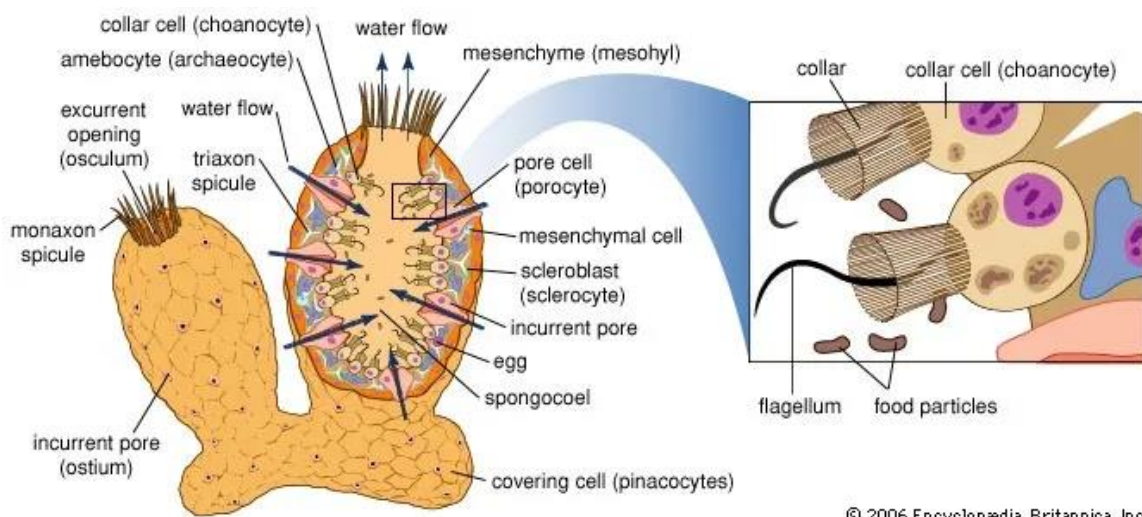
Figure 1.14. The body architecture and the aquiferous system of a sponge (left). Structure of choanocytes, the typical feeding cells of Porifera (right). These flagellated cells are able to produce water flow within the aquiferous system and catch very small (a few micrometers) suspended organic particles. Ingested food is then transferred to mobile archeocytes (Manconi and Pronzato, 2015) (Image adopted from *Encyclopedia Britannica, 2006. https://www.britannica.com/animal/sponge-animal/Form-and-function*).

Choanocytes are very small (2–10 mm in diameter), specialized cells with a flagellum surrounded by a 'collar' of microvilli (i.e., finger-like actin-filled extensions that arise from the cell membrane). Food particles as small as 1 μm are captured via phagocytosis

[57]

by pseudopodia at the choanocyte. After uptake by pinacocytes and choanocytes, food particles in incipient digestion stage are passed to the mesohyl cells by transcytosis. Once inside the mesohyl, particles are phagocytosed by the mobile sponge cells, termed archaeocytes (amebocytes), which complete digestion and deliver the resulting assimilable compounds to other cells in the sponge body.

c) Particles down to 0.1 μm (e.g., bacteria) are sieved by microvilli (Kowalke, 2000; Leys and Hill, 2012; Maldonado et al., 2012; Manconi and Pronzato, 2015).

Water flow through the sponges is achieved by the coordinated beating of many thousands of flagella along the choanosomal tissue. The beat of flagellum actively generates a low pressure at its base, driving a unidirectional water current from the inhalant sponge surface (ostia), through the entire body, up to the exhalant apertures (oscula) (Leys and Hill, 2012; Manconi and Pronzato, 2015).

From an ecological viewpoint, sponges constitute key components of aquatic communities given their multiple functional roles in marine ecosystems. Their substantial water filtering capabilities allow constant circulation and clean-up of the water column, by efficiently retaining particulate and dissolved organic matter. Meanwhile, they serve as living shelters for several organisms, while contributing to primary production through their symbiosis with autotrophic microorganisms. Moreover, their siliceous skeleton is involved in the formation of sediments after the death of sponges (Manconi and Pronzato, 2015).

1.3.3.2. Bioremediation capacity

Figure 1.15. Experimental sponge mariculture deployed adjacent to a fish farm in the Mediterranean Sea for bioremediation purposes (Pronzato et al., 1998).

The filter-feeding properties of sponges have attracted much research interest during the last century (Jørgensen, 1955, 1949; Henry M. Reiswig, 1971; H. M. Reiswig, 1971; Riisgård et al., 1993). In the light of their immense water processing rates (which can reach up to 50 L per gram of sponge tissue per day; Weisz et al., 2008), their high retention efficiencies (ranging from 75–99%; Pile and Witman, 1996; Henry M. Reiswig, 1971; Reiswig, 1974; Wilkinson et al., 1984), as well as their large natural distribution in marine environments, combined with their tolerance in extreme climate scenarios (Bell et al., 2017; Pawlik et al., 2016), they can considerably improve water quality of oceans and seas. Indeed, they have been proved to be efficient suspension feeders on a wide variety of organic waterborne substances, ranging from multi-size particles (0.1–50 μm;

Maldonado et al., 2010; Osinga et al., 1999; Pile and Witman, 1996; Henry M. Reiswig, 1971; Ribes et al., 1999), to dissolved organic matter (de Goeij et al., 2013; Goeij et al., 2008; Yahel et al., 2003). Remarkably, carbon uptake rates of sponges were found to be within the same range or even exceed the uptake rates of other, well-established filter-feeders, such as bivalves (29–1970 mg C m^{-2} d^{-1} for bivalves vs 9–3621 mg C m^{-2} d^{-1} for sponges; Riisgård and Larsen, 2000). This prompted the idea to use sponges for the remediation of organic pollution caused by aquaculture cages and urban environments (e.g., sewage). Relevant studies include results from either laboratory-based or *in situ* farming experiments, both demonstrating the added value of sponges, as it will be further investigated in the present thesis.

The ability of the Redbeard sponge to ameliorate microbial polluting assemblages associated with faecal contamination is well known sixty years now (Claus et al., 1967). In 1998, Pronzato and co-workers were the first to consider sponges as an extractive component in integrated aquaculture. Their idea was implemented by the cultivation of bath sponges near floating-cage fish farms in Kalymnos Island, with encouraging growth and survival rates reported (Pronzato et al., 1998). Later on, a plethora of lab-scale studies investigated sponges as selective bioremediators in integrated aquaculture by testing them against different biological pollutants and experimental setups.

Specifically, the Mediterranean sponge species *Chondrilla nucula* was able to retain up to 70 billion *Escherichia coli* cells per m^2 of sponge surface area, by efficiently clearing 14 L of water per hour (Milanese et al., 2003). Similarly to the previous study, Fu et al. (2006) reported removal of the bacteria *E. coli* and *Vibrio anguillarum* by the sponge *Hymeniacidon perlevis*, which could filter up to 8 million *E. coli* cells per gram of fresh sponge within an hour. Moreover, the Mediterranean sponge *Spongia officinalis* var. *adriatica*, commonly known as bath sponge, was highly capable of feeding on bacterioplankton, with a maximum clearance rate of 210 mL h^{-1} g^{-1} and retention efficiency of 61% within 2 hours, when used for bioremediation purposes (Stabili et al., 2006). The same sponge species was later proved to efficiently accumulate different bacterial groups (culturable heterotrophic bacteria, total culturable bacteria at 37 $\circ$C, culturable vibrios, total and fecal coliforms and fecal streptococci) by rearing specimens in sites with diverse levels of microbial contamination (Stabili et al., 2008). Similarly, the Mediterranean sponge species *Aplysina aerophoba* retained high numbers of bacterial isolates and microbial seawater consortia in a flow-through system (Wehrl et al., 2007). *Hymeniacidon perlevis* was indicated as a good bioremediator of bacteria pollution generated in a 1.5-m^3 turbot (*Scophthalmus maximus*) aquaculture system, by removing 60.0–90.2% of faecal coliform bacteria, 37.6–81.6% of pathogenic *Vibrio* spp. and 45.1–83.9% of the total bacteria (Zhang et al., 2010). In a similar laboratory study, *H. perlevis* was capable of accumulating, remediating, and metabolizing halophilic *Vibrio* spp., heterotrophic bacteria, total culturable bacteria, faecal coliforms and faecal *Streptococci* (Longo et al., 2010). Contrarily, *H. perlevis* was found to be a selective filter-feeder among three common pathogenic

[59]

microbes, with higher preference on *E. coli* and the yeast *Rhodotorula* sp., rather than *V. anguillarum* (Maldonado et al., 2010). Promising bioremediation findings were also reported for the sponge *Sarcotragus spinosulus*, as it could remove more than 99% of suspended *Vibrio parahaemolyticus* from seawater, while contributing to the nutrient load (Trani et al., 2021). Besides the uptake of harmful bacteria, sponges have been proposed as a tool for mitigating (harmful) algal blooms, the presence of which has been associated with the loss of suspension feeders in the aquatic environments (Peterson et al., 2006; Wall et al., 2012).

Field sponge farming experiments have further demonstrated the beneficial effects of mariculture/urban effluents on sponge survival and growth due to the increased food availability. For instance, Ledda and colleagues (2014) reported the highest particle retention and clean-up capacity of the western Mediterranean sponges *Ircinia variabilis* and *Agelas oroides* when cultivated at microbially polluted sites, affected by wastewater, urban runoff and marine recreational activities. Other pilot-scale *in situ* studies have confirmed that sponges can benefit from the increased availability of food in the vicinity of mariculture farms. Cultivations of *Dysidea avara* explants (i.e., regrown clones cut from parent sponges) placed under seabass aquaculture cages in Southwest Turkey exhibited 100% survival and doubled in size within four months, whereas explants cultured under pristine conditions showed decreased growth (up to 0–50% per year) (Osinga et al., 2010). Similarly, *Chondrosia reniformis* explants cultured on suspended PVC plates in the proximity of fish farms wastewater achieved a better annual survival and growth rate (86% and 170%, respectively) than explants grown at a pristine site (39% survival and 79% growth) (Gökalp et al., 2019).

In addition to capturing organic particles related to anthropogenic pollution, studies have also indicated the ability of marine sponges to uptake individual hazardous chemical pollutants. For example, an aquaria-based experimental study revealed a 10-times faster reduction of the detergent 1-(p-sulfophenyl)nonane in the presence of *S. officinalis* sponges, rather than in the presence of only marine bacteria alone (Perez et al., 2002). Furthermore, in-field measurements demonstrated the capacity of the abovementioned sponge to accumulate polychlorinated biphenyls (Perez et al., 2003), while a similar behavior was observed for the species *H. perlevis*, which was also found efficient in bioaccumulating PAHs and metallic trace elements (MTEs) (Gentric et al., 2016). Interestingly, the latter study reported that heavy metals, such as Al, Co, Cr, Fe, Pb, and Ti, as well as the PAH member benzo(a)pyrene were more effectively bioaccumulated in *H. perlevis* compared to the oyster *Crassostrea gigas*. Additional studies have also confirmed the bioremediation properties of marine sponges against heavy metals (Cebrian et al., 2007; Gravina et al., 2022; Hansen et al., 1995; Olesen and Weeks, 1994; Orani et al., 2018; Patel et al., 1985; Perez et al., 2005; Philp, 1999).

Intriguingly, recent laboratory experiments revealed a remarkable remediating capacity of sponges against pesticides as well, with *H. perlevis* removing up to 97% of lindane from seawater (1 μg L^{-1}) within 8 days (Aresta et al., 2015). However, lindane-degrading bacteria isolated from *H. perlevis* were suggested to contribute to the removal of this pesticide (Aresta et al., 2015; Loredana et al., 2017). An increasing number of studies also show that sponges are capable of uptaking dissolved organic substrates. These include conventional organic nutrients, such as amino acids (e.g., glycine; Stephens and Schinske, 1961) and sugars (e.g., fructose; Camacho et al., 2006) or substrates passing through 0.2-μm filter (technically included in the dissolved organic pool), such as 0.1 μm nanospheres (Leys and Meech, 2011) and viruses (Hadas et al., 2006). However, no data exist to date to suggest that sponges can uptake dissolved organic pollutants generated from intensive fish farm activities.

1.3.3.3. Bioproduction potential

The inclusion of sponges in IMTA applications has become even more appealing in recent years, since they hold great biotechnological potential and have their own commercial value as bioproducts, in addition to their high bioremediation efficiency. Reports indicate the use of sponges in medicine since ancient times (Voultsiadou, 2005a), while its application in surgery was pretty common until the end of the nineteenth century (Müller et al., 2004). For instance, sponges soaked with extracts of opium were used to anaesthetize patients prior to surgery, and extracts of *Spongia tosta* were found to be effective in the treatment of scrofula (Müller et al., 2004).

Figure 1.16. Commercial mariculture systems for "bath sponges" production in Zanzibar (top) and Micronesia (bottom). Adopted from maricultures.org (*https://www.marinecultures.org/en/projects/spongefarming/spongefarming/*) and MERIP (*https://www.meripmicronesia.org/sponge-farming/*).

Besides their biomedical applications, sponges also hold commercial value as bath utensils due to their absorptive properties, and their exploitation for this purpose dates back thousands of years (Pronzato and Manconi, 2008; Schippers et al., 2012). The so-called "bath sponges" concern species with spongin skeleton, such as the widely distributed Mediterranean sponges *Hippospongia communis*, *S. officinalis*, *S. lamella*, *S. mollissima* and *S. zimocca* (Duckworth, 2009; Milanese et al., 2008). For over 100 years, researchers have been investigating potential cultivation techniques to develop successful and economically viable farming structures, with a particular focus on "bath sponges" (Bierwirth et al., 2022). Until 1960, commercial bath sponge farming was a lucrative business spreading from the Mediterranean to the Atlantic and Pacific Ocean (Bernard, 1968; Duckworth, 2009). From 1960 onwards, the invention of the low-cost,

synthetic sponge materials decelerated the mariculture production of natural bath sponges. Nowadays, it only occupies a small niche in the market for high-quality applications (Hogg et al., 2010), by offering specimens of increasingly high price. Interestingly, the retail price of a medium-size specimen (20–25 cm in diameter) can easily exceed 30 Euros, while the gross market price of Mediterranean bath sponges is calculated to be around 500–1000 Euro per kg dry weight, depending on the quality level (Milanese et al., 2008).

Current biotechnological applications and research on sea-based sponge farming are more directed towards the production of marine natural products (Bierwirth et al., 2022; Duckworth, 2009). Indeed, these sessile metazoans, as seemingly primitive and morphologically defenseless organisms, have developed chemical mechanisms aiding in their communication, growth and protection from a multitude of external environmental pressures (e.g., fouling organisms, predators, spatial competitors, pathogenic microorganisms). In detail, these ingenious survival strategies involve the production and accumulation of numerous physiologically active metabolites, with some of them presenting antibacterial, anticancer, antifungal, anti-HIV, anti-inflammatory, and antimalarial properties. Screening of sponge extracts has revealed an array of biologically active compounds belonging to the class of alkaloids, terpenoids, peptides, polyketides, steroids and macrolides. More than 9500 of them have been identified and isolated to date, rendering sponges, along with their host microbiome, great contributors in the natural products market (~30%) (Chu et al., 2022; Duckworth, 2009; Han et al., 2019).

Research upon sponge bioactive compounds has been initiated since the mid-twentieth century. Bergmann and Feeney were the first to isolate the antiviral nucleosides from the Caribbean sponge *Cryptotethya crypta* in 1951 (Bergmann and Feeney, 1951), which served as scaffolds for the synthesis of the derivatives Ara-A (active against herpes) and Ara-C (effective in leukaemia treatment). This prompted pioneering research on marine natural product drug discovery during the mid-1970s, with marine sponges continuously being the most prolific source of newly discovered bioactive compounds ever since (Schippers et al., 2012). For instance, *Dysidea avara*, *Lissodendoryx sp* and *Mycale hentscheli* are just three of the most famous sponges producing molecules with strong antitumor activity (i.e., avarol, halichondrin B and peloruside A) (Müller et al., 1985; Munro et al., 1999; Page et al., 2011).

In addition to their pharmaceutical potential, a plethora of other applications have been identified for sponge-derived natural products. Sponges, such as *Chondrosia reniformis*, *Axinella cannabina* and *Suberites carnosus* have shown to produce particularly high levels of collagen (>20% of sponge dry weight), which has been documented as one of the most promising sponge-derived biomaterials, with multiple advantages in medical and cosmetic applications. Compared to the conventional collagens derived from terrestrial sources (e.g., bovine or porcine origin), the sponge-

derived collagen has been regarded equivalent or even safer, because it is free of risks associated with Bovine Spongiform Encephalopathy (BSE) (Heinemann et al., 2007; Swatschek et al., 2002; Tziveleka et al., 2017).

Despite their enormous biotechnological potential, only a limited number of sponge-derived natural compounds have been successfully developed into commercially bioproducts (See **Table 1.6**). A major obstacle is the lack of sufficient supply of biological material required for preclinical and clinical development, which is widely recognized as "The Supply Problem". Although controlled synthesis of bioactive molecules or their related analogues (e.g., via chemical or microbial processes) has always been the preferred method for drug discovery, most marine metabolites are characterized by high structural complexity and their synthesis is often challenging and economically unfeasible, even for the purpose of preclinical testing (Carballo et al., 2010; Schippers et al., 2012; Sipkema et al., 2005).

Table 1.6. List of sponge metabolites resulting from drug discovery, that have advanced to clinical trials (adopted from Schippers et al., 2012).

Metabolite	Source	Status	Target	Developed by
In clinical use				
E7389 (halichondrin B derivative)	*Lissodendoryx* sp. and *Halichondria okadai*	Phase III (cancer)	Tubulin	Eisai
HMR–4011A (aka IPL–576,092, contignasterol derivative)	*Neopetrosia contignata*	Phase II (anti-asthmatic)	Histamine	Aventis
Zalypsis (renieramycin derivative)	*Xestospongia* sp.	Phase I (cancer)	DNA	Pharmamar
E7974 (hemiasterlin derivative)	*Cymbastela* sp. and *Auletta* sp.	Phase I (cancer)	Tubulin	Eisai
Taltobulin (aka HTI–286, hemiasterlin derivative)	*Cymbastela* sp. and *Auletta* sp.	Phase I (cancer)	Tubulin	Wyeth-Andersen
KRN–7000 (agelasphin derivative)	*Agelas mauritianus*	Phase I (cancer)	NKT	Koezuka-Kirin
LBH 589 (psammaplin derivative)	*Psammaplinaplysilla* sp.	Phase I (cancer)	HDAC	Novartis
Debromohymenialdisine	*Stylotella aurantium*	Phase I (Alzheimer's)	Kinases	Genzyme Tissue Repair
Peloruside A	*Mycale hentscheli*	Preclinical (cancer)	Tubulin	Reata Pharmaceuticals
Discontinued from clinical trials				
Cryptophycin 52 (=arenastatin)	*Dysidea arenaria*	Phase II (cancer)	Tubulin	Lilly, Valeriote
Manoalide	*Luffariella variabilis*	Phase II (antipsoriatic)	Calcium channel	Allergan
Discodermalide	*Discodermia dissoluta*	Phase I (cancer)	Tubulin	Novartis, HBOI
LAF 389 (bengamide derivative)	*Jaspis* sp.	Phase I (cancer)	MetAP	Novartis, Crews
LAQ 824 (psammaplin derivative)	*Psammaplinaplysilla* sp.	Phase I (cancer)	HDAC	Novartis, Crews
Girolline (aka girodazole)	*Cymbastela cantharella*	Phase I (cancer)	Protein synthesis	Potier

Investments in establishing a synthesis route for complex molecules are usually not done until the potency of the compound has been sufficiently proven (i.e., after completion of preclinical development studies or early clinical trials). As a result, material obtained from natural sources is often needed for preclinical development. Since many of these natural compounds are commonly found at trace levels within a sponge (e.g., concentrations of milligrams per kilogram of sponge biomass), the amount of sponge biomass required is simply too high to provide sufficient biomolecule quantities. Consequently, wild harvesting entails valid concerns for the conservation of sponges diversity in marine ecosystems (e.g., disturbance of population density, extinction of the respective species), rendering this

[63]

strategy as unsuitable (Belarbi et al., 2003; Carballo et al., 2010; Duckworth, 2009; Page et al., 2011; Schippers et al., 2012). Other methods, such as cell lines, primmorphs and *ex situ* cultures have extensively been investigated as economically feasible approaches for obtaining sufficient quantities of drug-lead sponge molecules, but they have shown a number of limitations (e.g., high time and resources consumption, poor growth rates) (De Rosa et al., 2003; Hadas et al., 2005; W. E. Müller et al., 2000).

1.3.3.4. The need for sponge mariculture

Among the tested strategies, cultivation of marine sponges that produce the biotechnologically interesting compounds emerges as a viable method to overcome the supply issue. To date, a multitude of mariculture trials has been reported for the production of sponge biomass and further extraction of bioactive compounds for drug development (Duckworth, 2009; Pronzato and Manconi, 2008).

Sponge farming is a simple, low-cost, ecologically and environmentally sustainable solution to exploit the plant-like regeneration properties and resilience of sponges to overcome physical damage. In combination with their innate bioremediation capacity, sponges inclusion in IMTA systems has become even more appealing during the last decade (Bierwirth et al., 2022; Giangrande et al., 2020; Gökalp et al., 2022, 2019; Pronzato and Manconi, 2008).

A typical sponge mariculture (also termed as *in situ* culture) is conducted by growing sponges on man-made constructions under the sea, such as longlines with scallop lanterns, vertical ropes, underwater platforms, frames and cages. To set up a sponge cultivation, donor sponges obtained from natural populations are usually fragmented artificially, by cutting them into smaller pieces (i.e., explants). The explants are further secured to an artificial substrate, such as, ropes, nylon lines or plastic plates, or are positioned into nets or cages (Duckworth, 2009; Schippers et al., 2012). By this mean, the conservation of targeted species can be promoted, by minimizing the extraction of biological resources and, thus, the exertion of pressure to natural populations (Pronzato and Manconi, 2008). Furthermore, the cultured explants have been shown to be equally capable of producing the metabolites of interest, in a similar way to their natural donors (Bergman et al., 2011; Carballo et al., 2010; Osinga et al., 2010; Ternon et al., 2017).

Regardless of the exact purpose (i.e., bath sponges, bioactive compounds supply or bioremediation), a number of about 20 countries has investigated sponge farming in coastal areas around the world (Table A1). Twenty-seven of these studies have been carried out in New Zealand, Australia, Caribbean, Mexico, India, Indonesia, Philippines, Colombia, Tanzania and the Netherlands. Remarkably, a considerable amount of sponge mariculture attempts concerns also Mediterranean countries, such as Italy (i.e., 7), Turkey (i.e., 4), France (i.e., 3), Israel (i.e., 2), Spain (i.e., 1), Croatia (i.e., 1), and Greece (i.e., 1). Among these, only three studies from Italy (Giangrande et al., 2020; Pronzato et

[64]

al., 1998; Stabili et al., 2008) and two from Turkey (Gökalp et al., 2019; Osinga et al., 2010) have applied experimental sponge cultures close to fish farms highlighting the novelty of "Sponge-driven Bioremediation/Bioproduction" concept.

With regard to Greece, research about sponge cultivation, either for biomass exploitation or bioremediation purposes, is practically absent. Considering the thriving aquaculture sector of Greece, as well as the vast diversity of sponges existing in Aegean Sea (Voultsiadou et al., 2016), investigations on the potential role of marine sponges in integrated fish aquaculture systems are rather warranted.

Chapter 2. Thesis Objectives

Integration of sponges in fish aquaculture systems has not been adequately demonstrated and systematically investigated under real-life conditions, especially in Greece. The present doctoral thesis investigates the potential integration of four Cretan demosponges, suitable for cultivation, in fish aquaculture for the improvement of water quality and the delivery of high added-value products. The targeted species were the widely distributed Mediterranean demosponges *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*. Their selection for experimental open-sea cultivation was based on their high natural abundance across East-Mediterranean habitats, as well as their excellent rearing performance. These results were obtained from a prior 2-year field study conducted by diving scientists of HCMR. By deploying an operational multi-species sponge culture in the proximity of fish farm cages in Chania (Crete), this thesis aims:

1) **To evaluate the bioremediation capacity of native sponge species, suitable for integrated aquaculture (Chapters 3 & 4).** The standardization of sponge farming as a bioremediation technology requires extensive primary data regarding the cleanup capacity of the cultivated species. To this end, a part of the explants that were collected for initial farm seeding, were tested through controlled laboratory experiments across an array of typical aquaculture pollutants and conditions.

   More specifically, **Chapter 3** focuses on the *in vitro* bioremediation potential of candidate sponges for aquaculture-related biological pollutants. The specific study involved the exposure of sponge fragments to phytoplanktonic cells, in an effort to simulate sponges' response to eutrophic algal blooms resulting from fish farm discharges. The "model" microorganisms involved an array of microalgae belonging to different taxonomic groups with particular characteristics. Consequently, the following questions were sought to be addressed:

   a. **Are sponges able to be used as a tool for mitigating (harmful) algal blooms? And if so, are they capable of maintaining their filtering activity for a sufficient time span?**

   b. **Are sponges selective filter-feeders on a variety of microalgae exhibiting different cell sizes and motility traits?**

   c. **What is the effect of the initial cell concentration on sponges uptake kinetics? What is the maximum cell concentration that sponges can tolerate without losing much of their cleanup efficiency?**

   d. **What is the effect of day-night changes on sponges filtering performance? Is there an increased feeding activity in the absence of light?**

The *in vitro* bioremediation dataset concludes with **Chapter 4**, in which the uptake of typical aquaculture-related dissolved organic pollutants is examined. This series of *in vitro* experiments involved the exposure of sponge explants to individual harmful chemical pollutants (e.g., antibiotics, antifouling booster biocides, PAHs), and complex organic mixtures (e.g., filtrates of uneaten fish feed and fish excreta), mimicking DOM encountered in aquaculture effluents. Similarly to Chapter 3, corresponding questions were desired to be resolved:

   a. **What is the kinetics dictating the uptake of dissolved organic pollutants by sponges? Are sponges effective filter-feeders on dissolved organic pollutants?**

   e. **Are sponges selective filter-feeders on a variety of pollutants covering different levels of lipophilicity?**

   f. **Do sponges similarly uptake complex dissolved organic mixtures?**

   g. **What is the key mechanism ruling the removal of dissolved organic matter (DOM) from seawater by sponges? Are pollutants being adsorbed on the sponge pinacoderm or digested by choanocytes?**

   h. **Does the DOM uptake constitute a reversible process? Do sponges release the uptaken pollutants back to the environment via desorption or excretory processes?**

2) **The final criteria to evaluate sponge's suitability for integrated aquaculture is the valorisation potential of their cultivated biomass (Chapters 5 & 6).** Based on the *in vitro* bioremediation efficiency across an array of aquaculture-related pollutants, the two best biofilters, *A. oroides* and *S. foetidus*, were selected to be investigated for their biotechnological applications.

In detail, **Chapter 5** discusses the repertoire of targeted bioactive metabolites that were traced and quantified using mass-spectrometry-based methods in farmed sponges. Additionally, the bioactivity of crude sponge extracts was screened within an array of bioassays (e.g., antimicrobial, anticancer). Bioproduction and bioactivities of farmed sponges were compared to those observed for their wild counterparts, in order to evaluate the ability of sponge farming to retain the "natural" production of the metabolites of interest. Furthermore, a primary literature review on the current bioassays used for screening marine extracts (e.g., for antimicrobial, antibiofilm, cytotoxic, antiviral, antioxidant, and anti-ageing acitivity) is provided in **Chapter 6**, with the aim to be employed for the discovery of sponge-derived bioactive compounds as well.

To answer all the addressed questions, a series of traditional analytical methods (HPLC, TOC-L Analysis), in conjunction with high-resolution (LC-MS/MS), and high-throughput ones (microplate-based fluorescent analysis) were performed.

The main novelty of this dissertation comes with the *in situ* application of Greek marine sponges in integrated aquaculture. By taking advantage of the thriving aquaculture sector of Greece, as well as the vast diversity of sponges existing in Aegean Sea, this study intends to establish a solid basis for the investigation of sponge cultivation in Greece and the benefits it could bring to aquaculture enterprises through bioremediation/bioproduction applications.

This is the first study to systematically assess bioremediation capacity over a broad range of sponges, aquaculture-related pollutants, and different experimental setups. Moreover, it involves pioneering research upon dissolved organic pollutants uptake, by scrutinizing the associated kinetics and relations between sponges' filtering performance and pollutants lipophilic nature. The respective patterns are further translated into mathematical expressions, and mechanistic features of DOM uptake are also unraveled.

Moreover, the commercial value of the cultivated sponges *Agelas oroides* and *Sarcotragus foetidus* is firstly demonstrated here, by scrutinizing their metabolic profile and bioactivities. In this context, a comprehensive metabolites/ions/mass fragments library was created, including all the previously reported compounds for both study sponge genera. This massive database served as a tool for the detection of targeted biomolecules in the sponge extracts.

Overall, the present study provides the first tangible results on bioremediation and bioproduction capacity of Greek marine sponges, that can serve as a reference for the promotion of "sponge-driven bioremediation/bioproduction" concept in fish farms. Based on these results, specific native species are proposed as suitable candidates for future large-scale farming in integrated systems.

Chapter 3. Bioremediation capacity: Microalgae
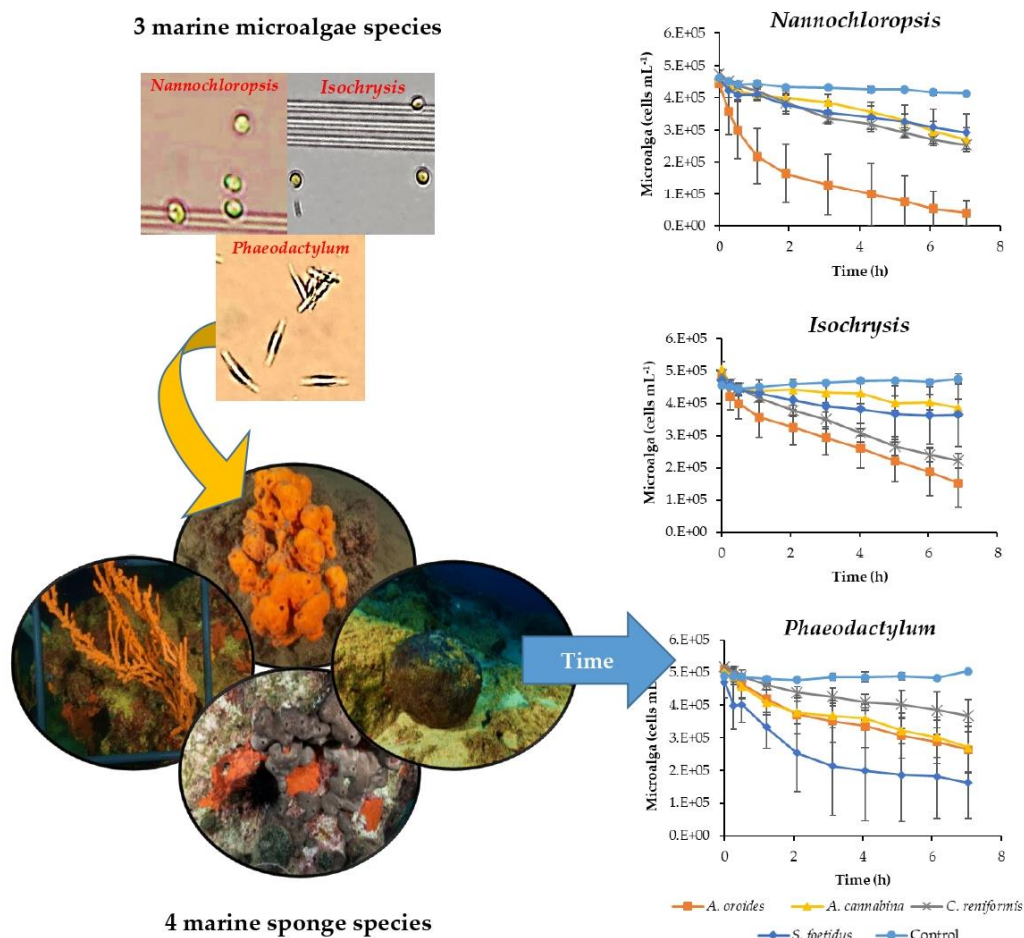
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Despoina Varamogianni-Mamatsi: Formal analysis, Investigation, Writing – original draft, Visualization. **Thekla I. Anastasiou:** Investigation. **Emmanouela Vernadou:** Investigation. **Nikos Papandroulakis:** Resources, Writing—review and editing. **Nicolas Kalogerakis:** Writing – review & editing. **Thanos Dailianis:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Manolis Mandalakis:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

3.1. Abstract

Chronic discharge of surplus organic matter is a typical side effect of fish aquaculture, occasionally leading to coastal eutrophication and excessive phytoplankton growth. Owing to their innate filter-feeding capacity, marine sponges could mitigate environmental impact under integrated multitrophic aquaculture (IMTA) scenarios. Herein, we investigated the clearance capacity of four ubiquitous Mediterranean sponges (*Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*) against three microalgal substrates with different size/motility characteristics: the nanophytoplankton *Nannochloropsis* sp. (~3.2 μm, nonmotile) and *Isochrysis* sp. (~3.8 μm, motile), as well as the diatom *Phaeodactylum tricornutum* (~21.7 μm, nonmotile). *In vitro* cleaning experiments were conducted using sponge explants in 1 L of natural seawater and applying different microalgal cell concentrations under light/dark conditions. The investigated sponges exhibited a wide range of retention efficiencies for the different phytoplankton cells, with the lowest average values found for *A. cannabina* (37%) and the highest for *A. oroides* (70%). The latter could filter up to 14.0 mL seawater per hour and gram of sponge wet weight, by retaining 100% of *Isochrysis* at a density of 10^5 cells mL^{-1}, under darkness. Our results highlight differences in filtering capacity among sponge species and preferences for microalgal substrates with distinct size and motility traits.

Keywords: Mediterranean sponges; microalgae; filtering capacity; bioremediation; integrated aquaculture; cell retention

3.2. Introduction

With the gradual increase of global population, and consequently of fish food demand, the aquaculture industry has rapidly expanded over the last few decades (FAO, 2020). As a result, high organic and nutrient loadings, generated from aquaculture activities (e.g., feed wastage, fish excretion, and fecal production), are continuously being released into the seawater (Wu, 1995), occasionally causing detrimental effects in the surrounding environment, such as toxic algal blooms, eutrophication and anoxia (Buschmann et al., 2008). Such effects are particularly likely at aquaculture settings located in sheltered areas such as gulfs and bays (Belias et al., 2003).

The Integrated Multi-Trophic Aquaculture (IMTA) concept is a newly introduced approach, firstly developed in Asia (Neori et al., 2004), in which the by-products from one species are recycled to become input for another, thus minimizing the environmental impact caused by intensive aquaculture practices. These systems include the aquaculture unit of a main farmed species (e.g., finfish) in proximity with the rearing of secondary species, typically belonging to lower trophic levels, such as filter-feeders (Barrington et al., 2009).

Sponges (Porifera), the oldest extant filter-feeding macroinvertebrates (Reitner and Wörheide, 2002), have recently been viewed as promising candidates for IMTA scenarios (Milanese et al., 2003; Stabili et al., 2006) due to their capability to filter large volumes of water (Osinga et al., 1999; H. M. Reiswig, 1971; Vogel, 1977) and retain microorganisms or other particles of various sizes, ranging from 0.1 to 50 μm (Larsen and Riisgård, 1994; Pile and Witman, 1996; Ribes et al., 1999), with high efficiency (75–99%) (Pile and Witman, 1996; H. M. Reiswig, 1971; Reiswig, 1975; Wilkinson et al., 1984). Having developed intricate patterns of symbiotic associations with microbial communities, sponges are among the most diverse and complex holobionts in the marine environment (Pita et al., 2018) and possess unique feeding mechanisms. Associated microbiomes render sponges capable of distinct nutritional strategies that extend beyond the standard heterotrophy described above. Hence, depending on the species and environmental parameters, sponges can benefit from photosynthesis (Wilkinson et al., 1984), or feed on dissolved organic matter (DOM) (Goeij et al., 2008). In addition to these appealing characteristics, sponges do sustain a "gold mine" of bioactive compounds with pharmaceutical (Sipkema et al., 2005) and cosmetic potential (Swatschek et al., 2002), while the biomass of some species can be exploited for the production of bath sponges (Brümmer and Nickel, 2003). By offering several valorization opportunities, cultivation of sponges can become an extra source of profit for fish farmers and, thus, their inclusion in IMTA systems is rather tempting.

The first study investigating microalgae as test particles in sponge filtering experiments was conducted by Frost (Frost, 1978). Therein, the clearance effect of the freshwater sponge *Spongilla lacustris* was examined upon the unicellular green microalga *Chlamydomonas reinhardtii*, among other microbial species, and it was found to be capable of filtering up to 0.055 mL of water per second and per gram of sponge wet weight. In a later study, Riisgård et al. (1993) related the filtering activity and pumping energy cost of the marine sponge *Halichondria panicea* with temperature, after experimenting with flagellated cells of the microalgal species *Rhodomonas* sp. as test particles (Riisgård et al., 1993). By using flow cytometry, Pile and Witman (1996) investigated *in situ* feeding of the boreal sponge *Mycale lingua* on heterotrophic and autotrophic plankton and found that 86% of the autotrophic eucaryotes of 3 to 10 μm, can be efficiently retained (Pile and Witman, 1996). Similarly, Ribes et al. (1999) studied *in situ* the natural diet of the marine sponge *Dysidea avara* (Schmidt) throughout an annual cycle, and they concluded that microalgae constitute a significant percentage of the marine sponge diet, with pico- and nanoeucaryotes contributing 11 ± 3% and larger phytoplankton accounting for 11 ± 10% (Ribes et al., 1999). An interesting finding was also reported by Osinga et al. (2001), who tested the tropical sponge *Pseudosuberites* (aff.) *andrewsi* with a wide range of microalgal cell concentrations of the marine species *Dunaliella tertiolecta* (5–8 μm) under laboratory conditions. It was demonstrated that the sponge filtration

rate dropped dramatically at concentrations higher than approximately 4×10^5 cells mL^{-1} (Osinga et al., 2001).

However, none of these cases conceptualized marine sponges as living bioremediation agents and specifically aimed to compare the filtering power of various species for the reduction of phytoplanktonic biomass near aquaculture facilities. Until now, the majority of research was focused on the removal of bacteria from seawater (Fu et al., 2006; Ledda et al., 2014; Longo et al., 2010; Milanese et al., 2003; Stabili et al., 2006). In this study, we investigate the inherent filtering capacity and selectivity of four Mediterranean marine demosponges thriving in Greek waters, namely *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*, against three representative marine microalgae species of different size and motility characteristics: *Nannochloropsis* sp., *Isochrysis* sp. and *Phaeodactylum tricornutum*. In addition, we assess the reproducibility of sponges' cleaning performance, and we examine how the initial cell concentration in seawater and light intensity can affect filtering capacity. Our results provide valuable insights into the suitability of sponge species as bioremediators in IMTA systems, or other impacted environments with high microalgae loading. To the best of our knowledge, this is the first report to systematically assess filtering activity over a broad range of sponges, microalgal substrates and different experimental setups.

3.3. Materials and Methods

3.3.1. Sponge Species Studied

Four Mediterranean sponge species were examined for their filtering activity: (a) *Agelas oroides*, (b) *Axinella cannabina*, (c) *Chondrosia reniformis*, and (d) *Sarcotragus foetidus*. The selection of these species was based on their high abundance (occurrence at high densities) in local natural populations, as well as their massive or erect growth form, since body size has been found to be the major determinant of pumping rates in sponges (Morganti et al., 2019).

Agelas oroides Schmidt, 1864 (Agelasida: Agelasidae) is a common massive Mediterranean demosponge, with vivid orange colour and irregular to lobate-digitate shape. Its height varies from 5–25 cm and it can be typically found in 2–40 m water depths, preferably in habitats with low light intensity (Ferretti et al., 2009; Idan et al., 2020).

Axinella cannabina Esper, 1794 (Axinellida: Axinellidae) is an erect-form sponge, with irregular branches emerging from its body and inner canals 1−3 mm in diameter. It can reach 55 cm in height (Koukouras et al., 1996). It is native in the Mediterranean Sea, with increasing occurrence along its eastern basin (Gerovasileiou et al., 2015).

Chondrosia reniformis Nardo, 1847 (Chondrosiida: Chondorosidae) is acknowledged for its unusual collagenous texture and regenerative properties, which are of biotechnological interest. In contrast to other demosponges, this particular species lacks of skeletal siliceous spicules and spongin fibers (Nickel and Brümmer, 2003). It can generate outgrowths that can extend from the parental body for up to 3 m (Bonasoro et al., 2001). It inhabits shaded rocky cliffs or caves at a depth of 1–50 m (Fassini et al., 2012).

The sponge *Sarcotragus foetidus* Schmidt, 1862 (Dictyoceratida: Irciniidae) is a medium grey, black or brown demosponge species, which approximates a globular body form. It is quite abundant and one of the largest sponges in Mediterranean coastal ecosystems, typically reaching 1 m in diameter and 50 cm in height with large oscules (0.5–1 cm in diameter) (Manconi et al., 2013).

The selected species represent distinct sponge growth forms (lobate/digitate for *A. oroides*, erect for *A. cannabina*, thickly encrusting for *C. reniformis* and massive for *S. foetidus*). Moreover, they reflect different symbiotic patterns: *A. oroides*, *C. reniformis* and *S. foetidus* are high microbial abundance (HMA) sponges, while *A. cannabina* is a low microbial abundance (LMA) sponge (Moitinho-Silva et al., 2017). Finally, some contrasting ecological traits occur among the species. For example, *S. foetidus* is predominantly photophilous (Enrichetti et al., 2020), while *A. oroides* strictly sciaphilous (Gerovasileiou et al., 2017; Grenier et al., 2018; Idan et al., 2020). Moreover, *A. cannabina* inhabits deeper water (>20 m depth) (Katagan et al., 2015), while the distribution of *C. reniformis* and *S. foetidus* starts from shallow waters (Fassini et al., 2012; Manconi et al., 2013).

3.3.2. Sponge Sampling

Sponge specimens of the four species were collected in February 2020 from natural populations in two locations in NW Crete, Greece: Stavros (35.588°; 24.075°) for *C. reniformis* and *S. foetidus*, and Souda bay (35.478°; 24.107°) for *A. oroides* and *A. cannabina*. Collection was performed selectively by diving scientists and care was taken to partially collect excess biomass, thus leaving the donor individuals to regenerate. Identification was performed *in situ* during collection, based on external morphological characters typical for the species. Tissue samples from the candidate species were examined to confirm identification with observation of skeletal features under an optical microscope. Photos of representative individuals of the four studied species at the collection sites are provided in **Figure 3.1**.
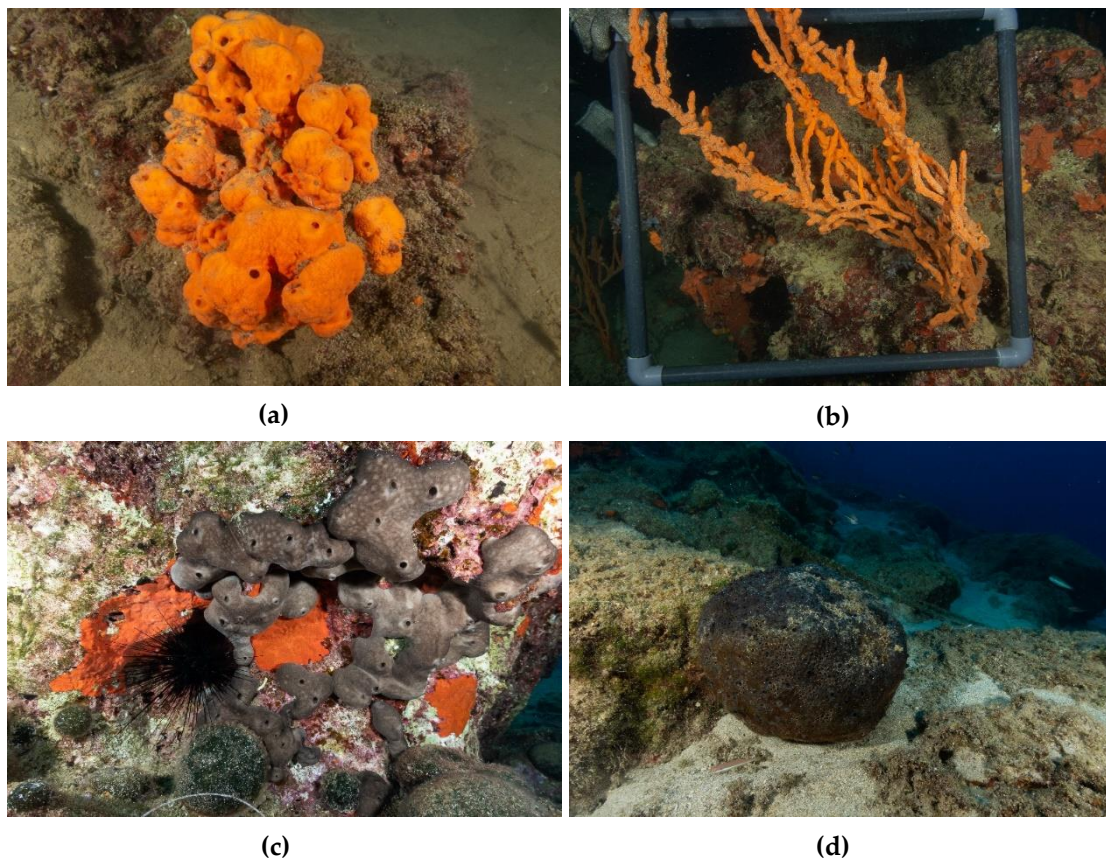
Figure 3.1. Photos of representative individuals of (a) *Agelas oroides*, (b) *Axinella cannabina*, (c) *Chondrosia reniformis* and (d) *Sarcotragus foetidus* taken from their collection sites.

Collected specimens were transported live in cool boxes with controlled temperature to the Underwater Biotechnological Park (UBPC) of the Hellenic Centre for Marine Research (35.346°; 25.278°), an underwater experimental facility in the open sea. Subsequently, sponges were cut in fragments of approximately 100 g and kept for 3 months in UBPC for regeneration and healing. After the regeneration phase, they were transferred in land-based tanks with continuous flow of filtered NSW (**Figure 3.2**) under controlled conditions, resembling local environmental characteristics at the time of transfer (T = 20 °C, pH = 7.6–7.9, Salinity = 39). NSW was passing through a UV disinfection system prior to entering the tank to keep microorganisms at low levels. Before the onset of experiments, the sponges were acclimatized in the tanks for 2 weeks, during which temperature, pH, and salinity were daily monitored to ensure stable conditions. In addition, the wet weight of each sponge explant was measured at the nearest 0.1 g prior and after the conclusion of the experiments to verify that their weight remained consistent throughout the experiments (see Table A2).
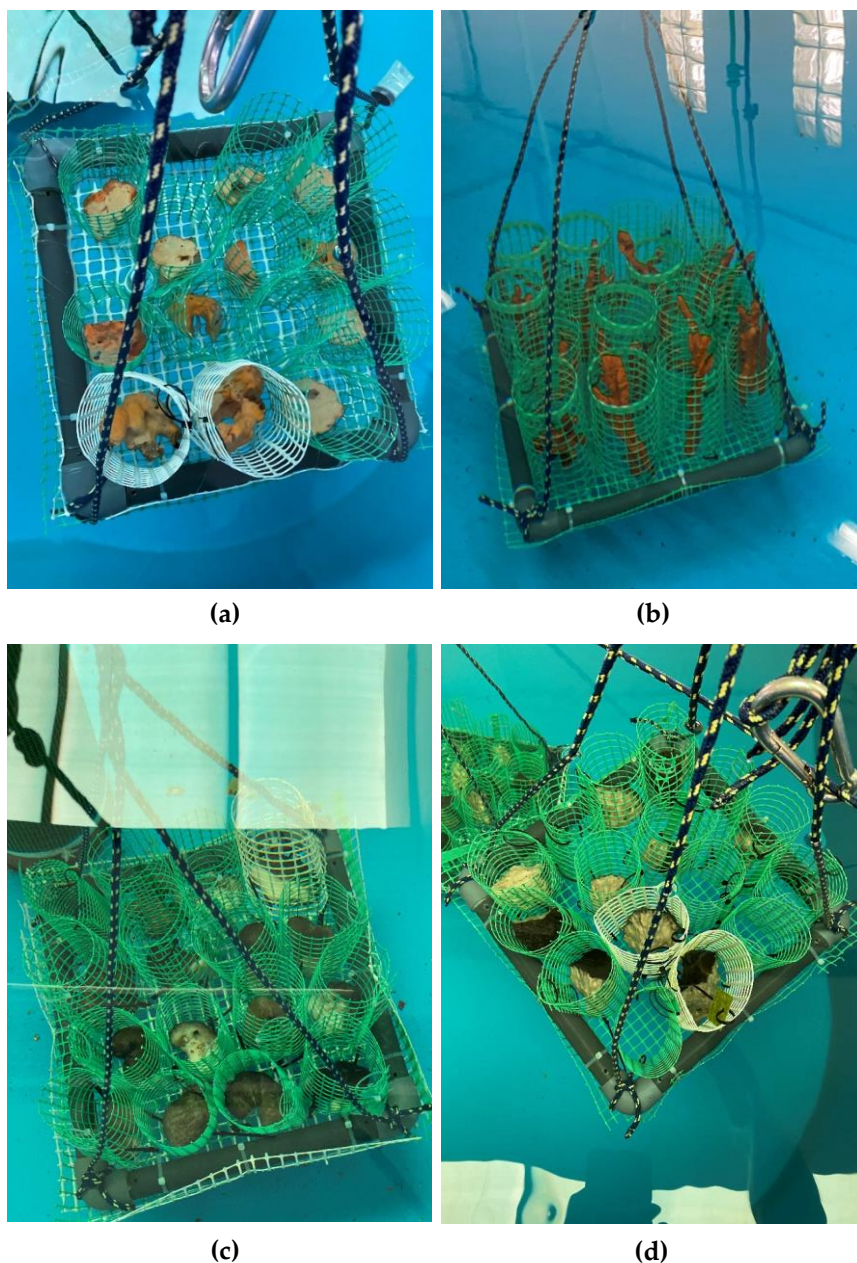
Figure 3.2. Photos of the regenerated fragments of (a) *Agelas oroides*, (b) *Axinella cannabina*, (c) *Chondrosia reniformis* and (d) *Sarcotragus foetidus* in the land-based tanks.

3.3.3. Biological Substrates

The lab-based experiments were conducted with the aim to investigate sponges' filtering activity against three marine microalgae species, each one having different sizes and motility characteristics (**Figure 3.3**). One of them was *Nannochloropsis*, which is a green, unicellular, nonmotile microalga belonging to the class Eustigmatophyceae, order Eustigmatales, and family Eustigmataceae. According to Ma et al., the shape of its cell is oval to round, varying in size from 2 to 8 μm, and it has plastids similar to plant cells (Ma et al., 2016). In addition, the golden-brown marine flagellated alga *Isochrysis* (class Prymnesiophyceae, order Isochrysidales, family Isochrysidaceae) was selected as being

motile, yet having a small size similar to that of *Nannochloropsis* (4–6 μm) (Martínez-Fernández et al., 2004; Sadovskaya et al., 2014). It is characterized by fast growth rate, as well as wide temperature and salinity tolerance (Matos et al., 2019). The much larger *Phaeodactylum tricornutum* (18–26 μm), a unicellular alga belonging to the class of Bacillariophyceae, order Phaeodactylineae and family Phaeodactylacea, was also included in the experiments. It is a pleiomorphic diatom that can be found in three morphotypes of different size (oval, fusiform or triradiate) depending on the environmental or growth conditions (Tesson et al., 2009). According to its shape, it can be either slowly motile, or nonmotile. Fusiform cells of a pelagic, nonmotile morphotype were used in our study.

The three aforementioned microalgal species were used as phytoplankton models for simulating extreme eutrophic scenarios and wild cultures of them were provided by the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) with no prior incubation. For each species, cell size and motility were determined via optical microscopy, as it follows: *Nannochloropsis* sp. (3.2 ± 0.2 μm, non-motile; **Figure 3.3**a), *Isochrysis* sp. (3.8 ± 0.4 μm, motile; **Figure 3.3**b), *Phaeodactylum tricornutum* (21.7 ± 1.2 μm, non-motile; **Figure 3.3**c).
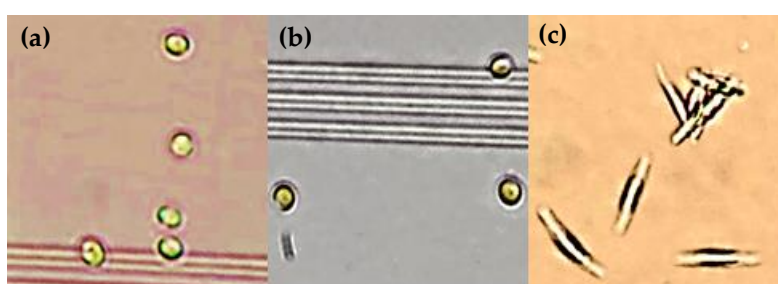
Figure 3.3. The model microalgae selected to assess sponges' cleanup capacity for the biological pollution typically encountered in fish aquaculture settings; (a) *Nannochloropsis* sp., (b) *Isochrysis* sp., (c) *Phaeodactylum tricornutum*.

3.3.4. Experimental Procedures

The protocol for determining the clearance rates of microalgae by marine sponges was based on the methodology proposed by Stabili et al., with minor modifications (Stabili et al., 2006). More specifically, the lab-scale experiments were performed in 2-L glass jars filled with 1 L of NSW collected from the storage tanks and supplemented with the microalgae of interest. In order to achieve exactly the same cell concentration in all jars, a 26-L suspension of microalgae was initially prepared in a plastic carboy by mixing a specific volume of the original microalgae culture with NSW, followed by gentle stirring. After transferring 1-L aliquots into the jars, a single sponge explant was subsequently immersed in each one of them. For each substrate tested, a total of 20 jars were prepared with explants of the four sponges (five replicates for each sponge species), while another five jars containing NSW and substrate (without sponge) were used as controls.

[78]

During an experiment, triplicate samples of 350 μL were collected from each jar at ten different points in time (after 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 7 h) and loaded onto a 96-well opaque microplate for measuring microalgal cell concentration (a total of 750 samples collected in each experiment). The quantitation of microalgae was based on the direct fluorometric detection of cellular chlorophyll a using a microplate reader (Infinite F200 Pro; Tecan GmbH, Grödig, Austria) with appropriate optical filters and under high-sensitivity settings (Exc: 435 ± 40 nm, Em: 676 ± 29 nm, settling time: 200 ms, detector gain: 50, temperature: 25 °C, number of flushes: 10) (Mandalakis et al., 2017). NSW blank samples were also collected and analysed prior to the initiation of each individual experiment. Although, blank values were close to the detection limit, all measurements of microalgae were subjected to blank subtraction to eliminate the background fluorescence signal of NSW.
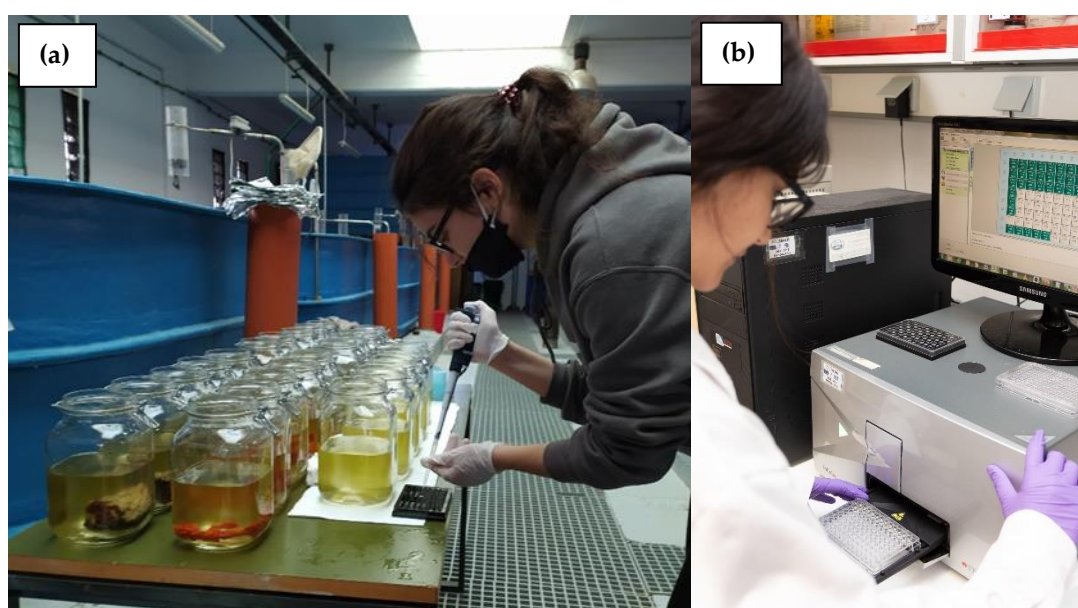
Figure 3.4. (a) The experimental set-up and sampling procedure established for the *in vitro* investigation of sponges bioremediation capacity against aquaculture-related algal blooms. (b) The fluorescence microplate reader used for the analysis of microalgae-enriched seawater samples.

A calibration curve correlating fluorescence signal with cell density was prepared for each microalgae species and used for cell quantification in the samples. Throughout the experiments, the liquid inside the jars was gently stirred at regular intervals to eliminate potential biases due to gravitational settling of cells and the localized reduction of cell concentration around the feeding sponge.

The cleaning capacity of the sponges was examined against a broad range of microalgal concentrations ranging from 0.5 to 10×10^5 cells mL^{-1}, which approximate those experienced in highly eutrophic systems (Moncheva et al., 2001). More specifically, the initial concentrations of microalgae were 1, 5, 10×10^5 cells mL^{-1} for *Nannochloropsis*, 0.5, 1, 5 and 10×10^5 cells mL^{-1} for *Isochrysis* and 0.5, 2.5 and 5×10^5 cells mL^{-1} for *Phaeodactylum*.

For each microalgal substrate, we also tested sponges' filtering activity in the presence and absence of light. This refers to the hypothesis that sponges harboring phototrophic microsymbionts may reduce their pumping rate in the presence of light, while they might shift to increased heterotrophy in darkness. In this case, the experiments were performed in the same way as described above, but the jars were totally covered with aluminum foil.

The effect of cell size, initial cell concentration and light intensity on the sponges' cleaning performance was determined by performing one-way ANOVA analysis, to track any differences. The significance level (α) was set to 0.05 ($p \leq 0.05$).

3.3.5. Preliminary Experiments

To ensure that the filtering capability of sponges under laboratory conditions remained constant from day to day, we conducted a series of preliminary experiments using a single marine microalga (i.e., *Isochrysis*) at a concentration of 10^5 cells mL^{-1}. In particular, we monitored the daily filtering capacity of sponges over five consecutive days and the reproducibility of the results was evaluated. Five explants were used from each sponge species and repetitive experiments were performed following the same procedures as described in the previous section. To check differences in the microalgae retention capacity of sponges between days, a one-way ANOVA was performed on the average removal capacity of the five fragments for each species. The significance level (α) was set to 0.05 ($p \leq 0.05$).

3.3.6. Data Analysis

Clearance rate (*c*) is a measure that indirectly quantifies the filtering activity of marine suspension feeders (Coughlan, 1969), including sponges (Simpson, 1984). It represents the volume of water cleared of particles per unit time and sponge weight. The depletion of particle concentration driven by sponges' filtering activity over time follows the exponential function described by Coughlan (Coughlan, 1969):

$$C_t = C_0 \times e^{-c \cdot w \cdot t / V} \quad (3.1)$$

where C_0 and C_t represent particle concentrations (cells mL^{-1}) at time 0 and t, respectively, V represents the volume of NSW (i.e., 1000 mL) in the jars, t is the time (hours) and w is the wet weight of sponge (g). For each experiment, the clearance rate was derived by fitting **Equation (3.1)** to cell concentration data, as proposed by Riisgård et al. (1993) and Turon et al. (1997), and by dividing the resulting constant in the exponent by NSW volume and sponge wet weight. The results from the five biological replicates were averaged to obtain the final *c* value for each sponge species.

Retention rate (r) is another common term, widely used to describe sponges' filtering activity in the literature (Fu et al., 2006; Turon et al., 1997; Wehrl et al., 2007). It is defined as the number of particles retained by the sponge, normalized to sponge wet weight (g) and time (hours). This parameter was calculated according to Wehrl et al. (2007), using the following equation:

$$r = \frac{1-(10^{(y60)})}{w} C_0 V \quad (3.2)$$

where y is the slope of the semi-logarithmic graph C_t versus t for the linear time interval, multiplied by 60 to give retention rates per hour.

In the same context, the retention efficiency (RE) can be calculated as the percentage removal of microalgae from seawater at a specific sampling point (Stabili et al., 2006):

$$\% \, RE = 100 * \frac{(C_0 - C_t)}{C_0} \quad (3.3)$$

In our study, we calculated the overall retention efficiency of the sponges by using cell concentration from the last sampling point ($t_9 = 7$ h).

To offset weight differences between sponge species and replicates, we also used the term *Removal Capacity*, which designates the number of microalgal cells removed within specific time period (i.e., $t_9 = 7$ h) per unit of sponge wet mass (Fu et al., 2007):

$$Removal \, Capacity = \frac{(C_0 - C_t)}{w} V \quad (3.4)$$

3.4. Results and Discussion

3.4.1. Assessment of Reproducibility

The depletion of *Isochrysis* cells by the four sponge species *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus* over the series of five consecutive experiments, conducted for seven hours each, is shown in **Figure 3.5**. The initial concentration of *Isochrysis* was 10^5 cells mL^{-1} and presented results correspond to the average depletion derived for each species using data from five biological replicates. The average wet weight of sponge fragments was 71.6 ± 6.7 g for *Agelas oroides*, 55.5 ± 4.7 g for *Axinella cannabina*, 82.6 ± 6.2 g for *Chondrosia reniformis* and 113.3 ± 29.1 g for *Sarcotragus foetidus*. These values were assumed to remain constant throughout the experiments.

Microalgae concentration in natural seawater (NSW) decreased markedly within the tested seven hours, following Coughlan's exponential model (Coughlan, 1969). This depletion was clearly the result of sponges' filtering activity and not of other causes, such as cell settling or lysis, as the concentration of microalgae in the control group remained rather constant over time (**Figure 3.5**e). The

pattern of results obtained from each sponge species over the five consecutive experiments demonstrated high similarity, implying a rather stable filtration performance. However, it is worth noticing that the consumption of microalgal cells on the last day of the experiments (Day 5) tended to be lower for all sponges (**Figures 3.5** and **3.6**). It is likely that sponge's aquiferous system experienced partial saturation effects (e.g., clogging) as a result of continuous and excessive intake of microalgae, which eventually led to reduced pumping activity (Osinga et al., 2001). The discrepancy observed for *C. reniformis* was more pronounced, but still of marginal importance. Even in this case, the concentration decrease of microalgae measured over the course of Day 5 deviated only 14% from those detected during the previous four days.
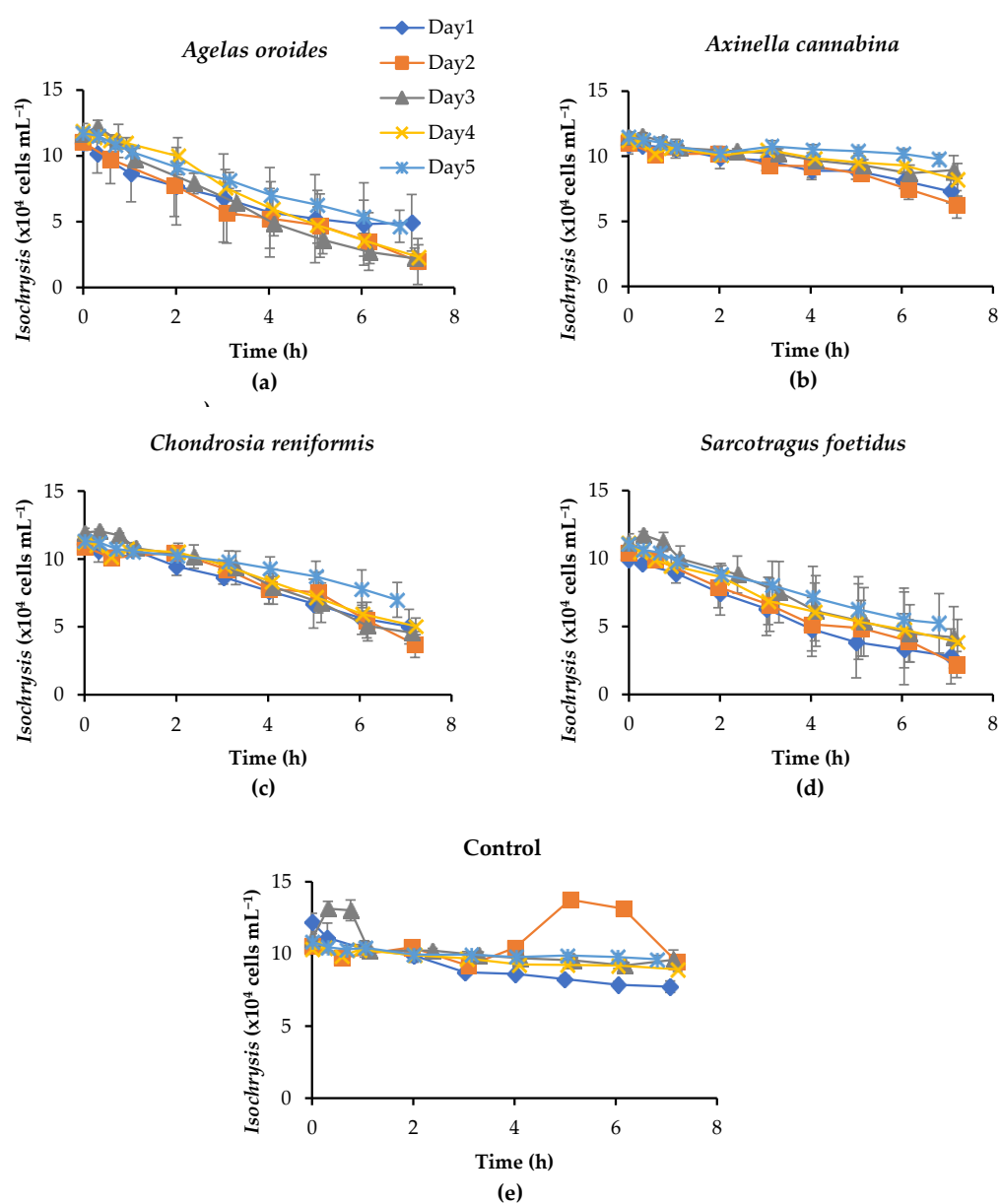
Figure 3.5. Removal of the marine microalga *Isochrysis* by the four study sponge species over five consecutive days. The 7-h decrease of *Isochrysis* by (a) *Agelas oroides*, (b) *Axinella cannabina*, (c) *Chondrosia reniformis*, (d) *Sarcotragus foetidus* and (e) control samples (without sponge explants) are presented. In each experiment, the initial concentration of *Isochrysis* was set at 10^5 cells mL^{-1}. The error bars represent the standard deviation obtained from the five biological replicates of each sponge species.

Another interesting aspect of these preliminary experiments was the considerable biological variation (i.e., among different explants) in the results of the species *A. oroides* and *S. foetidus* throughout the 7-h experiment, as illustrated by the large error bars in **Figure 3.5**a,d. This is probably the result of weight differences between sponge fragments or of physiological variations (i.e., different number of oscules among explants). Nevertheless, this finding comes as no surprise, given the extensive within-sponge variances that have also been reported in other studies (e.g., Frost, 1978).

Figure 3.6 demonstrates the removal capacity of the examined sponge species against *Isochrysis* cells after 7-h of exposure and over five consecutive days. In general, all sponges were able to efficiently retain significant quantities of microalgal cells, with a daily average exceeding 5×10^5 cells per gram of sponge wet weight. The performance of all species demonstrated some day-to-day fluctuation, but *A. oroides* was clearly the most efficient filter-feeder, as it could retain the highest amounts of *Isochrysis* cells on all days (up to 10^6 cells per gram wet weight). The highest cell removal capacity achieved by this species was 1.3×10^6 cells g^{-1} and it was observed on Days 3 and 4. During the second experiment, the capacity of *A. oroides* was slightly reduced, while the lowest values were observed during the first and the fifth day. Concerning *C. reniformis*, the second most efficient sponge with an average removal capacity of $7.5 \pm 1.5 \times 10^5$ cells g^{-1}, its performance maximized on Days 2 and 3 (8.6 and 8.8×10^5 cells g^{-1}, respectively). Slightly lower performance was evident on Days 1 and 4 (7.6 and 7.4×10^5 cells g^{-1}, respectively), and this was further reduced on Day 5 (5.1×10^5 cells g^{-1}). A similar day-to-day variability was also observed for *S. foetidus*, which exhibited an average removal capacity of $6.5 \pm 0.9 \times 10^5$ cells g^{-1}. Among the four study species, *A. cannaniba* demonstrated the lowest removal capacity with an average daily value of $5.7 \pm 2.1 \times 10^5$ cells g^{-1} and the most striking day-to-day fluctuation. Indeed, the capacity of this species on Day 2 (8.5×10^5 cells g^{-1}) decreased almost 3-fold on Day 5 (3.0×10^5 cells g^{-1}).
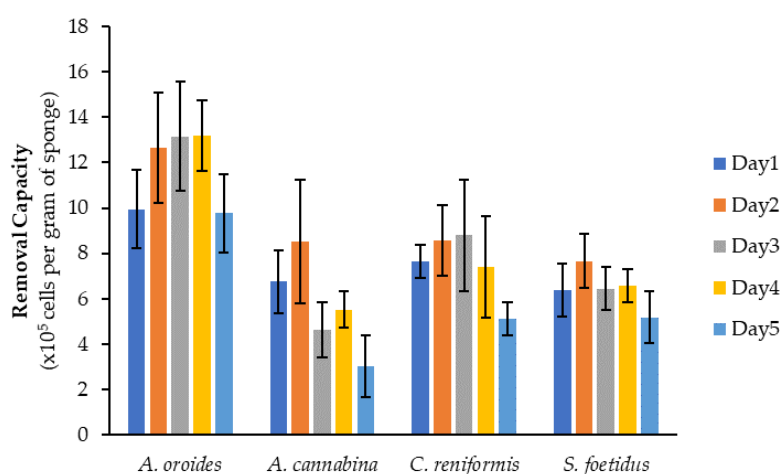
Figure 3.6. Average retention of *Isochrysis* cells by *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus* after 7 h of exposure and over five consecutive experiments. Error bars indicate the standard deviation obtained from the five biological replicates of each sponge species.

Over the first four days, the variance in the removal capacity of all sponge species was of no statistical significance (*A. oroides*: $p = 0.16$, *A. cannabina*: $p = 0.06$, *C. reniformis*: $p = 0.19$, *S. foetidus*: $p = 0.46$). Despite the compromised performance observed for all sponges during Day 5, one-way ANOVA revealed that the variation during the entire 5-day experimental period was significant only for the species *A. cannabina* ($p = 0.004$) and *C. reniformis* ($p = 0.0005$). Although a previous *in situ* study reported that sponges' filtering activity can vary substantially on a time scale of a few days (H. M. Reiswig, 1971), our lab-based investigation showed that all examined species were capable of maintaining their filtering performance for at least four or five consecutive days of 7-h exposure to microalgae. The fairly stable performance of sponges might be indicative of their successful adaptation to tank conditions and their healthy physiological status, while it could be also attributed to the highly-controlled experimental conditions.
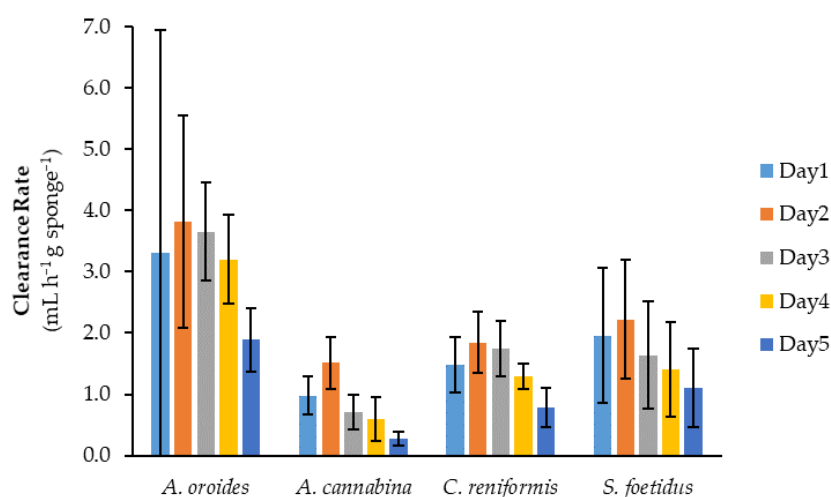
Figure 3.7. Calculated clearance rates of *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus* for a fixed initial concentration of *Isochrysis* (10^5 cells mL^{-1}) over five consecutive experimental days. Error bars indicate the standard deviation obtained from the five biological replicates of each sponge species.

In **Figure 3.7**, the clearance rates (*c*) of the four sponge species derived for *Isochrysis* cells using **Equation (3.1)** are presented for the five experimental days. Similarly with the removal capacity (**Figure 3.6**), *A. oroides* showed the highest clearance rates, as it was capable of cleaning almost 4 mL of NSW per hour and per gram of sponge wet weight, while the lowest values were found for *A. cannabina* species (<1 mL h^{-1} g^{-1}). Most species exhibited the highest clearance rates on Day 2, followed by a gradual decrease thereafter. Once again, the lowest values were observed on Day 5 for all species. The variance of clearance rate values over the 5-day experimental period was significant only for *A. cannabina* ($p < 0.0001$) and *C. reniformis* ($p = 0.003$), while no significant variation was found for *A. oroides* and *S. foetidus* ($p = 0.52$ and $p = 0.33$, respectively).

It should be also noted that the daily *c* values of some species (e.g., *A. oroides* and *S. foetidus*) were accompanied by high biological variation. This is not surprising as similar findings have been

reported in previous studies (Frost, 1978; Fu et al., 2006; Milanese et al., 2003). In general, the comparison of clearance rates between different species, and particularly those obtained from different studies, is a tricky task due to the inherent variability of each species, the dissimilarities in the size of sponge used and their morpho-physiological features, the different types of microalgae tested, as well as the diverse units in which c are expressed (Ribes et al., 1999; Trani et al., 2021). Nevertheless, the clearance rates that we measured for the four sponge species were consistently lower than the rate reported by Frost for the freshwater sponge *Spongilla lacustris* (198 mL h^{-1} g^{-1}), which was tested with the microalga *C. reinhardtii* (6.6 μm) at the same initial cell concentration (Frost, 1978). A possible explanation could be that *S. lacustris* is a freshwater sponge dwelling in lakes, where eutrophication events and high concentrations of microalgae are much more common than in open-sea systems, especially the oligotrophic ones at the Eastern Mediterranean (Azov, 1991). In addition, the microalga used in that study was almost twice the size of the *Isochrysis* cells used in our experiments. Furthermore, much higher rates have also been observed by Turon et al. (1997), who tested *Dysidea avara* (426 mL h^{-1} g^{-1}) and *Crambe crambe* (432 mL h^{-1} g^{-1}) with latex spheres of the same size (4 μm) and at a similar initial concentration as in our experiments. However, these rates were derived using the dry weight of sponges, while it is questionable whether microalgae retention can be approximated by latex beads.

Another possible explanation for this apparent discrepancy could be the significantly larger sponge fragments used in our experiments, compared to those tested by Frost (0.97 to 2.22 g; Frost, 1978) and Turon (approximately 0.63 g for *D. avara* and 0.49 g for *C. crambe*; Turon et al., 1997). Decreasing clearance rates are typically observed for sponges of increasing size and this is mainly due to the lower number of living choanocytes present per unit weight in larger sponges. This trend was acclaimed by Ribes et al. (1999), who additionally argued that the use of larger sponges may lead to water refiltration in the experimental chamber and cause a shift of rates towards lower values.

3.4.2. Effect of Cell Size on Cleaning Capacity

Several studies have shown that sponges are either capable (Duckworth et al., 2006; Ribes et al., 1999; Turon et al., 1997) or incapable (Frost, 1978; Fu et al., 2007; Pile and Witman, 1996) of discriminating food particles based on their size. While this issue is not entirely clear, sponges are indeed suggested to use different mechanisms for selectively feeding on bigger or smaller particles (Bergquist, 1978). In particular, particles with size smaller than approximately 5 μm are captured by the choanocytes, the flagellated cells of choanoderm, which are also responsible for the creation of sponges' water current. The larger particles are primarily ingested through phagocytosis by pinacocytes, which line the incurrent canals (Hill and Hill, 2009; H. M. Reiswig, 1971; Weissenfels, 1992).

In our study, we tested two nanophytoplankton with minimal size differences and a diatom of much larger size as feed particles for sponges, all suspended at the same initial concentration of 5×10^5 cells mL^{-1}. **Figure 3.8** presents a comparison regarding the filtering performance of the four study sponge species against the different types of cells. All performance metrics suggested that the investigated sponges have different food preferences. With regard to clearance rates, Coughlan's model demonstrated a perfect fit to the time-series of microalgae concentration data, with the coefficient of determination being higher than 0.96 in most cases (**Table 3.1**). According to one-way ANOVA, each sponge presented statistically significant variance in its clearance rates among the three microalgae tested (*A. oroides*: $p = 0.016$; *A. cannabina*: $p = 0.0008$; *C. reniformis*: $p = 0.0002$; *S. foetidus*: $p = 0.036$).

The species *A. oroides* was more keen to retain small cells, and it presented retention efficiencies as high as 91 ± 8% for the smallest cells investigated (i.e., *Nannochloropsis*, 3.2 μm) (**Table 3.1**). For the slightly larger *Isochrysis* cells (3.8 μm), the retention efficiency dropped significantly down to 69 ± 14%, while the clearance and retention rates decreased more than half compared to *Nannochloropsis*. However, this difference in clearance rates was not enough to reach statistical significance ($p = 0.08$). Being almost six times lower in size, *Phaeodactylum* cells (21.7 μm) showed a further decrease in retention efficiency, which approached 48 ± 13%.

Table 3.1. Wet weight of the sponge specimens used in the experiments and results obtained for the retention rate (*r*), the clearance rates (*c*) derived from Coughlan's exponential model (together with the coefficient of determination R^2), the removal capacity, and the retention efficiency of the sponges tested against three different types of marine microalgae. Standard deviations are reported in parentheses.

Microalgae	Cell Size (μm)	Sponge Species	Wet Weight (g)	Retention Rate ($\times10^5$ cells h^{-1} g Sponge^{-1})	Clearance Rate (mL h^{-1} g Sponge^{-1})	R^2	Removal Capacity ($\times10^5$ cells g Sponge^{-1})	Retention Efficiency (%)
Nannochloropsis	3.2 (0.2)	*A. oroides*	67.9 (5.4)	25.4 (5.4)	5.4 (2.0)	0.98	59.4 (9.1)	91 (8)
		A. cannabina	50.4 (7.6)	9.9 (3.6)	1.3 (0.3)	0.96	38.5 (8.2)	41 (8)
		C. reniformis	84.2 (12.4)	5.0 (1.2)	1.1 (0.2)	0.99	26.6 (4.2)	46 (3)
		S. foetidus	106.5 (26.8)	3.1 (1.2)	0.6 (0.3)	0.97	16.4 (6.7)	36 (13)
Isochrysis	3.8 (0.4)	*A. oroides*	67.9 (5.4)	10.4 (3.8)	2.3 (0.9)	0.97	49.4 (10.0)	69 (14)
		A. cannabina	50.4 (7.6)	2.5 (0.3)	0.6 (0.1)	0.80	23.9 (7.0)	24 (8)
		C. reniformis	84.2 (12.4)	6.2 (1.4)	1.4 (0.2)	1.00	32.2 (5.5)	55 (6)
		S. foetidus	106.5 (26.8)	1.8 (1.3)	0.4 (0.3)	0.96	9.3 (6.3)	30 (24)
Phaeodactylum	21.7 (1.2)	*A. oroides*	67.9 (5.4)	8.3 (4.3)	1.4 (0.6)	0.96	36.5 (10.1)	48 (13)
		A. cannabina	50.4 (7.6)	13.7 (4.3)	1.6 (0.5)	0.96	46.1 (10.7)	46 (15)
		C. reniformis	84.2 (12.4)	4.5 (1.6)	0.5 (0.3)	0.98	17.5 (5.4)	29 (10)
		S. foetidus	106.5 (26.8)	14.3 (11.2)	1.8 (1.3)	0.81	30.0 (9.1)	66 (21)

All performance metrics of *A. cannabina* were consistently higher for both the smallest and biggest cells under investigation (i.e., *Nannochloropsis* and *Phaeodactylum*), with the retention efficiencies reaching 41 ± 8 and 46 ± 15%, respectively. Surprisingly, *Isochrysis* was deemed to be the least preferable substrate, as its retention efficiency dropped to half. Considering that *Isochrysis* was the only motile species among those investigated, it is tempting to speculate that cell motility plays a role in the filtration performance of *A. cannabina*.

The species *C. reniformis* exhibited the highest clearance rate and retention efficiency for *Isochrysis* (1.4 ± 0.2 mL h^{-1} g^{-1} and 55 ± 6%, respectively), closely followed by *Nannochloropsis* (1.1 ± 0.2 mL h^{-1} g^{-1} and 46 ± 3%, respectively). A much lower retention efficiency was observed for the large

diatom cells (29 ± 10%). In addition, the clearance rate measured for *Phaeodactylum* (0.5 ± 0.3 mL h^{-1} g^{-1}) was statistically lower than those for *Nannochloropsis* and *Isochrysis* (*Nannochloropsis* vs. *Phaeodactylum*: $p = 0.004$; *Isochrysis* vs. *Phaeodactylum*: $p = 0.0006$).
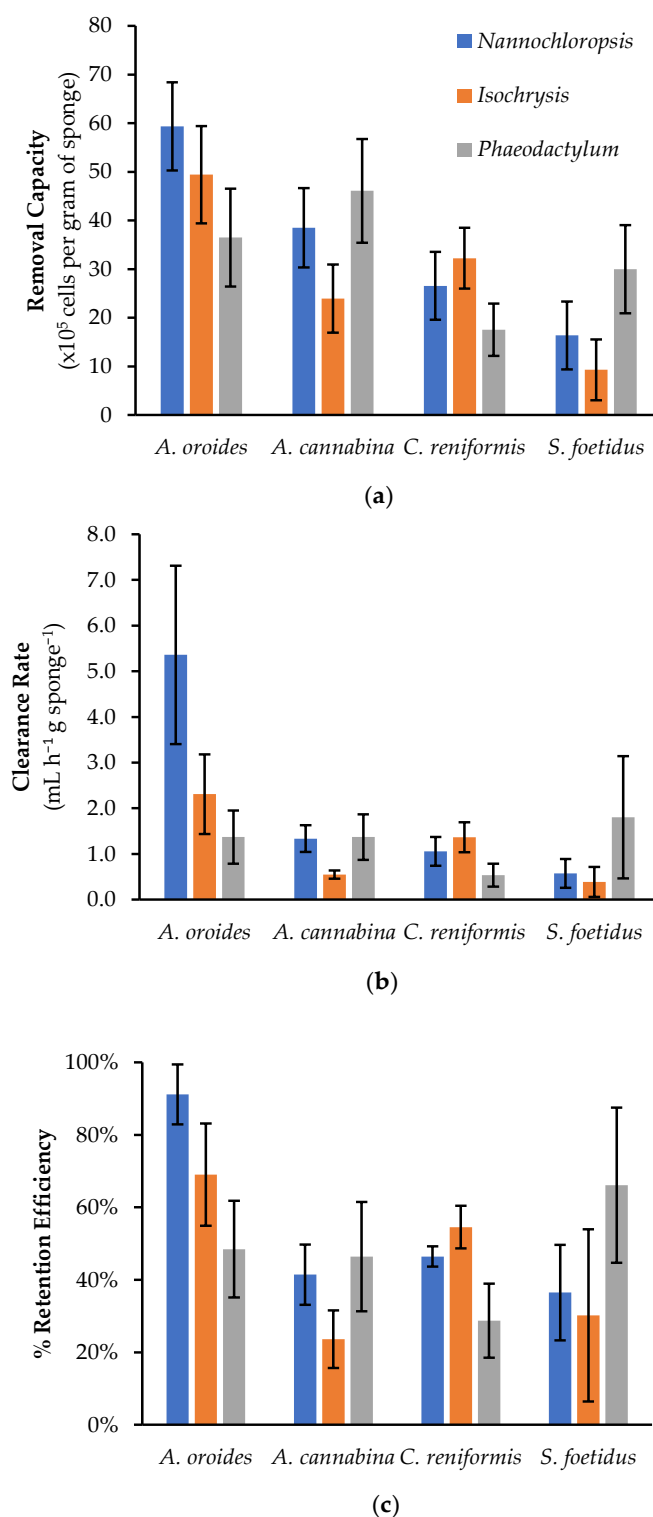
Figure 3.8. (a) Cell removal capacity, (b) clearance rate and (c) retention efficiency of sponges *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus* for three different types of marine microalgae prepared at the same initial concentration (5 × 10^5 cells mL^{-1}).

With regard to *S. foetidus*, all performance metrics were considerably higher when tested against the larger cells of the pelagic diatom *Phaeodactylum* (**Table 3.1**). Indeed, its retention efficiency for *Phaeodactylum* (66 ± 21%) was the highest observed among the examined sponge species, implying that *S. foetidus* might feed more efficiently on big particles. On the contrary, *S. foetidus* demonstrated less preference for cells of smaller size, as the retention efficiency for *Nannochloropsis* (36 ± 13%) and *Isochrysis* (30 ± 24%) were 46% and 55% lower, respectively. Similarly with *A. cannabina*, it was evident that the filtering activity was not necessarily limited by an increase of particle size. This comes in contrast to Duckworth et al., who examined three tubular sponges (i.e., *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis*) *in situ* and found that particle retention by all of them decreased as particle size increased from 0.7 to 18 μm (Duckworth et al., 2006).

Overall, the examined sponges exhibited widely disparate retention efficiencies for the different microalgae, which ranged from 24% up to 91% when the initial cell concentration was 5×10^5 cells mL^{-1}. This range is much broader than the one reported by Pile and Witman, who investigated a single boreal sponge (i.e., *Mycale lingua*) against several types/classes of planktonic cells <10 μm present in the ambient seawater (72–93%; Pile and Witman, 1996), or the range of values obtained in various studies for nano- and pico-plankton (75–99%; Larsen and Riisgård, 1994; Reiswig, 1975; Riisgård and Larsen, 1995; Simpson, 1984; Wilkinson, 1978). On the basis of clearance rates, the best performance in our study was observed for *A. oroides* exposed to *Nannochloropsis* cells (5.4 ± 2.0 mL h^{-1} g^{-1}), while the worst one was noticed for *S. foetidus* with *Isochrysis* cells (0.4 ± 0.3 mL h^{-1} g^{-1}). In view of the overall feeding efficiency of sponges on phytoplankton (i.e., all three tested microalgae as a total), *A. cannabina* had the lowest performance (37 ± 12%), followed with slightly higher values by *C. reniformis* (43 ± 13%) and *S. foetidus* (44 ± 19%). On top of them, *A. oroides* displayed the highest retention levels, with an overall feeding efficiency of 70 ± 21%. However, the variances in overall retention efficiencies and clearance rates among the different sponges were determined as not significant (p = 0.170 and 0.165, respectively) via one-way ANOVA analysis.

What is more noteworthy is the divergence in the cleaning performance of two examined sponges against cells of similar size, but of different microalgal species (i.e., *Isochrysis* and *Nannochloropsis*). Statistical analyses confirmed that this is the case for *A. cannabina* (p < 0.001) and *C. reniformis* (p = 0.035). In particular, the clearance and retention rates of *A. cannabina* were almost three times higher for *Nannochloropsis* than for *Isochrysis*. This is in sharp contrast to the results reported by Duckworth et al., who investigated the retention efficiency of three coral reef sponges against diverse microbial substrates dwelling in the surrounding waters and concluded that sponges are unselective feeders for a given particle size (Duckworth et al., 2006). On the other hand, a feeding selectivity for specific microbial substrates among others of comparable size was observed by Maldonado et al.

(Maldonado et al., 2010). The latter study showed that two similarly sized bacteria, namely *E. coli* (1 μm in length × 0.4 μm in diameter; non-flagellated strain) and *V. anguillarum* (1.1 μm × 0.6 μm; flagellated), were retained by different rates from the marine sponge *Hymeniacidon perlevis*. This distinctive difference was attributed to the capability of sponges to readjust the intake rate of each microorganism in response to other features rather than size alone. It was further argued that the phagocytosis process in choanocyte chambers might be more complicated for flagellated *V. anguillarum* cells, as flagellum beating in choanocytes can make the engulfing more laborious and, thus, less efficient. This theory could explain the higher cleaning efficiency of non-motile, non-flagellated *Nannochloropsis* cells compared to the motile, flagellated *Isochrysis* that was observed in our study. Moreover, differences in the retention rates of similarly sized cells are also likely to occur as a result of choanocytes' ability to discriminate microorganisms based on the chemical entities present on their exterior surface, as Wehrl et al. have previously shown for bacteria (Wehrl et al., 2007).

In our study, we showed that a range of marine sponges are able to exhibit special food preferences on different microalgae. This can be of particular importance for aquaculture applications, as the best-performing bioremediators for the development of IMTA systems could be selected and applied by taking into account the characteristics of microalgae present in each particular area.

3.4.3. Effect of Initial Cell Concentration on Cleaning Capacity

In this series of experiments, we examined how the initial abundance of microalgae in the medium affects the cleaning performance of sponges. In particular, **Figure 3.9**a–c presents the clearance rate as well as the total removal capacity of the four sponges under investigation in relation to the initial cell concentration of three microalgae species.

When the four examined sponge species were subjected to *Nannochloropsis* cells (**Figure 3.9**a), the increase of cell concentration did not have such a dramatic effect in their clean-up capacity. In particular, *S. foetidus*' activity did not show any systematic variation with cell concentration, and it steadily provided the lowest c values among the four sponges (~0.6 ± 0.1 mL h^{-1} g^{-1}). A similar behavior was observed for *A. cannabina*, which provided fairly stable c values of ~1.1 ± 0.3 mL h^{-1} g^{-1} over the range of cell concentrations tested. The respective values of *A. oroides* presented a slight decrease (~38%) at concentrations higher than 5×10^5 cells mL^{-1}, but it remained the best-performing species with a maximum c of 4.5 ± 1.1 mL h^{-1} g^{-1}. A clearer trend was evident only for *C. reniformis*, which presented a limited but steady decrease of c with increasing cell concentration. All the results were further evaluated by one-way ANOVA, and it was revealed that *C. reniformis* was the only sponge species indicating a significant variance in its clearance rate across the different concentrations of *Nannochloropsis* cells ($p < 0.00001$).
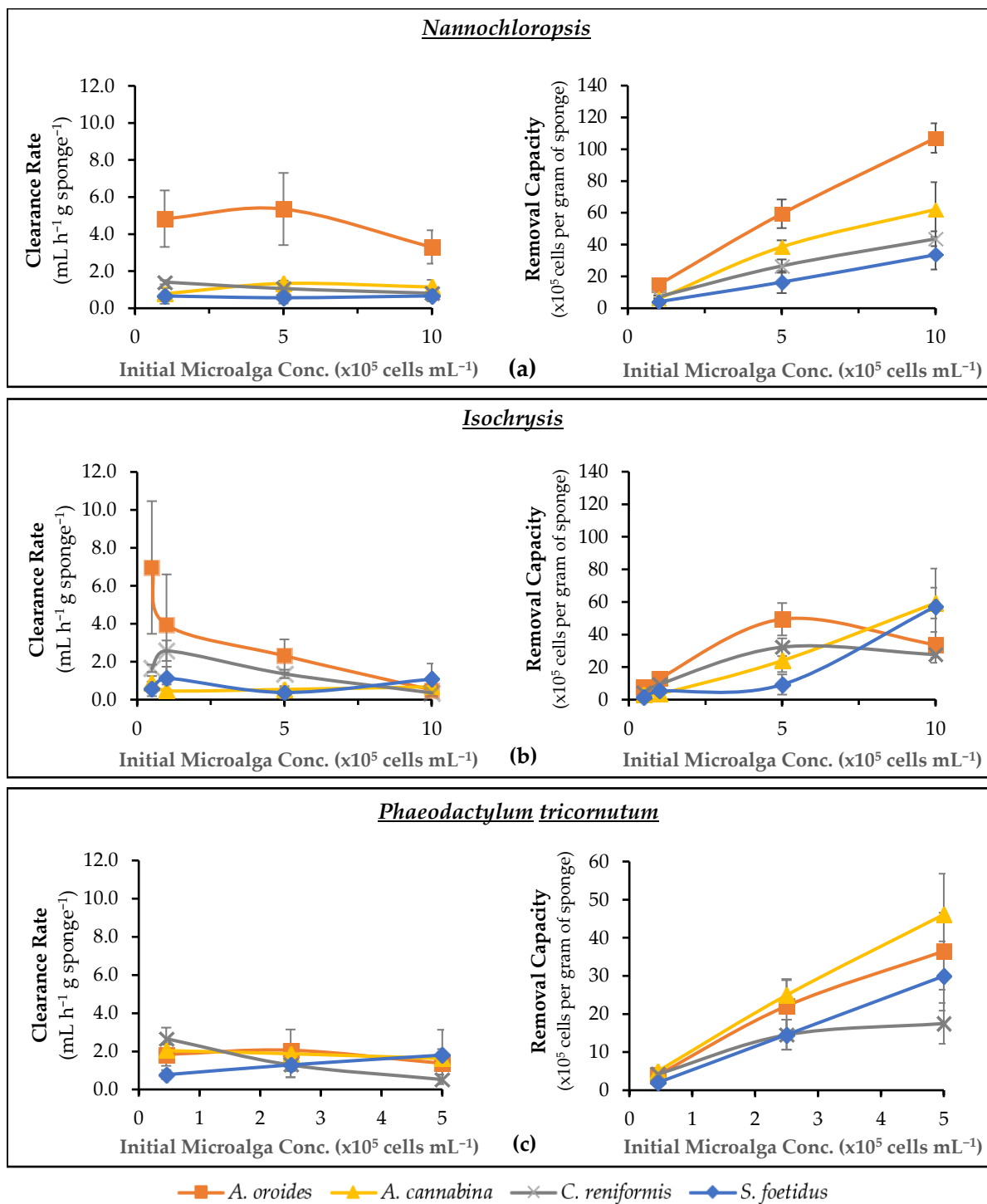
Figure 3.9. Average cell removal capacity (right) and clearance rate (left) of sponges *A. oroides*, *A. cannabina*, *C. reniformis*, and *S. foetidus* after a 7-h exposure to different initial cell concentrations of microalgae (a) *Nannochloropsis* sp.; (b) *Isochrysis* sp.; and (c) *Phaeodactylum tricornutum*.

As a result of the relatively stable clearance rates reported above, the removal capacity of all four sponges against *Nannochloropsis* demonstrated an almost linear increase with cell concentration (i.e., the more cells in seawater, the more cells retained by sponges). This finding suggests that all sponges will be able to remove microalgal cells similar to *Nannochloropsis* from aquaculture settings,

while their filtration capacity will remain relatively unaffected by temporal or seasonal increases in phytoplankton abundance. Nevertheless, the performance of *A. oroides* looks superior on a quantitative basis, and it would clearly be the best option followed by *A. cannabina*, *C. reniformis*, and *S. foetidus*.

With regard to *Isochrysis* (**Figure 3.9**b), the four sponges responded differently to concentration changes. In particular, *A. cannabina* and *S. foetidus* were generally characterized by relatively low clearance rates, which remained fairly stable over the range of cell concentrations tested. As a consequence, the removal capacity exhibited a steady increase with cell concentration for both species, but this trend was more prominent for *A. cannabina*. These two sponges attained the same maximum removal capacity at the highest cell concentration tested (i.e., 1×10^6 cells mL^{-1}), and they were able to retain $\sim 5.8 \times 10^6$ cells per gram of sponge wet weight within 7 h of exposure. It could therefore be inferred that the specific sponges could respond efficiently even in marine systems with rather high microalgae loadings.

The cleaning effect of *A. oroides* was considerably faster at the lowest cell density tested (5×10^4 cells mL^{-1}), providing an average clearance rate of 7.0 ± 3.5 mL h^{-1} g^{-1}, but it slowed down sharply at higher cell concentrations. It is worth stressing that one individual fragment of *A. oroides* exhibited the highest *c* value among all four sponges, and it reached 12.5 mL h^{-1} g^{-1} at *Isochrysis* concentration 5×10^4 cells mL^{-1}. This was almost five times higher than the *c* of the next best-performing species, *C. reniformis*, which showed a maximum value of 2.6 ± 0.5 mL h^{-1} g^{-1} at a concentration of 10^5 cells mL^{-1}. Regarding removal capacity, the same two sponge species (*A. oroides* and *C. reniformis*) followed a similar parabolic pattern in relation to *Isochrysis* concentration and exhibited maximum retention at 5×10^5 cells mL^{-1} ($4.9 \pm 1.0 \times 10^6$ and $3.2 \pm 0.6 \times 10^6$ cells retained per gram of *A. oroides* and *C. reniformis*, respectively). This trend suggests that, above a critical concentration of *Isochrysis*, the aquiferous system of the two sponges may gradually become engulfed and saturated by microalgal cells. Similar parabolic patterns were previously observed for the removal capacity of the marine sponge *Hymeniacidon perleve* tested against different concentrations of total organic carbon (Fu et al., 2007). Moreover, Osinga et al. observed the same pattern by testing the tropical sponge *Pseudosuberites aff. Andrewsi* against microalga *Dunaliella tertiolecta* ($\sim 5-8$ μm) at a concentration range of $1 \times 10^4 - 1.3 \times 10^6$ cells mL^{-1} (Osinga et al., 2001). The phenomenon of engulfing was also reported by Scheffers et al., who measured the removal of bacteria by encrusting sponges (Demospongiae and Calcarea) (Scheffers et al., 2004).

Investigating the effect on the cleaning performance of sponges by the concentration of *Phaeodactylum* cells was more complicated due to the relatively large biological variation in the experimental data (**Figure 3.9**c). This was particularly evident in the cases of *A. oroides*, *A. cannabina*, and *S. foetidus*, for which it was not possible to identify any statistically significant influence of cell

concentration on clearance rates. However, the clearance rate of *C. reniformis* exhibited a steady decline with increasing cell concentration. The same species also demonstrated a distinctly different behavior with regard to the effect of cell concentration on removal capacity. With the exception of *C. reniformis*, which appeared less capable to cope with *Phaeodactylum* concentrations higher than 2.5×10^5 cells mL^{-1}, all other sponges presented the characteristic increase of removal capacity with cell concentration. The faster filter-feeder for *Phaeodactylum* was *S. foetidus*, which was able to clean 3.5 mL seawater per gram of sponge wet weight, per hour at the highest initial concentration of 5×10^5 cells mL^{-1}.

Grazing and retention rates have been reported to be independent of cell abundance (Ribes et al., 1999; Wehrl et al., 2007), but many other studies have come to the opposite conclusion (Fu et al., 2007; Gerrodette and Flechsig, 1979; Maldonado et al., 2010; Milanese et al., 2003; Osinga et al., 2001). Our results highlighted that both theories can stand for certain sponge species. More specifically, the species *A. cannabina* and *S. foetidus* exhibited a concentration independence regarding their clearance rate (**Table 3.2**). On the other hand, the increase of the microalgae concentration had a significant impact on the cleaning performance of the sponges *A. oroides* and *C. reniformis*. However, this was more obvious for *C. reniformis*, as its clearance rates were considerably affected with concentration changes of all tested substrates (i.e., *Nannochloropsis*; $p < 0.0001$, *Isochrysis*; $p < 0.00001$, *Phaeodactylum*; $p = 0.00002$), meaning that the particular sponge species is very susceptible to microalgal concentration changes. For *A. oroides*, this was only the case for *Isochrysis* loadings ($p = 0.0024$). Remarkably, higher concentrations of these small-sized particles (e.g., 10^6 cells mL^{-1}) led to significantly decreased clearance rates that were almost 93% lower than the ones recorded at low concentration (e.g., 5×10^4 cells mL^{-1}). As Osinga et al. suggest, it is more likely that the high concentrations of smalls cells are blocking the aquiferous system, leading to a reduction in pumping activity (Osinga et al., 2001).

Table 3.2. *p*-values derived from one-way ANOVA test of sponges' clearance rates resulted from different microalgal concentrations.

	Nannochloropsis	*Isochrysis*	*Phaeodactylum*
A. oroides	0.196	0.0024	0.405
A. cannabina	0.051	0.106	0.245
C. reniformis	0.0001	<0.00001	0.00002
S. foetidus	0.893	0.127	0.221

3.4.4. Effect of Light Intensity on Cleaning Capacity

An additional series of *in vitro* cleaning experiments were performed to investigate if light conditions can exert an effect on the cleaning performance of sponges based on a hypothetical scenario of increased feeding activity in darkness, as well as to test whether significant day-night differences should be expected in a real-use scenario in IMTA systems. The "light vs. dark" experiments were performed at a specific cell concentration for each tested microalga. More specifically, a concentration

of 5×10^5, 10^5 and 2.5×10^5 cells mL^{-1} was applied for *Nannochloropsis*, *Isochrysis*, and *Phaeodactylum*, respectively. The concentration selected for each microalga was the lowest possible to minimize potential stress on sponges during the experiments, whilst providing high signal intensity with the fluorometric method to keep analytical errors at minimum. To the best of our knowledge, this is the first time the effect of light intensity is examined as a factor affecting the feeding intensity of sponges, using microalgae as a reference substrate.

The results for *Nannochloropsis* (**Figure 3.10**a) demonstrated no significant variance in clearance rates between light and dark conditions for all examined sponge species (*A. oroides*: $p = 0.879$; *A. cannabina*: $p = 0.897$; *C. reniformis*: $p = 0.218$ and *S. foetidus*: $p = 0.144$). This shows that at least the selected candidates retain a consistent filtering activity regardless of daily light cycles when the seawater is enriched with the particular microalga. Concerning the clearance of *Isochrysis* (**Figure 3.10**b), significant differences were observed only in the filtering performance of *A. cannabina*, which tended to be more efficient in darkness ($c_{dark} = 1.1 \pm 0.5$ mL h^{-1} g^{-1}) than in light ($c_{light} = 0.5 \pm 0.3$ mL h^{-1} g^{-1}) ($p = 0.0403$). Notably, the best performance under darkness was displayed by *A. oroides*, with one of its explants exhibiting both the highest measured clearance activity ($c = 14.1$ mL h^{-1} g^{-1}) and retention efficiency (100%) in the present study. However, this extreme case was not sufficient to make a significant difference between light and dark conditions for this species, as the variance in the performance across replicates was particularly high. Lastly, when *Phaeodactylum* is used as a substrate (**Figure 3.10**c), significant variances in clearance rates were received for *A. cannabina* and *C. reniformis* ($p = 0.0407$ and 0.0222, respectively), with better performances observed in light ($c = 1.9 \pm 0.3$ and 1.8 ± 0.4 mL h^{-1} g^{-1}, respectively). However, these values were higher only by 22% for *A. cannabina* and 35% for *C. reniformis* than the ones in darkness.

In general, out of the twelve cases we examined (four sponge species against three microalgae under two different light intensities), only three were associated with significant variances. In particular, the filtering activity of *A. oroides* and *S. foetidus* was not found to be influenced by light conditions. Given that *A. oroides* is a sciaphilic species, which dwells in sheltered places with low light availability (i.e., cave entrances, overhangs, or mesophotic habitats; Idan et al., 2020), it is remarkable that it exhibits equally efficient performance under different light regimes for all tested microalgal cells (best performance 12.5 and 14.1 mL h^{-1} g^{-1} in light and darkness, respectively). This suggests an adaptability of the particular sponge to fluctuating irradiance levels. On the other hand, this was rather expected for *S. foetidus*, which is commonly found in shallow habitats exposed to light, but also in darker zones up to 400 m in depth (Manconi et al., 2013). Nevertheless, this species exhibited the least efficient clearance effects in all experiments (<2.0 mL h^{-1} g^{-1}) regardless of light conditions.

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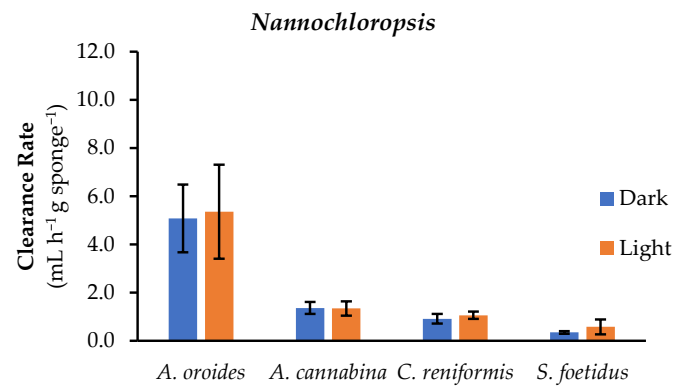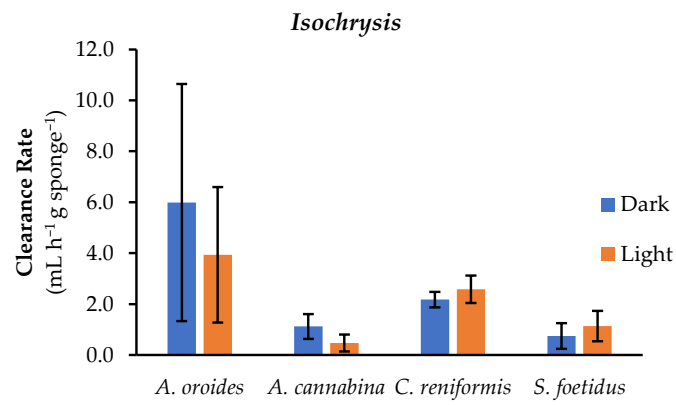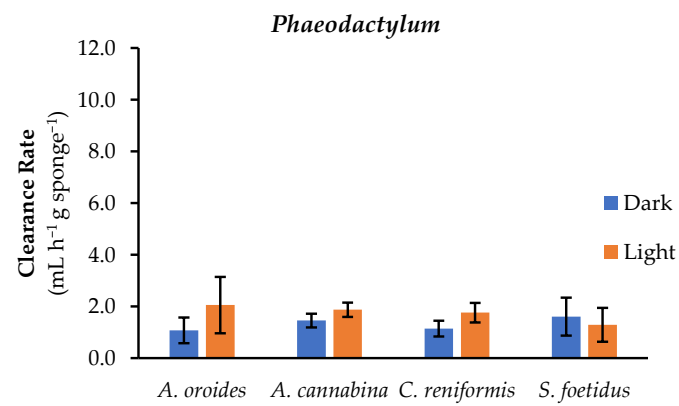
Figure 3.10. Average clearance rates of sponges *A. oroides, A. cannabina, C. reniformis,* and *S. foetidus* after a 7-h exposure to the three tested microalgae (a) *Nannochloropsis*; (b) *Isochrysis*; and (c) *Phaeodactylum* under light and dark conditions.

Notably, *A. cannabina* can exhibit different filtering performance in the presence and absence of light, depending on the substrate available in the surrounding seawater. Typically, *A. cannabina* can be

found in semi-dark habitats (e.g., caves) (Gerovasileiou et al., 2017), but also in places exposed to light (e.g., rocks, stones and calcareous algae at 15–55 m depth) (Voultsiadou, 2005a). Thus, we assume the perceived difference in its cleaning performance under dark and light conditions is probably attributed to substrate characteristics. For example, *Isochrysis* are single-celled marine autotrophic microalgae with enhanced cell motility driven by two flagellar systems (Garces et al., 2015). Studies have shown that their motility is strongly affected by environmental conditions such as light intensity, pH and nutrients (Quarmby, 2004). Recent findings indicate that, during low light periods, *Isochrysis* cells are less motile (Ishikawa Ishiwata et al., 2019). Hence, according to Maldonado et al., their capture would require less strain (Maldonado et al., 2010).

Contrastingly, a tendency for increased filtering activity in light rather than in dark conditions was observed for *C. reniformis* and partially for *A. cannabina*, but this difference is significant only when *Phaeodactylum* is used as a substrate. Since *C. reniformis* is inhabiting the littoral zone, with preference on shaded spots (Fassini et al., 2012; Lazoski et al., 2001), increased efficiency in the dark would be expected. Our finding may suggest that at least some sponge species can be more active in increased light intensity. Indeed, Reiswig reported diurnal variances in the pumping rates of the massive, shallow-water marine sponge *Tethya crypta*, with its pumping activity being higher under the regime of light (H. M. Reiswig, 1971). However, that divergence was attributed to the synchronization of the pumping activity with local water circulation patterns, using the light as a stimulus. Our results are rather inconclusive towards the identification of specific patterns of diurnal differences to filtration rates, suggesting that a more targeted investigation is required to address this concept in future studies.

3.5. Conclusions

In the present study, we were able to compare the filtering capacity and selectivity of four demosponges thriving in the Eastern Mediterranean, namely *A. oroides*, *A. cannabina*, *C. reniformis*, and *S. foetidus*, against three representative marine microalgae of different size and motility characteristics. This multiparametric investigation was made possible largely by using a high-throughput, microplate-based method for the fluorometric detection of microalgal cells in a large number of samples. To the best of our knowledge, this is the first time that such a methodological approach is applied in studies of this kind. Moreover, this is the first study to systematically assess filtering activity over a broad range of sponges, microalgal substrates, and different experimental setups.

The examined four sponge species showed distinct preferences regarding the filtering of microalgal substrates of different cell size. While *A. oroides* and, in part, *C. reniformis* followed the expected trend for increased clearance rates with decreasing particle size, this was inverted for *A.*

cannabina and *S. foetidus*, which clearly shows that preference to particle size is an innate trait that can show substantial variability and should be further examined without strict adherence to expectations.

Motility of the particulate substrate is another parameter to be considered when dealing with sponge filtering capacity, since an evident preference for non-motile substrates (*Nannochloropsis*) was observed in our experiments at least for two candidate sponges (*A. oroides* and *A. cannabina*) as compared to motile microalgae of the same size class (*Isochrysis*). This could imply that certain sponge species have optimized their aquiferous system for reduced-mobility (e.g., detritic) substrates, being less effective with microorganisms that are able to escape the inhalant flow.

Abundance of microalgal substrates in the surrounding medium was not found to play a prominent role to the filtration efficiency of sponges in our experiments, when addressing a wide range of concentrations approximating the gradient from oligotrophic to highly eutrophic systems, simulating the conditions prevailing in the vicinity of fish farms. Taking aside the fact that substrate concentrations were experimentally tested excluding other phenomena commonly associated with eutrophic conditions, such as enrichment in nutrients or presence of pollutants, this finding suggests that sponges retain an optimal filtering capacity along a broad spectrum of microalgal concentrations in the seawater.

No evidence for a potential effect of the presence or absence of light on the filtering performance of the examined sponges was observed. This could be expected based on the different preferences of the selected candidates to illumination conditions in their natural habitats. This implies that even sciaphilic species, such as *A. oroides* and *A. cannabina*, can effectively be used in adjacence to fish farms, which are commonly located in the open sea and, thus, exposed to light during the day. It remains to be shown, however, that sciaphilic species can be successfully reared in environments where daylight prevails.

All four candidate sponge species, commonly abundant in Eastern Mediterranean coastal habitats, showed the capacity to feed on microalgal cells. Taking aside variations of performance in the presence of substrates of different size and mobility characteristics, *A. oroides* appears as the most efficient filter-feeder, followed by *S. foetidus*. Hence, both species emerge as interesting candidates for bioremediation applications in IMTA scenarios. However, this evidence regards exclusively microalgae, which are a single component of the spectrum of microorganisms constituting the sponge diet. Similar experiments with viruses, bacteria, protists, and other pico- and nanoplanktonic organisms are still essential. Moreover, the *ex situ* experimental evidence presented herein should be supplemented by *in situ* experimental approaches, more closely approximating real-life conditions.
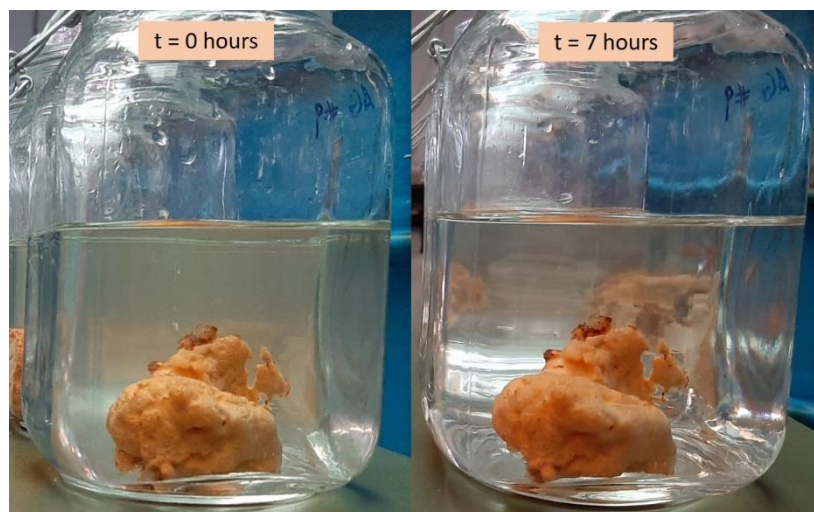
Figure 3.11. Purification of microalga-enriched seawater driven by *A. oroides* filtering activity over the course of 7 h.

Chapter 4. Bioremediation capacity:

Dissolved organic pollutants
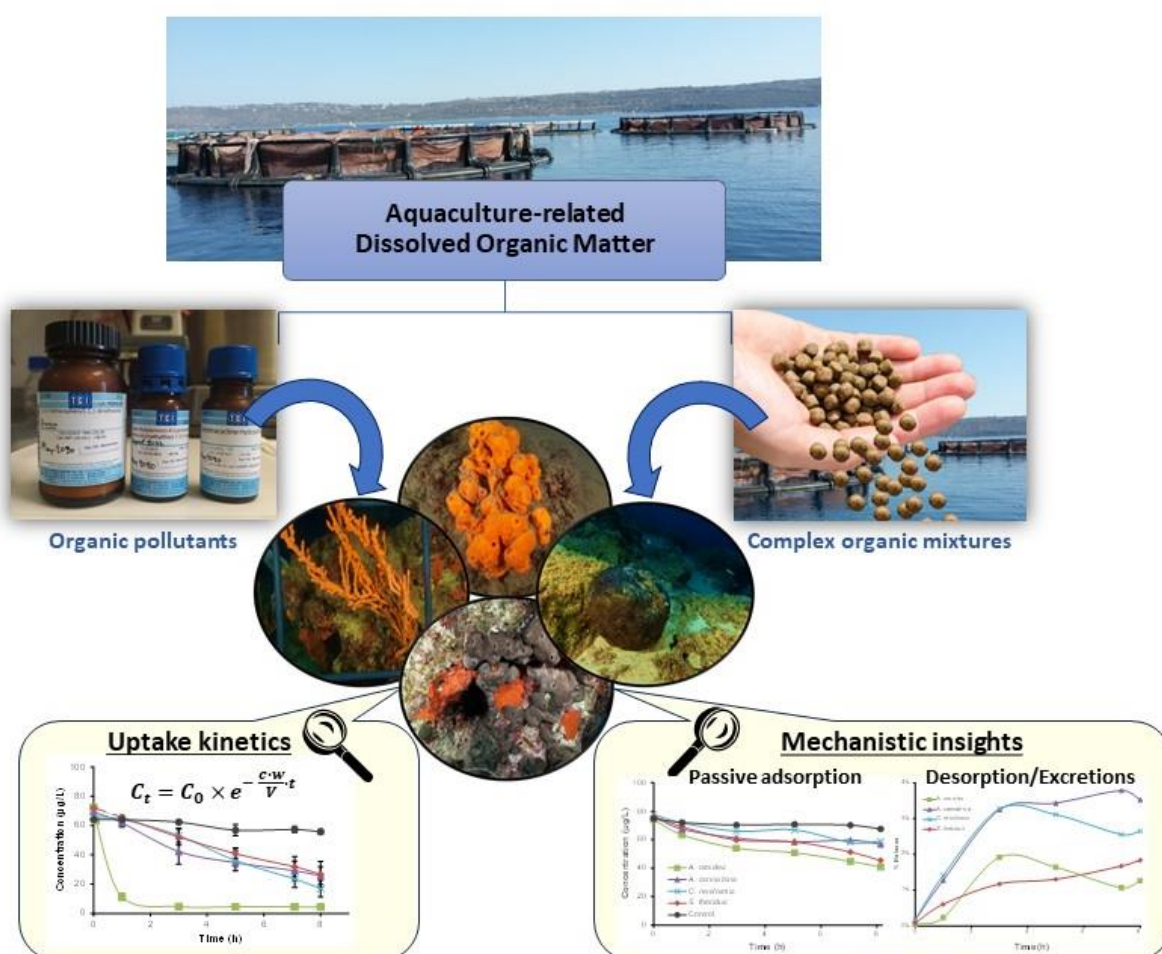
CRediT authorship contribution statement

Despoina Varamogianni-Mamatsi: Investigation, Formal analysis, Writing – original draft, Visualization. **Thekla I. Anastasiou:** Investigation. **Emmanouela Vernadou:** Investigation. **Nikos Kouvarakis:** Investigation. **Eirini Kagiampaki:** Investigation. **Nicolas Kalogerakis:** Writing – review & editing. **Thanos Dailianis:** Conceptualization, Resources, Writing – review & editing. **Manolis Mandalakis:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision.

4.1. Abstract

Besides the release of organic matter from uneaten feed and fish excreta, a considerable amount of deleterious chemicals may also end up into the marine environment from intensive aquaculture. A fraction of these pollutants remains freely dissolved and pose a threat to marine life due to increased bioavailability. Given the filter-feeding ability of sponges, we investigated the capacity of four ubiquitous Mediterranean species (*Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*) in removing aquaculture-related dissolved organic pollutants. These included individual chemicals belonging to antibiotics (i.e., oxytetracycline), antifouling biocides (i.e., diuron and Irgarol 1051) and polycyclic aromatic hydrocarbons (i.e., 2,6-dimethylnapththalene, phenanthrene). The uptake of pollutants was assessed *in vitro* by exposing small sponge explants to each chemical for a period of 8 h. Additional "cleanup" experiments were performed for complex mixtures mimicking the dissolved organic material encountered in fish farms, such as filtrates of fish feed and excreta. All sponges exhibited a pronounced preference for lipophilic pollutants and a strong positive correlation was revealed between clearance rate and substrate hydrophobicity. Our best filter-feeder (i.e., *A. oroides*) was able to clear 10.0 ± 1.3 mL of seawater per hour and per gram of sponge, when exposed to 2,6-dimethylnapththalene. Active pumping was found to be the predominant mechanism dictating the assimilation of dissolved pollutants in all sponge species, as it was 3–10 times faster than pollutants' passive adsorption on sponges' pinacoderm. Additionally, the uptaken pollutants were shown to be strongly retained by sponges and they were hardly released back to seawater as a result of desorption or sponge excretory mechanisms. Our study corroborates that sponges are highly efficient in uptaking dissolved organic compounds and it offers new insights into the kinetics and mechanisms ruling this process.

Keywords: Aquaculture wastes; Organic pollutants; Dissolved organic matter (DOM); Mediterranean sponges; Bioremediation; Uptake kinetics

4.2. Introduction

Aquaculture constitutes the driving force behind the growth in global fish production. Given the rising urgency for food sustainability, as well as the current dietary trends towards a better health, the specific economic sector has experienced great demand over the last few decades and will continue to expand, reaching 109 million tonnes in 2030 (FAO, 2020). However, intensification of fish farming has raised a series of environmental concerns. These are mainly associated with the release of large quantities of organic waste into the marine environment, predominantly generated from the dispersion of uneaten feed, fish faeces and soluble excretory products (Rosa et al., 2020). To enhance productivity and growth, a wide range of chemicals are used in fish farms, a considerable amount of which ends up

in the water column with sound threats engendered for the marine ecosystem. Such compounds include antibiotics, which are administrated to farmed species to control disease, pesticides to control parasites and algae, as well as antifouling agents and booster biocides to prevent the development of epibionts (i.e., marine biofouling) on the submerged infrastructures (Tornero and Hanke, 2016; Yebra et al., 2004). What is more, increased boating activity in proximity to fish farm facilities, can potentially pose an additional source of organic effluent through the release of petroleum-related combustion byproducts (Nasher et al., 2013).

Sponges (Phylum: Porifera) have recently been viewed as promising bioremediators in integrated aquaculture systems (Fu et al., 2006; Gökalp et al., 2021; Longo et al., 2022; Milanese et al., 2003; Pronzato et al., 1998). The innate capability of these sessile invertebrates to filter large volumes of water, by retaining efficiently different types of particulate organic matter (POM) including bacteria (Claus et al., 1967; Longo et al., 2010; Maldonado et al., 2010; Stabili et al., 2008, 2006; Wehrl et al., 2007; Zhang et al., 2010), phytoplankton (Frost, 1978; Riisgård et al., 1993; Varamogianni-Mamatsi et al., 2021) and even synthetic latex microspheres (Turon et al., 1997), has been well-documented. Many studies have provided further insights into the mechanisms by which sponges are able to capture suspended particles (Maldonado et al., 2010; Henry M. Reiswig, 1971; Van Well, 1949; Weissenfels, 1992).

The ability of sponges to feed on dissolved organic matter (DOM) has also been postulated (de Goeij et al., 2013), but the few existing *in vitro* attempts to assess this capacity are limited to conventional organic nutrients, such as amino acids (e.g., glycine; Stephens and Schinske, 1961) and sugars (e.g., fructose; Camacho et al., 2006). Additional laboratory studies examining small particles, such as 0.1 μm nanospheres (Leys and Eerkes-Medrano, 2006) and viruses (Hadas et al., 2006), have been used in support to the sponges' capacity for DOM feeding (considering that these substrates pass through 0.2-μm filter and they are technically included in the dissolved organic pool). Furthermore, Ribes et al. (1999) measured the *in situ* grazing rates of the temperate sponge *Dysidea avara* over an annual cycle, only to discover that the specific species was more a producer, rather than a consumer of dissolved organic carbon (DOC) (Ribes et al., 1999). It was Yahel et al. (2003) who revealed that most of the carbon gained by the reef sponge *Theonella swinhoei* was in dissolved (<90%) rather than in particulate form, highlighting the importance of DOC in metazoan nutrition and the role of these animals in carbon cycling (Yahel et al., 2003). In the same context, Goeij et al. (2008) reported the highest ever DOC removal rates for three encrusting reef sponges under in situ conditions (Goeij et al., 2008).

Although enlightening, the above findings do not focus on the bioremediation capability of these filter-feeding animals to reduce marine pollution resulting from aquaculture operation. It was Pronzato and his co-workers (Pronzato et al., 1999, 1998) who first attempted to cultivate the

Mediterranean bath sponges *Spongia officinalis* and *Hippospongia communis* near floating-cage fish farms in Kalymnos Island for the mitigation of the aquaculture organic wastes. However, their study was only limited to growth and survival rates of sponges, rather than the dynamics of the organic matter uptake. Furthermore, Fu et al. (2007) conceptualized the marine sponge *Hymeniacidon perlevis* as a potential bioremediator in integrated aquaculture ecosystems and they investigated the capacity of this species to remove organic detritus and excretions from the fish *Fugu rubripes* (Fu et al., 2007). Though, it is worth stressing that their study was targeted only to total organic carbon (TOC) removal. Later on, the same sponge was proved to be able to remove the organochlorine pesticide lindane from seawater under laboratory conditions (Aresta et al., 2015). Other studies have further indicated the bioaccumulation of organic pollutants in marine sponges, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (Gentric et al., 2016; Perez et al., 2003).

While the uptake of dissolved organic compounds by sponges has been previously investigated in a handful of studies, the kinetics of this process were scrutinized only in one of them (e.g., fructose; Camacho et al., 2006). Relevant research on the uptake kinetics of organic pollutants is completely absent. In the present study, a series of *in vitro* experiments was performed using four ubiquitous Mediterranean sponge species and their uptake kinetics against aquaculture-related pollutants, as well as complex organic mixtures, were assessed. We further aimed to clarify some key mechanistic aspects related to DOC uptake by sponges. In this context, we investigated passive adsorption of pollutants onto sponge biosurface and we assessed the contribution of this process to the overall uptake rate. We also examined whether the pollutants uptaken by sponges can be released back to seawater (i.e., via desorption or sponges excretory mechanisms). To the best of our knowledge, this is the first study to examine the uptake of various organic pollutants by several sponge species and provide comprehensive kinetic information about this process, accompanied by valuable mechanistic insights.

4.3. Materials and methods

4.3.1. Study sponge species

The filtering activity towards dissolved organic compounds was examined over four demosponge species that are commonly distributed in high abundances along eastern Mediterranean habitats (Voultsiadou, 2005b), namely: (a) *Agelas oroides*, (b) *Axinella cannabina*, (c) *Chondrosia reniformis*, and (d) *Sarcotragus foetidus*. The selection, sampling, transportation, acclimation and maintenance of the particular sponge specimens has been thoroughly described in a previous study (Varamogianni-Mamatsi et al., 2021). The main characteristics of those species are only briefly discussed below.
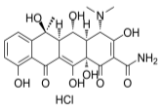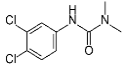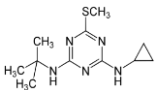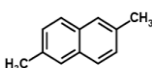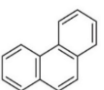
Agelas oroides (Schmidt, 1864) is a massive, variably lobate-digitate, vivid orange-colour demosponge, generally 5–25 cm in height and common in 2–40 m water depth. It preferably dwells in habitats with low light intensity (Ferretti et al., 2009; Idan et al., 2020). The erect, branching *Axinella cannabina* (Esper, 1794) mainly occurs along sciaphilous hard-bottom assemblages of the Mediterranean basin (Idan et al., 2018). The particular metazoan owns many short inner canals and can reach 55 cm in height (Gerovasileiou et al., 2015; Koukouras et al., 1996). The demosponge *Chondrosia reniformis* (Nardo, 1847) lacks typical skeletal structures (i.e., siliceous spicules or spongin fibers) and is particularly known for its unusual collagenous texture and regenerative properties, which have attracted great biotechnological interest (Nickel and Brümmer, 2003). Shaded environments of the littoral zone (0–50 m) commonly host this species (Wilkinson and Vacelet, 1979). *Sarcotragus foetidus* (Schmidt, 1862) is a variably dark-colour, rather common Mediterranean keratose demosponge, which approximates an irregularly globular to massive growth form, generally reaching 1 m in diameter and 50 cm in height. It is commonly found in shallow habitats exposed to light, but also in darker zones up to 400 m in depth (Manconi et al., 2013).

4.3.2. Substrates tested for sponge uptake

4.3.2.1. Chemical pollutants

The organic substrates selected for the "cleanup" experiments were covering different levels of lipophilicity and represented typical pollutants of various aquaculture settings. These are presented in **Table 4.1** along with some information about their use/origin and their octanol-water partition coefficients (Daghrir and Drogui, 2013; Konstantinou and Albanis, 2004; Loos, 2012; Miller et al., 1985).

Table 4.1. The studied organic substrates and their role in aquaculture settings along with their octanol-water partition coefficients (LogK$_{OW}$).

Chemical pollutant (Trivial name)	Chemical structure	Use/Origin	logK$_{ow}$
Oxytetracycline		Antibiotic with worldwide use in fish farming	-1.12 (Daghrir and Drogui, 2013)
3-(3,4-dichlorophenyl)1,1-dimethylurea (Diuron)		Non-metallic organic booster biocide widely used in marine antifouling paints	2.82 (Konstantinou and Albanis, 2004)
2-methylthio-4-butylamino-6-cyclopropylamine-s-triazine (Irgarol 1051)		Non-metallic organic booster biocide widely used in marine antifouling paints	3.95 (Loos, 2012)
2,6-Dimethylnaphthalene		Dicyclic aromatic hydrocarbon arising from combustion or oil anthropogenic activities	4.31 (Miller et al., 1985)
Phenanthrene		Tricyclic aromatic hydrocarbon arising from combustion or oil anthropogenic activities	4.57 (Miller et al., 1985)

The assimilation capacity of sponges was further examined against two complex mixtures of dissolved organic compounds, which mimicked aquaculture wastes and resulted from the filtration of water-solubilized fish feed and fish excreta. In the first case, solid feed pellets were mixed with ultrapure water and subsequently homogenized at ambient temperature using a conventional mixer. The solution was centrifuged (9500 g for 15 min) and the supernatant was then passed through a 0.45 μm polyethersulfone (PES) filter to remove any remaining submicron-sized particles. In the second case, fresh, crude samples of faeces were collected from fish breeding tanks of *Sparus aurata* and subsequently, homogenized at ambient temperature using a conventional mixer. The mixture was further centrifuged (7000 g for 15 min) and dissolved organic fraction was obtained by filtrating the supernatant through a 0.45 μm PES filter. Stock solutions of the two DOC substrates were stored at 4 ∘C until their use in sponge "cleanup" experiments.

4.3.3. Sponge-based experiments

4.3.1. Uptake of chemical pollutants and DOM by sponges

The experimental protocol followed for investigating the uptake of typical aquaculture pollutants was based on the methodology proposed by Varamogianni-Mamatsi et al. (2021) with a few modifications, depending on the applied substrate (Varamogianni-Mamatsi et al., 2021). In detail, the lab-scale experiments were performed in 2-L glass jars filled with 1 L of natural seawater (NSW) collected from sponge storage tanks and supplemented with the pollutant of interest. To ensure complete and fast dissolution of organic pollutants, crystals of each compound (~2 mg) were initially mixed with a small volume of organic solvent (e.g., 400 μL methanol) and then added in a carboy containing 20 L of NSW to reach a final concentration of ~100 μg L^{-1}. Jars were filled with 1-L aliquots of the solution and sponges were subsequently immersed in. For each chemical reagent tested, a total of twelve jars were prepared with explants of the four sponges (three replicates for each sponge species), while another three jars containing NSW and substrate (without sponge) served as controls. Over the experimental time course, aeration was applied by an air pump to achieve adequate water mixing and maximize oxygen levels. For the more volatile substrates, such as phenanthrene and 2,6-dimethylnapththalene (2,6-DMN), gentle rod-stirring was applied instead of aeration to minimize evaporation losses. Water samples of 1 mL were collected at regular time intervals (0, 1, 3, 5, 7, 8 h) and subjected to HPLC analysis. Regarding the DOM derived from fish feed and excreta, aliquots of their stock solution were diluted in NSW to reach a final concentration of 20–30 mg L^{-1} before sponge experiments. Water samples of 8 mL were collected from each jar at the same time intervals as described above and subjected to organic carbon analysis. In addition, the wet weight of each sponge explant was

measured at the nearest 0.1 g prior and after the experiments to verify weight stability and the average value was used in subsequent calculations.

Figure 4.1. The experimental set-up established for the *in vitro* investigation of sponges bioremediation capacity against aquaculture-related dissolved organic pollutants.

4.3.2. Assessment of pollutants desorption/release from sponges to seawater

Best-performing explants in terms of pollutants uptake (i.e., one specimen of each sponge species) were immersed in seawater containing ~100 μg L⁻¹ of phenanthrene and exposed to the chemical for 16 h. The wet weight of the specific explants was 151 g for *A. oroides*, 23 g for *A. cannabina*, 67 g for *C. reniformis* and 70 g for *S. foetidus*. Water samples collected before and after exposure were analyzed by HPLC to quantify the amount of phenanthrene uptaken by sponges. Subsequently, the explants were transferred in jars with fresh, pollutant-free NSW and 1-mL aliquots were collected at the same time intervals as previously described. The samples were analyzed by HPLC to assess the release of phenanthrene that may occur due to desorption or sponge excretory processes.

4.3.3. Assessment of passive adsorption of pollutants onto sponge biosurfaces

Best-performing explants of each sponge species were sacrificed by overnight freezing at −20 ∘C. Dead sponges lacking filtration/uptake activity were subsequently immersed in NSW containing ~100 μg L⁻¹ of phenanthrene. Water samples were collected and analyzed as described above to track phenanthrene removal due to passive adsorption on sponge biosurfaces.

4.3.4. Sample analysis

4.3.4.1. Chemical pollutants

Concentrations of the contaminants were measured in seawater over the course of sponge-uptake experiments using an Agilent 1260 Infinity Binary Pump HPLC system coupled with a UV–Vis diode array detector (Agilent Technologies; **Figure 4.2**a). Chromatographic separation of analytes was achieved with a Zorbax Eclipse Plus column (EC-C18, 2.1 × 50 mm, Agilent Technologies), by setting injection volume to 20 μL, temperature at 35 $\circ$C and flow rate of mobile phase at 0.5 mL min^{-1}. Mobile phase A was a mixture of water: methanol (95:5 v/v), while phase B was pure methanol. Mobile phase additives were not used with the exception of oxytetracycline (OTC) analysis, where formic acid was added in phase B (0.1% final concentration) to enhance chromatographic performance. A gradient elution program with a run time of 12 min was used as follows: 0 min: 10% B, 5 min: 100% B, 9 min: 100% B, 9.5 min: 10% B, 12 min: 10% B. The detection of pollutants was achieved spectrophotometrically at wavelengths of maximum absorbance (with 8 nm bandwidth), as follows: OTC, 356 nm; diuron, 250 nm; Irgarol 1051, 225 nm; 2,6-DMN, 224 nm; phenanthrene, 254 nm.
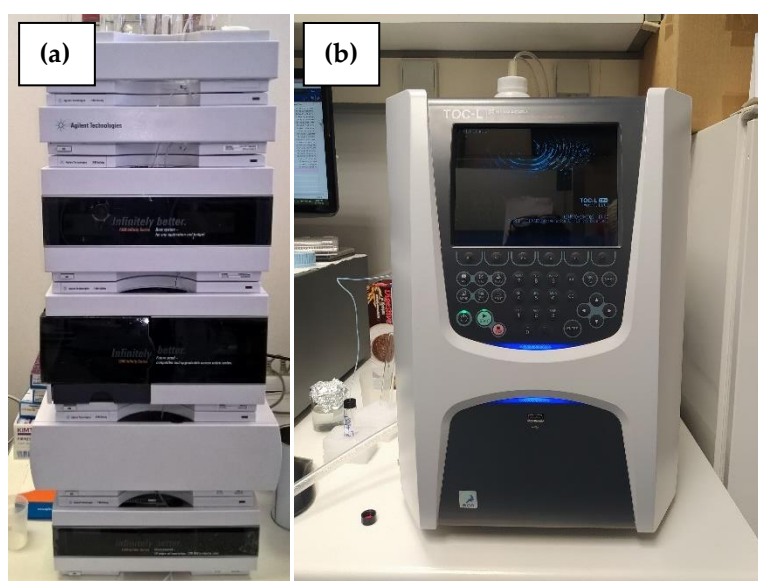
Figure 4.2. Analytical instrumentation employed for the analysis of organic contaminants in seawater samples; (a) The High-Performance Liquid Chromatography system (1260 Infinity with Binary Pump, Agilent Technologies) used for the detection of individual chemical pollutants and (b) the Total Organic Carbon analyzer (Shimadzu TOC–L$_{CSN/CSH}$, Japan) used for DOM measurements.

4.3.4.2. Dissolved organic matter

For the quantification of DOM levels over the course of sponge "cleanup" experiments with complex organic mixtures, seawater samples were subjected to a total organic carbon analyzer (Shimadzu TOC–L$_{CSN/CSH}$, Japan; **Figure 4.2**b) equipped with a high-salt combustion tube kit. The method of non-purgeable organic carbon (NPOC) was employed following manufacturers' default

settings. The analyses were performed by setting an injection volume of 50 μL, three replicates per sample and maximum coefficient of variation at 1.5%. A calibration curve was generated using standard solutions of potassium hydrogen phthalate. All NPOC measurements were subjected to blank subtraction to adjust for the minimal background levels of DOC in NSW.

4.3.5. Data analysis

4.3.5.1. Uptake kinetics and clearance rate (c)

The following exponential decay equation proposed by Coughlan (1969) and later on by Ferguson (1980), for the uptake of dissolved organic compounds by filter-feeders was used for the calculation of pollutants clearance rates:

$$C_t = C_0 \times e^{-c \cdot w \cdot t / V} \quad (4.1)$$

where C_0 and C_t are the concentrations of each pollutant in the treatment jar at the beginning of the experiment and at time t, respectively, c is the clearance rate (mL h^{-1} g^{-1}), V represents the volume of NSW (i.e., 1000 mL) in the jars, t is the time (h) and w is the wet weight of sponge (g).

Apart from the uptake driven by sponge's filtering activity, other processes may also contribute to the overall loss of chemical compounds in NSW, such as photooxidation (ECHA, 2018; Ogura, 1972; Sakkas et al., 2002; XiaoWu and Shao, 2017), adsorption on plastic materials (e. g., pipette tips/glass surfaces) (Buma et al., 2009), or volatilization (ECHA, 2018). Pollutants removal caused by those external factors was assumed to follow first-order kinetics, as shown in the equation below:

$$C_t = C_0 \times e^{-k \cdot t} \quad (4.2)$$

where k (h^{-1}) is the corresponding pollutant kinetic constant.

The depletion of organic substrates in our study systems was assumed to result from both sponge's filtering activity and external factors. Thus, the overall removal can be described by the following model, which derives from the sum of the **Equations (4.1)** and **(4.2)**:

$$C_t = C_0 \times e^{-\left(\frac{c \cdot w}{V} + k\right) \cdot t} = C_0 \times e^{-k_{total} \cdot t} \quad (4.3)$$

where k_{total} (h^{-1}) is the kinetic constant governing the overall substrate removal over the experimental time course. For each sponge system, k_{total} kinetic constants were obtained by fitting **Equation (4.3)** to the experimental C-t data. The respective k values describing pollutants removal by external factors were also derived after fitting **Equation (4.2)** to experimental data from control systems (experiments without sponges). Finally, the clearance rates (c) corresponding to sponge's filtering activity alone were calculated from the following equation:

$$c = \frac{V}{w}(k_{total} - k) \quad (4.4)$$

4.3.5.2. Retention rate (r)

According to Wehrl et al. (2007), retention rate is the term used to describe the quantity of a substrate that is uptaken by sponges, normalized to their wet weight (g) and time of exposure (minutes):

$$r = \frac{1 - (10^{y60})}{w} C_0 V \quad (4.5)$$

where y is the slope of the semi-logarithmic graph of C_t versus t for the linear time interval, multiplied by 60 to give retention rates per hour.

4.3.5.3. Retention efficiency (RE)

To estimate the ability of sponges to remove dissolved organic substrates from NSW in a percentage scale, we used the term *Retention Efficiency* (*RE*), as proposed by Stabili et al. (2006):

$$RE = \left(\frac{C_0 - C_t}{C_0}\right) \times 100\% \quad (4.6)$$

4.3.6. Statistical analysis

Analysis of variance (ANOVA) was used to test the differences between different experimental groups. Statistically significant differences were identified by setting the level of significance at $p < 0.05$.

4.4. Results and discussion

4.4.1. Kinetics of organic pollutants removal by marine sponges

A total of 75 laboratory experiments was performed in order to investigate the removal of five organic pollutants from seawater using four different species of marine sponges. The experiments were conducted in five independent sets, where three explants of each sponge species were immersed in seawater containing a pollutant of interest, and three extra jars were sponge-free (i.e., containing only the pollutant solution) to serve as control systems. Water samples were analyzed at regular intervals to determine the change in pollutants level over time. In all cases, the initial concentration of each compound was set to 100 μg L⁻¹. It is worth stressing that toxicity effects on sponges were not visually observed during their exposure to pollutants. **Figure 4.3**A− E shows the concentration-time profiles of OTC, diuron, Irgarol 1051, 2,6-DMN and phenanthrene over an 8-h treatment with *Agelas oroides*, *Axinella cannabina*, *Chondrosia reniformis* and *Sarcotragus foetidus*, as well as under the absence of any sponge (controls). Plotted data points represent the average values of three biological replicates (three sponge explants), while the error bars indicate standard deviation. The concentration of all chemicals showed a time-dependent decrease in the presence of sponges, the actual magnitude of which varied among the different species. More specifically, 2,6-DMN and phenanthrene exhibited the most striking

[108]

decrease, particularly when these compounds were subjected to *A. oroides*, while the change in OTC levels was hardly noticeable after 8-h exposure to any sponge species. An intermediate behavior was evident in the kinetic profiles of diuron and Irgarol 1051.
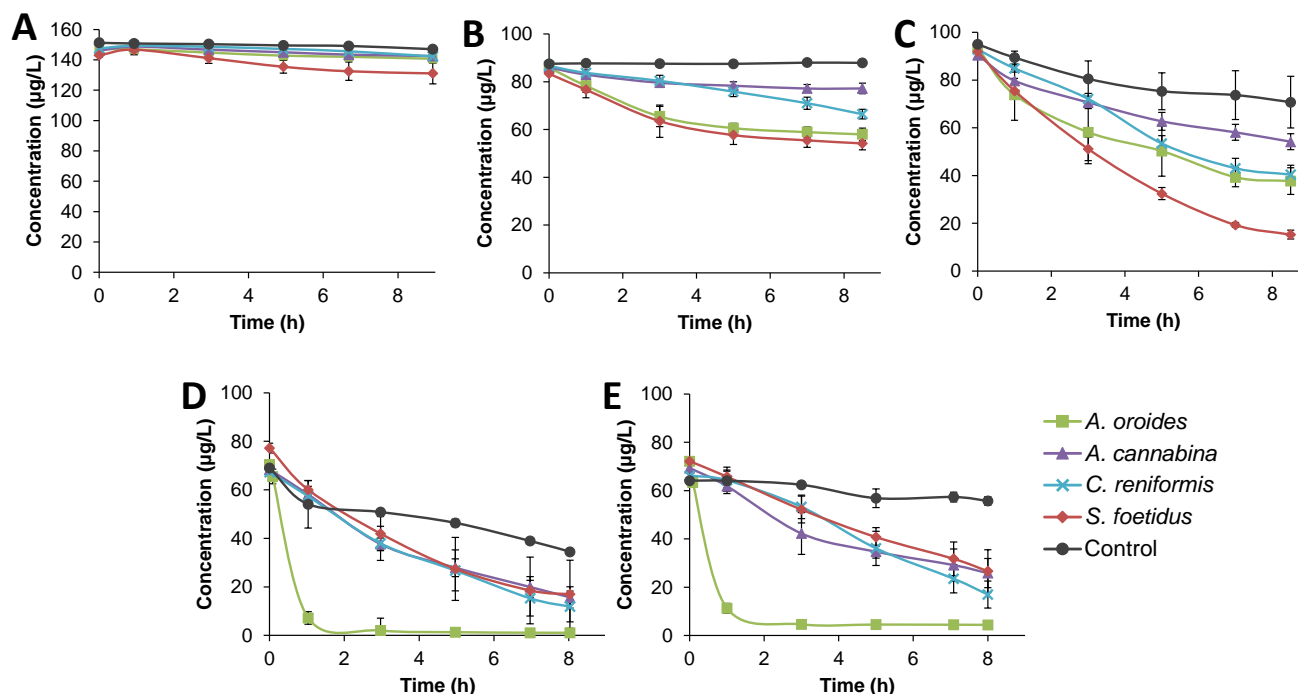
Figure 4.3. Concentration-time profiles (mean values ± SD) of (A) OTC, (B) diuron, (C) Irgarol 1051, (D) 2,6-DMN and (E) phenanthrene in the treatment (i.e., under the presence of the species *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus*) and control experiments (i.e., without sponges) over the course of 8 h.

A progressive but less profound decrease of concentration was also observed in the control experiments of Irgarol 1051 and 2,6-DMN, as well as for phenanthrene to much lesser extent, which complicated the evaluation of sponges' efficiency on pollutants removal. This finding implied that, in addition to sponge uptake, the decline of chemical substrates was also modulated by other processes, such as degradation, evaporation, adsorption or a combination of them. Indeed, Irgarol 1051 has been shown to be susceptible to photodegradation in natural waters (Sakkas et al., 2002), while adsorption on plastic materials (e.g., pipete tips) or glass surfaces has been also suggested to cause quick losses of this compound (Buma et al., 2009). On the other hand, 2,6-DMN was by far the most volatile compound among those tested (followed by phenanthrene) and dimethylnaphthalenes have been reported to undergo rapid volatilization and/or photodegradation when present in aqueous systems (ECHA, 2018). Regardless of the process dictating the results in control experiments, the concentration-time profiles of all five pollutants in the absence of sponges were highly reproducible across replicates (average RSD of analyte concentrations was <10%) and well described by first-order kinetics (R^2: 0.91–0.95).

The data from sponge experiments were also fitted to first-order kinetics (**Equation (4.3)**) and the influence of controls was subtracted to derive models describing the net effect of sponges on pollutants removal (**Equation (4.1)**). The latter were used to simulate the decrease of pollutants in seawater solely driven by the filtration activity of sponges (Figure A1). Based on this approach, all four types of sponges retained only 2–8% of OTC within 8 h, while the respective retention efficiency for diuron varied from 10 to 35%. A high retention efficiency of Irgarol 1051 was found for *S. foetidus* (79%), while moderate values were observed for the rest of the sponges (18–45%). With regard to 2,6-DMN and phenanthrene, *A. cannabina*, *C. reniformis* and *S. foetidus* demonstrated a similar behavior and removed 57–68% and 56–70% of those two pollutants, respectively. A more impressive effect was evidenced for *A. oroides*, which caused complete removal of 2,6-DMN and phenanthrene within ~4 h.

Previous studies dealing with the uptake of individual organic compounds by sponges are very scarce. Aresta et al. (2015) exposed *H. perlevis* to the organochlorine pesticide lindane and they observed a 50% decrease within 2 days (Aresta et al., 2015). Given the differences in sponge species and substrates, a detailed comparison with our results is of limited value, but overall lindane removal seems to follow slower kinetics. Interestingly, lindane-degrading bacteria were isolated from *H. perlevis* and they were suggested to contribute to the removal of this pesticide. Another study from early '60s examined amino acids uptake by 35 marine invertebrates including sponges (Stephens and Schinske, 1961). By performing laboratory experiments with *Cliona celata* and *Microciona prolifera*, a decrease in glycine levels by 38% and 90% was observed respectively, within ~20 h. Since amino acids are essential nutrients readily assimilated by all types of organisms, seawater was amended with antibiotics to minimize glycine uptake by microorganisms. Still, an effect from microbial growth cannot be ruled out in that study, as sponges and other seawater organisms have been frequently reported to host antibiotic-resistant bacteria (Laport et al., 2016; Phelan et al., 2011; Smaldone et al., 2014). In a similar investigation, faster kinetics were observed for fructose uptake by *Crambe crambe*, as the concentration of the specific substrate decreased by ~42% within 6 h (Camacho et al., 2006). Though, sponge excretions were suspected to interfere with fructose measurements, which were implemented with a universal carbon analyzer.

Clearance and retention rates are more appropriate measures for assessing the kinetics of pollutants removal by sponges, because the mass of the filtering organism is taken into account. The specific parameters were calculated using the wet weight of each sponge explant, while the average values were 81.5 ± 31.4 g, 32.3 ± 7.0 g, 67.7 ± 23.5 g and 86.1 ± 10.2 g for *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus*, respectively. Highly varying results were obtained for both clearance (0.03 to 10.0 mL h^{-1} g^{-1}) and retention rate (4 to 383 ng$_{chemical}$ h^{-1} g^{-1}) across sponges and chemical substrates (**Table 4.2**). The one-way ANOVA test indicated significant differences between the clearance rates of

the four sponge species for each pollutant investigated, while the same was also true for retention rates (Table A3). These differences resulted primarily due to the significantly higher values that *A. oroides* and *S. foetidus* presented in comparison to the other sponge species. More specifically, the clearance and retention rates of *A. oroides* for 2,6-DMN, phenanthrene and diuron were roughly 2–6 times higher compared to the other sponges, while *S. foetidus* exhibited 2–4 times higher rates for Irgarol 1051 and oxytetracycline.

Table 4.2. Clearance and retention rates of the four study sponge species derived for five organic pollutants with different levels of lipophilicity (i.e., LogK$_{OW}$).

	Clearance Rate, c (mL h^{-1} g$_{sponge}^{-1}$)				Retention Rate, r (ng$_{chemical}$ h^{-1} g$_{sponge}^{-1}$)			
Chemical	*A. oroides*	*A. cannabina*	*C. reniformis*	*S. foetidus*	*A. oroides*	*A. cannabina*	*C. reniformis*	*S. foetidus*
OTC	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	5 ± 2	8 ± 4	4 ± 0	15 ± 6
Diuron	0.8 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	125 ± 35	59 ± 12	30 ± 5	74 ± 27
Irgarol 1051	1.2 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	2.0 ± 0.4	105 ± 9	60 ± 22	76 ± 15	165 ± 32
2,6-DMN	10.0 ± 1.3	4.5 ± 1.2	3.1 ± 1.2	2.2 ± 2.1	383 ± 69	288 ± 80	196 ± 75	148 ± 122
Phenanthrene	7.8 ± 1.7	4.3 ± 0.7	3.3 ± 1.1	1.4 ± 0.3	324 ± 74	283 ± 45	200 ± 67	96 ± 17

With the exception of *S. foetidus*, ANOVA analysis also revealed significant differences among the five organic pollutants for each individual sponge investigated (Table A4). This outcome was largely driven by the significantly higher "cleanup" parameters obtained for phenanthrene and 2,6-DMN. Both of them demonstrated up to 284 times higher clearance rates and up to 76 times higher retention rates compared to all other compounds. By scrutinizing these results, a positive association between the "cleanup" parameters of the pollutants and their lipophilicity was suspected. By preparing log-log plot of clearance rates of the five pollutants versus their octanol-water partition coefficients (K$_{OW}$) (Figure A2), a linear relationship was observed for each one of the four sponge species (R^2: 0.84–0.95, $p < 0.05$). The equation describing the relationship of those two terms was Log(c) = 0.4017*Log(K$_{OW}$)-1.0959 for *A. oroides*, Log(c) = 0.3168*Log(K$_{OW}$)-1.0592 for *A. cannabina*, Log(c) = 0.3508*Log(K$_{OW}$)-1.2443 for *C. reniformis* and Log(c) = 0.2224*Log(K$_{OW}$)-0.7709 for *S. foetidus*. With the exception of *S. foetidus*, all other sponge species resulted in equations with almost identical slopes. Strong positive correlations with K$_{OW}$, accompanied by similar curve slopes, were also evident in the respective plots of retention rates (Figure A3 and Table A5). Despite the aforementioned dissimilarities in the magnitude of clearance/retention rates among sponges (**Table 4.2**), the consistency in curve slopes implies that the mechanism governing the kinetics of organic pollutants uptake by the different sponges is of similar nature and K$_{OW}$-dependent.

Bioaccumulation of pollutants in marine biota, representing the result of dynamic equilibrium between the uptake (both waterborne and dietary) and elimination processes taking place, has been extensively studied. In the case of persistent organic pollutants, which are hardly metabolized or degraded, bioaccumulation practically reflects the dynamics of pollutants uptake and this has been widely documented to be highly correlated with pollutants hydrophobicity in various types of aquatic organisms (Ding et al., 2019; Oliver and Niimi, 1988). Notably, strong positive correlations with Log K_{OW} have been reported for the bioconcentration of organic pollutants (i.e., waterborne-only uptake) in filter-feeders, such as mussels (Endicott et al., 1998; Geyer et al., 1991). In relation to those findings, our results suggest that the lipophilicity of organic pollutants has a dominant effect not only on the dynamics but also on the kinetics of their uptake by filter-feeder organisms and especially marine sponges.

Despite the significant variability observed in the present study for the "cleanup" metrics of sponges among different pollutants and species, the ranges of values obtained for clearance rates were similar to those that previously reported for the uptake of microalgae by the same sponge species (clearance rate: 0.4–7.0 mL h^{-1} g^{-1}; Varamogianni-Mamatsi et al., 2021). This means that the specific sponges can serve as efficient biofilters not only for the removal of POM and biological particles, but also for the mitigation of dissolved organic pollutants that may enter aquatic environments as a result of intensive aquaculture practices.

4.4.2. Removal of DOM by marine sponges

Apart from individual organic pollutants, we further investigated the "cleanup" capacity of sponges against complex mixtures of dissolved organic compounds. More specifically, two types of DOM, resulting from the 0.45 μm-filtrate of sea bream excreta and a typical fish feed, were selected in an effort to simulate the DOM present in the seawaters of fish aquaculture farms. **Figure 4.4**A-B shows the concentration-time profiles of those two DOM substrates over an 8-h exposure to the four sponge species. Although not as rapid as with individual pollutants, a gradual decrease of both substrates was also evident for the majority of species, while the loss of DOM in the control experiments was minimal. After 8-h of exposure, sponges retained 4–15% and 4–30% of the DOM originating from fish feed and fish excreta, respectively. Once again, *A. oroides* stood out as the best DOM uptaker among the four sponge species.

The decrease in DOM content was fairly described by the exponential model given in **Equation (4.3)** (R^2: 0.79–0.95 and 0.88–0.97 for DOM of fish excreta and fish feed, respectively). However, a deviation was evident when *A. oroides* was exposed to DOM from fish excreta. The latter showed a considerable decline over the first 5 h and then tended to an asymptotic value. Similar

saturation patterns have been observed in a previous *in vitro* study of Camacho et al. (2006), where explants of the poeciloclerid sponge *C. crambe* (Schmidt) were exposed to various fructose concentrations (Camacho et al., 2006).
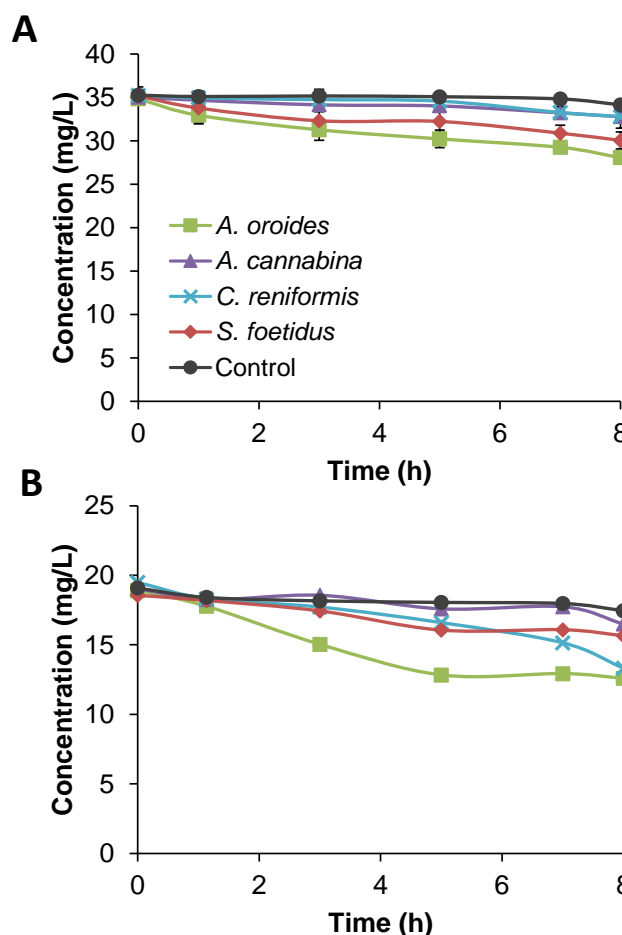
Figure 4.4. Concentration-time profiles (mean values ± SD) of two representative aquaculture DOM forms; (A) fish feed and (B) fish faeces, in the treatment (i.e., under the presence of the species *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus*) and control experiments (i.e., without sponges) over the course of 8 h.

The clearance and retention rates were calculated for the different sponge species and DOM substrates using the experimental results from three explants. The average values are presented in **Table 4.3**. Significant differences in the assimilation rates of the two substrates were tracked for *A. oroides* and *C. reniformis* (*t*-test, $p < 0.05$), with DOM from fish excreta providing higher values in both cases. In general, the clearance rates derived for organic mixtures were in the lower range of those measured for individual organic pollutants and approximated the values of pollutants of low hydrophobicity (i.e., OTC, diuron). Based on this finding, it was hypothesized that DOM substrates were mainly composed of hydrophilic components that can be barely assimilated by sponges. To test this possibility, the lipophilicity of both substrates was assessed using reversed-phase HPLC, which separates the different molecules on the basis of their hydrophobic interactions with the stationary phase. Indeed, the

chromatograms of both DOM types comprised intense unresolved peaks mainly eluting at retention times lower than 1.0 min (Figures A4 and A5). Considering that OTC and diuron eluted at 2.0 and 4.0 min, respectively, it can reasonably be inferred that DOM substrates were of hydrophilic nature, thus explaining their limited uptake from sponges.

Table 4.3. Clearance and retention rates of the four study sponge species for two different types of dissolved organic matter obtained from fish feed and faeces.

DOM source	Clearance Rate, c (mL h^{-1} g$_{sponge}^{-1}$)				Retention Rate, r (μg$_{DOC}$ h^{-1} g$_{sponge}^{-1}$)			
	A. oroides	*A. cannabina*	*C. reniformis*	*S. foetidus*	*A. oroides*	*A. cannabina*	*C. reniformis*	*S. foetidus*
Fish feed	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	6 ± 1	7 ± 1	4 ± 0	7 ± 3
Fish feces	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	9 ± 0	4 ± 0	9 ± 0	3 ± 0

To further investigate the cleanup behavior of sponges against complex organic mixtures and whether faster uptake can be achieved for DOM of greater lipophilic character, additional experiments were performed using a dilute solution of olive oil. The HPLC analysis of this substrate revealed major peaks eluting at higher retention times (2.25–3.71 min) than those observed in the chromatograms of fish feed and faeces (Figure A6). Due to resource constraints, the specific "cleanup" tests were limited to the *A. oroides* explant presenting the highest removal rates for individual organic pollutants. Figure A7 displays the concentration-time profile of olive oil in experimental systems with or without *A. oroides*. Under the filtering action of sponge, the organic substrate showed a sharp decline and it was totally consumed within 5 h. The corresponding clearance rate was estimated as 5.6 mL h^{-1} g^{-1}, outpacing the values measured for DOM of fish feed (0.2 mL h^{-1} g^{-1}) and fish excreta (0.5 mL h^{-1} g^{-1}), while it was close to the rate observed for the highly lipophilic phenanthrene (7.8 mL h^{-1} g^{-1}). These results corroborate that sponges are capable of uptaking multi-component organic mixtures from seawater and the speed of this process is determined by the overall lipophilic character of the mixture rather than its chemical complexity.

A comparison of our results with previous *in vitro* studies, dealing with complex mixtures uptake, is hard to be carried out. The primary reason concerns their limited number and their inconsistency with our target substrates. In the *in vitro* study of Fu et al. (2007), clones of the sponge *H. perlevis* were exposed to different concentrations of an algal/fish protein powder mixture for 24 h (Fu et al., 2007). However, this so-called dead organic matter diet was used as a representative of TOC pool in aquaculture settings, which means that besides DOC, the effect of particulate organic material was also taken under consideration. Such differences in the substrate nature of the latter study and ours, make the comparison of the results quite difficult. Leaving retention metrics aside, we can report that the

clearance rates obtained from our study (0.1–0.5 mL h^{-1} g^{-1} wet weight) fluctuated within the lower range of those measured for *H. perlevis* (0.33–7.64 mL h^{-1} g^{-1} wet weight).

Additionally, the uptake of natural seawater DOM has been investigated in a handful of *in situ* studies, but few of them actually provide numeric insights into the respective sponge rates. By placing sponges in incubation chambers and performing experiments at 12 m depth, de Goeij et al. (2008) investigated DOC removal from three encrusting species (Goeij et al., 2008). The clearance rates obtained from those *in situ* measurements were 126–702 mL h^{-1} g^{-1} for *Halisarca caerulea*, 180–330 for *Mycale microsigmatosa* and 168–210 for *Merlia normani* (assuming sponge density of 1 g mL^{-1}), which are three orders of magnitude higher than ours.

Yahel et al. (2003) measured *in situ* the DOC content of the water inhaled and exhaled by the Indo-West Pacific reef sponge *T. swinhoei* in an effort to unravel the "mystery" between metazoans and DOC cycling (Yahel et al., 2003). Their findings indicated an average carbon uptake of 26.0 nmol C mL$_{sponge}^{-1}$ min^{-1}, which is equivalent to 18.7 μg C h^{-1} g^{-1}, assuming 1 g mL^{-1} sponge density. The latter value is only two times higher than those observed for *A. oroides* and *C. reniformis* (9.0 μg C h^{-1} g^{-1}) in the present study.

By following a similar, yet more improved sampling technique, Ribes et al. (2023) intended to investigate the seasonal DOM uptake by four different sponges, including our case study species *A. oroides* and *C. reniformis* (Ribes et al., 2023). The average retention rates with respect to ambient DOC were estimated in the range of 17.9–107.3 μg h^{-1} g^{-1} (assuming 1 g mL^{-1} sponge density). Considering that sponges are likely to function optimally when present in their natural habitats, it is not surprising to receive lower cleaning metrics in our laboratory-based experiments.

4.4.3. Mechanistic insights into the uptake of dissolved organic pollutants by sponges

Although many studies have brought into light the mechanisms by which sponges uptake POM (i.e., bacteria, microalgae, latex spheres) (Henry M. Reiswig, 1971; Turon et al., 1997), little is known about DOM assimilation. Some microscopic observations suggested that sponge choanocytes are capable of capturing particles of very small size, such as 0.1 μm latex beads (Leys and Eerkes-Medrano, 2006), which are operationally classified as "dissolved" (i.e., material passing through 0.45 μm filters). The sieving function of choanocyte collars against microparticles was underscored in that study, but actual information about the interactions of water-soluble molecules with sponge surface was not provided. Other reports suggested the implication of microbial symbionts in the uptake and transformation of DOM by sponges (particularly in species with high microbial abundance; Olinger et al., 2021), but a symbiont-free uptake of DOC has been also supported (Gantt et al., 2019).

Regardless of microbes contribution, it is obvious that the capture of dissolved organic molecules is dictated by processes taking place in the aquiferous system of sponges. To the best of our knowledge, it is only Norman et al. (2014) who thoroughly investigated the adsorption kinetics of carmine dye on sponges, but this study aimed at staining dry demosponge skeleton (sponginin) for biomedical applications rather than elucidating the uptake of dissolved compounds in the biosurface of living sponges. Nevertheless, carmine was shown to be readily adsorbed on proteinaceous sponge skeleton, but a small percentage of it (5 to 19%) could be desorbed back to fresh water (Norman et al., 2014).

Considering this fact, we conducted additional experiments to ascertain if organic pollutants uptake is governed by the active pumping/filtering of sponges or by simple passive adsorption on sponge biosurfaces and whether assimilated pollutants can return back to water due to desorption/excretion mechanisms. This investigation was limited to phenanthrene, which was one of the most readily assimilated compounds by the different sponge species.

4.4.3.1. Assessment of passive adsorption of pollutants onto sponge biosurfaces

To evaluate the contribution of passive adsorption of organic pollutants by sponge biosurfaces, one explant from each sponge species was sacrificed and the dead specimens were exposed to a phenanthrene solution. **Figure 4.5** shows the concentration-time profile of phenanthrene in sponge and control experiments over the 8-h exposure period. In the presence of dead sponge explants, phenanthrene concentration showed a substantial decrease, which, after control subtraction, ranged between 16% (*A. cannabina*) and 37% (*A. oroides*) at the end of the experiments. However, these values are quite low when compared with the rapid removal of the same compound by living sponges (**Figure 4.3**E). For example, the decrease of phenanthrene by living *A. oroides* reached 93% and 99% after just 1 and 3 h of exposure, respectively.
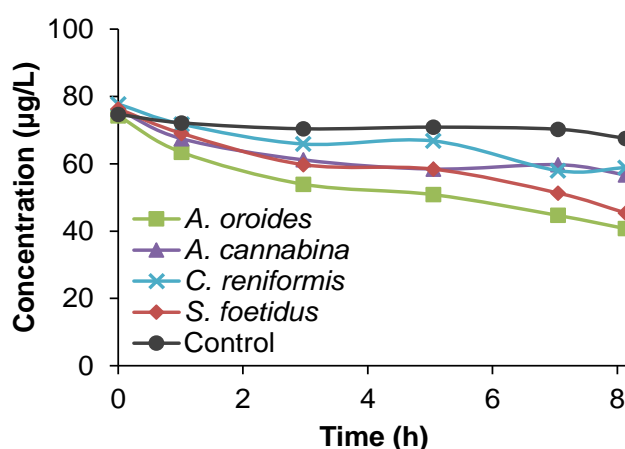
Figure 4.5. Concentration-time profiles of phenanthrene in the treatment (i.e., under the presence of dead *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus* fragments) and control experiments (i.e., without sponges) over the course of 8 h, when assessing passive adsorption phenomena onto sponges biosurface.

[116]

Similarly to the procedure described above, the data from sponge experiments were fitted to a first-order kinetic model (**Equation (4.3)**) and the influence of controls was subtracted to derive equations simulating the net effect of phenanthrene adsorption by sponges (Figure A8). In all cases, the model provided good fit to experimental data (R^2: 0.79–0.96). In analogy to retention rates, the adsorption rates of phenanthrene were derived using the modeled data. A comparison of retention rates resulting from passive adsorption effects and active pumping/filtration activity of sponges, is presented in **Table 4.4**. Since the active uptake of phenanthrene by *A. oroides* was almost completed within the first 1 h (**Figure 4.3**E), all rates were calculated for the same time period to maintain consistency. The retention rates obtained for phenanthrene adsorption on sponges were relatively low and indicated limited variability among the different species (33 to 58 ng of pollutant retained per hour and gram of sponge wet weight). For three of the study sponge species, these values were 6 to 10 times lower than the respective rates calculated above for active uptake (224 to 430 ng h^{-1} g^{-1}), while a 3-fold difference was observed for *S. foetidus* (active uptake: 112 ng h^{-1} g^{-1}).

Table 4.4. Retention rates (in ng$_{chemical}$ h^{-1} g_{sponge}^{-1}) attributed to passive adsorption and pumping activity of the four study sponge species against phenanthrene.

Retention process	*A. oroides*	*A. cannabina*	*C. reniformis*	*S. foetidus*
Passive adsorption	45	58	33	40
Active uptake	430	329	224	112

Based on our findings, DOM uptake by sponges is mainly a biological process that can be reasonably attributed to the function of choanocytes. These flagellated cells line the microcavities in the canal system of sponges and represent their basic pumping and filtering units. The morphological and functional similarities of choanocytes with their closest protistan relatives, the choanoflagellates, have been discussed for centuries (Mah et al., 2014). The latter have long been documented to ingest a variety of dissolved organic compounds, including carbohydrates, proteins, components of the colloidal fraction of DOM (Tranvik et al., 1993), as well as high molecular weight polysaccharide dextrans (Marchant and Scott, 1993; Sherr, 1988). Given this fact, it is not surprising that sponges are capable of removing DOM and individual chemicals from seawater. In both cell types, the capturing mechanism should involve flagellum-mediated diffusion of the organic solutes from the bulk liquid to the base of choanocyte collars and their subsequent pinocytosis through the formation of intracellular vacuoles (Hickman et al., 2003). Sponge microbial symbionts have been also suggested to play a role in the retention of heavy metals by sponges (Gravina et al., 2022), but their contribution to the uptake of dissolved organic pollutants remains unclear. Additional studies are required to reveal whether

diffusion, pinocytosis, sponge-associated microbiota activity, or a combination of all of them, control the overall kinetics of DOM uptake by sponges.

4.4.3.2. Assessment of pollutants desorption/release from sponges to seawater

Although active pumping/filtration was found to dictate dissolved pollutants uptake by sponges, it is not clear if this process is reversible and whether entrapped pollutants can return back to water through desorption or excretory phenomena. To investigate this aspect, explants of the four study sponges were exposed to phenanthrene solution for sufficient time (i.e., 16 h) to enable pollutant uptake and subsequently immersed in pollutant-free NSW. By monitoring pollutant levels for the following 8 h, release of phenanthrene was assessed. **Figure 4.6** illustrates the percentage of sponge-uptaken phenanthrene that was returned back to clean NSW over the course of experiments. For all sponges, the levels of desorbing/excreting phenanthrene demonstrated a gradual increase during the first 3 h, followed by quite steady values. The species *A. cannabina* and *C. reniformis* showed a more pronounce release, which approached 3% by the end of the experiments, while slightly lower values were observed for *A. oroides* and *S. foetidus* (~1.5 %). Despite minor discrepancies, our findings suggest that organic pollutants uptaken by marine sponges are strongly attached on their aquiferous system and they can be hardly released back to the surrounding environment.
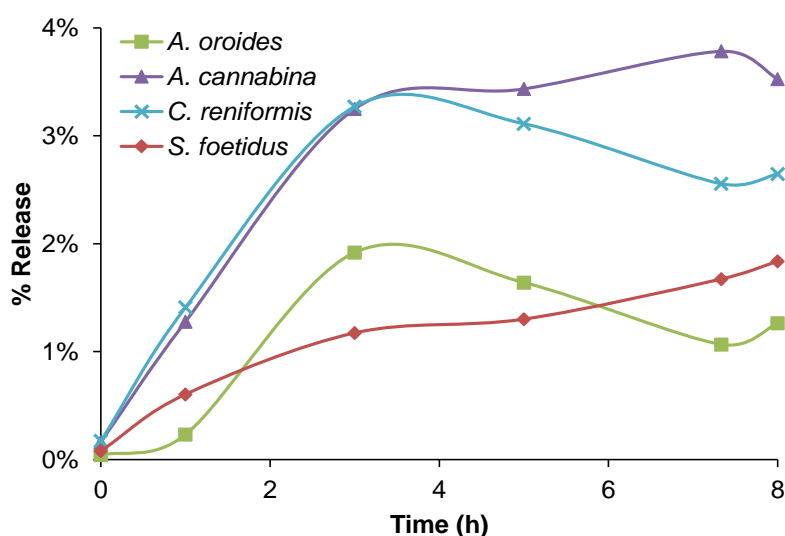
Figure 4.6. Release percentage of uptaken phenanthrene by *A. oroides, A. cannabina, C. reniformis* and *S. foetidus* over the course of 8 h.

4.5. Conclusion

The present study focused on the uptake of dissolved organic substances by marine sponges. The kinetics of this process were investigated for four widespread Mediterranean sponges against a series of aquaculture-related organic pollutants and complex mixtures, in an effort to explore the

potential of these biofiltering organisms for the alleviation of pollution near fish farms. All species were able to assimilate individual pollutants belonging to antibiotics, antifouling biocides and PAHs, but the speed varied significantly among compounds. With regard to multi-component organic mixtures, sponges retained DOM from fish feed and fish excreta at moderate rates, while a much faster DOM uptake was observed for a solution of olive oil composed of more lipophilic compounds. The species *A. oroides* exhibited the greatest filtering performance over all individual pollutants and DOM substrates, with the highest rates being recorded for the polyaromatic hydrocarbons 2,6-DMN and phenanthrene. In all four sponges, a pronounced preference for highly lipophilic compounds was discovered and all "cleanup" metrics demonstrated a strong positive correlation with the hydrophobicity of the compounds. To a further extent, we showed that active water pumping/filtration of sponges is the major driving force behind the removal of dissolved pollutants from seawater, as the uptake rates resulting from this mechanism were much higher than those derived for passive adsorption of pollutants onto the surface of dead sponges. Last, but not least, our results showed that the organic pollutants uptaken by sponges can barely be released back to seawater via desorption or excretory mechanisms, implying that the chemicals were strongly retained into the inner sponge body.

Sponges have been proved so far to be one of the most promising candidates for bioremediation in integrated multitrophic aquaculture systems. Apart from the well-known ability of sponges in removing POM, our study provides extensive evidence that these invertebrates are also very effective DOC uptakers and hold great potential as bioremediators in fish farms or other areas impacted by excessive organic/chemical loadings. Further field investigations and *in situ* trials are warranted to verify the fast uptake kinetics of sponges for dissolved organic pollutants under real-life conditions.

Chapter 5. Bioproduction Potential

CRediT authorship contribution statement

Footnote:

5.1. Abstract

Marine sponges are highly efficient in removing organic pollutants and their cultivation, adjacent to fish farms, is increasingly considered as a strategy for improving seawater quality. Moreover, these invertebrates produce a plethora of bioactive metabolites, which could translate into an extra profit for the aquaculture sector. Here, we investigated the chemical profile and bioactivity of two Mediterranean species (i.e., *Agelas oroides* and *Sarcotragus foetidus*) and we assessed whether cultivated sponges differed substantially from their wild counterparts. Metabolomic analysis of crude sponge extracts revealed species-specific chemical patterns, with *A. oroides* and *S. foetidus* dominated by alkaloids and lipids, respectively. More importantly, farmed and wild explants of each species demonstrated similar chemical fingerprints, with the majority of the metabolites showing modest differences on a sponge mass-normalized basis. Furthermore, farmed sponge extracts presented similar or slightly lower antibacterial activity against methicillin-resistant *Staphylococcus aureus*, compared to the extracts resulting from wild sponges. Anticancer assays against human colorectal carcinoma cells (HCT-116) revealed marginally active extracts from both wild and farmed *S. foetidus* populations. Our study highlights that, besides mitigating organic pollution in fish aquaculture, sponge farming can serve as a valuable resource of biomolecules, with promising potential in pharmaceutical and biomedical applications.

Keywords: porifera; demospongiae; marine sponge farming; chemical fingerprinting; marine natural products; secondary metabolites; primary metabolites; MS/MS dereplication; bioactive compounds supply; aquaculture and fish farming

5.2. Introduction

While aquaculture constitutes the fastest growing food production system (Garlock et al., 2022), it can exert pressure on adjacent marine habitats, mainly through the release of organic load and other substances (Taranger et al., 2015). Among bioremediation candidates to reduce organic pollution, marine sponges have attracted widespread interest (Fu et al., 2006; Gökalp et al., 2021; Longo et al., 2022; Milanese et al., 2003), due to their innate filter-feeding properties to remove particles (e.g., bacteria; Claus et al., 1967; Longo et al., 2010; Maldonado et al., 2010; Stabili et al., 2006; Wehrl et al., 2007; Zhang et al., 2010), phytoplankton (Frost, 1978; Osinga et al., 2001; Riisgård et al., 1993; Varamogianni-Mamatsi et al., 2021), and dissolved organic (Aresta et al., 2015; Camacho et al., 2006; Ribes et al., 2023)/inorganic substrates (Ferrante et al., 2018; Gravina et al., 2022), as well as fish farm wastes (Fu et al., 2007; Pronzato, 1999; Varamogianni-Mamatsi et al., 2023) from seawater. Besides their high cleanup capacity, marine sponges also hold great biotechnological potential, and their biomass can be turned into products of high added-value, serving as an additional source of profit for aquaculture-related enterprises. In fact,

to date, more than 5000 structurally characterized metabolites have been isolated from marine sponges, contributing to about 30% of all marine natural products (Han et al., 2019). Although a variety of sponges is currently known to produce an overwhelming array of secondary metabolites with pharmaceutical potential (Sipkema et al., 2005), the problem of their supply still remains a typical limiting factor for pre-clinical evaluation (Carballo et al., 2010) and further drug development (Munro et al., 1999).

Over the years, various techniques have been proposed to overcome this bottleneck. Synthesis of bioactive natural products or their related analogues (e.g., via chemical or microbial processes) has always been the preferred method for drug manufacture in pharmaceutical industry. Some of the few successful sponge-derived examples of this strategy are the well-known drugs adenine arabinoside (Ara-A, Vidarabine®) and cytosine arabinoside (Ara-C, Cytarabine®). Both synthetic products constitute derivatives of sponge nucleosides (Bergmann and Feeney, 1951, 1950), and they have been clinically approved for use as antiviral and antitumor drugs, respectively (Bodey et al., 1969; Buchanan and Hess, 1980; Oliver et al., 1994; Paintsil and Cheng, 2019). However, the development of synthetic strategies for the production of other marine metabolites with greater structural complexity is often challenging and economically unfeasible, even for the purpose of preclinical testing (Carballo et al., 2010; Sipkema et al., 2005).

Wild harvesting of sponges that are prolific sources of bioactive compounds has commonly been suggested as a method to supply novel therapeutic agents. However, the typically low naturally occurring concentrations of produced bioactive metabolites, combined with valid concerns for the conservation of sponge diversity in marine ecosystems, are the main reasons to consider this method unsuitable (Belarbi et al., 2003; Carballo et al., 2010; Page et al., 2011). This also aligns with the responsible research and innovation aspects that comply with environmental and societal values (Schneider et al., 2023, 2022). Nonetheless, a variety of novel marine drug-leads have proceeded to preclinical and clinical trials using materials from wild harvesting. This is the case for avarol, a novel sesquiterpenoid hydroquinone, which is uniquely found in the abundant Mediterranean species *Dysidea avara* (Müller et al., 1985). Being one of the most popular sponge-derived bioactive compounds, avarol exhibited strong anti-HIV activity during its preliminary testing (Müller and Schröder, 1991), but it was later withdrawn from human clinical trials. Moreover, avarol was patented as an anti-psoriasis agent (Müller, 1991), being used in paramedic medicine as one of the ingredients of topical ointments against psoriasis.

Halichondrin B, a metabolite isolated from the sponge *Lissodendoryx* sp., was reported by Hirata and Uemura (1986) as a strong antitumor agent, with a special effect against several melanoma types

and leukemia (Carballo et al., 2010). Although this compound has entered phase I of clinical trials (Munro et al., 1999), it was produced at very low concentrations by harvested sponges. The cytotoxic metabolite peloruside A, which is isolated from specimens of the sponge *Mycale hentscheli*, is another highly promising antitumor agent, since it operates in a similar way as the anticancer drug Taxol®, used to treat ovarian and breast cancers (Page et al., 2011). However, similarly to halichondrin B, wild sponge populations were unable to provide high yields of this compound to proceed in further drug development (M. Page et al., 2005).

Other methods, such as cell lines, primmorphs, and *ex situ* culture, have extensively been investigated as economically feasible approaches for obtaining sufficient quantities of drug-lead sponge molecules, but they have shown a number of limitations (e.g., high time and resources consumption, poor growth rates) (De Rosa et al., 2003; Hadas et al., 2005; W. E. G. Müller et al., 2000). By taking into account the plant-like regeneration capability of sponges, and their resilience to overcome physical damage (Wulff, 2006), mariculture of these metazoans merges as a promising, cost-effective outlook for the sufficient and sustainable supply of biologically active metabolites (A. Duckworth and Battershill, 2003). In combination with their filter-feeding characteristics, setting up a sponge farm in proximity to aquaculture operations is becoming highly appealing, since it can promote bioremediation applications through profitability (Dailianis and Mandalakis, 2019).

However, it is of great importance to reassure farmers that the cultivated sponge fragments are able to reproduce the targeted bioactive metabolites of their wild counterparts. In this context, we performed comparative chemical profiling of sponge crude extracts, obtained from wild and farmed populations. The study focused on two widely distributed Mediterranean species, namely *Agelas oroides* and *Sarcotragus foetidus*, which have already been distinguished for their *in vitro* bioremediation efficiency against various pollutants (Varamogianni-Mamatsi et al., 2023, 2021) and their production of metabolites with pharmaceutical and biotechnological importance (Chu et al., 2022; Liu et al., 2007; Richelle-Maurer et al., 2003; Wang and Shin, 2008). Chemical characterization of sponge extracts was performed by using high-resolution analytical techniques, such as liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), targeted to the bioactive metabolites reported in the literature. For this purpose, a comprehensive metabolites/ions/MS fragments library, including all the previously reported compounds for both species, was created. In addition, the sponge extracts were assayed for their biological activities, with a particular focus on their antibacterial and anticancer properties. Through our study, we aspire to demonstrate the bioproduction potential of farmed marine sponges and pave the way for their inclusion as prolific candidates in integrated aquaculture systems.

5.3. Materials and Methods

5.3.1. Sponge Material

Farmed individuals of *A. oroides* and *S. foetidus* were introduced in integrated aquaculture in June 2020, attached on cultivation structures (individual cages made from plastic mesh) in proximity (5–10 m distance) to the fish cages of an operating fish farm in Souda Bay, NW Crete, Aegean Sea (35.4801/24.1117), at 7–10 m depth. Farmed sponges originated from explants (i.e., biomass cuttings) collected from adjacent natural populations (Souda Bay, 35.4783/24.1091 for *A. oroides* and Stavros Bay, 35.5879/24.0785 for *S. foetidus*). For a continuous timespan of 19 months, the explants remained in open-sea cultivation, exhibiting negligible mortality and showing regeneration and positive growth (Vernadou et al., in preparation). Tissue sampling from three replicate individuals of each sponge species from wild and farmed sponge specimens (12 samples in total) were obtained in February 2022, according to Varamogianni-Mamatsi et al. (2021) (Varamogianni-Mamatsi et al., 2021). Sampling locations for the wild individuals corresponded to the locations of collection for the initial seeding of the experimental sponge cultivation. Sampling was performed selectively by diving, and care was taken to partially collect excess biomass, thus, allowing the donor individuals to regenerate. In both cases (farmed and wild sponges), tissue samples were extracted underwater from a parent sponge, using a razor blade, and held in individual labeled sterile bags, prior to preservation in cooler boxes with ice packs and transportation to the facilities of Hellenic Centre for Marine Research within 3 h, where they were kept in −20 °C until further analysis.

The studied demosponge species are commonly distributed in high abundances along eastern Mediterranean habitats (Voultsiadou, 2005b) with emerging bioremediation (Varamogianni-Mamatsi et al., 2023, 2021) and bioproduction potential (Blunt et al., 2005) to be included in integrated aquaculture systems. The first sponge of interest, *Agelas oroides* (Schmidt, 1864), is a massive, variably lobate-digitate, vivid orange-colored demosponge that can reach 25 cm in height. It typically occurs in 2–40 m water depth, preferably in habitats with low light intensity (Ferretti et al., 2009; Idan et al., 2020). Sponges of the genus *Agelas*, including the species *A. oroides*, are well-known alkaloid producers (e.g., pyrrole and terpenoid alkaloids) (Chu et al., 2022). The second case study demosponge is the species *Sarcotragus foetidus* (Schmidt, 1862). This concerns a variably dark-colored, rather common Mediterranean keratose sponge, which approximates an irregularly globular to massive growth form, generally reaching 1 m in diameter and 50 cm in height. It is commonly found in shallow habitats exposed to light, but also in darker zones up to 400 m in depth (Manconi et al., 2013). This demosponge is known to host numerous symbiotic bacteria (e.g., heterotrophic bacteria and cyanobacteria; Konstantinou et al. (2018)) and fungi (Altunok et al., 2015). *Sarcotragus* sponges are widely recognized to be prolific sources of a variety of bioactive compounds, such as terpenoids, indoles, as well as lipids (Liu et al., 2007).

5.3.2. Extraction

Freeze-dried specimens of wild and farmed sponges were ground in a conventional mixer to obtain a dry powder (1 to 4 g per specimen), which was subsequently extracted three times in an ice-cold sonication bath (15 min each round) using a solvent mixture of methanol/dichloromethane (20 mL of MeOH:DCM 1:1, *v/v* per gram of sponge). After each extraction, sponge suspensions were centrifuged (8000 × *g*, 7 min, 20 °C), and collected supernatants were passed through a filter paper and evaporated to dryness using a centrifugal vacuum evaporator (EZ-2 Plus; Genevac, United Kingdom). The dry extracts were redissolved in 4 mL MeOH:DCM 1:1, transferred into 50 mL Falcon tubes, and mixed with 16 mL acetonitrile. After overnight protein precipitation at −20 °C, the samples were centrifuged (10,000 × *g*, 10 min, 4 °C) and collected supernatants were evaporated to dryness. For the removal of neutral lipids, each sample was applied onto a glass column (8 mm i.d.) packed with 1.5 g of silica gel (silica gel 60, particle size: 0.060–0.200 mm, Merck; activated at 300 °C for 3 h) and elution was performed using 2% *v/v* ethyl acetate in hexane (20 mL), ethyl acetate (15 mL), and MeOH (15 mL). The latter two fractions were combined, evaporated to dryness, and stored at +4 °C until further analysis.
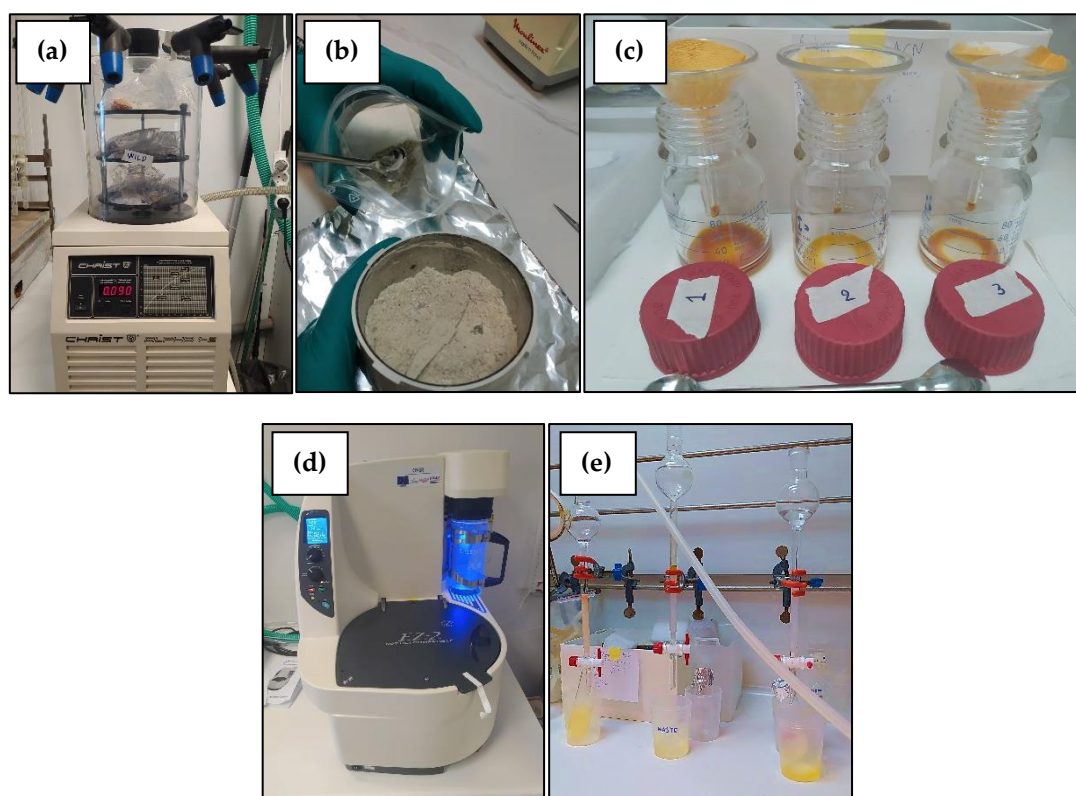
Figure 5.1. Experimental procedure followed for the delivery of crude sponge extracts; (a) freeze-drying of sampled sponge tissues, (b) grinding, (c) solvent extraction, (d) evaporation and (e) purification.

5.3.3. LC–MS/MS Analysis

5.3.3.1. Chemicals and Reagents

Analytical solvents, MeOH, and acetonitrile solvents at Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC–MS) grade and formic acid at LC–MS grade were supplied from Carlo Erba® Reagents S.A.S (Le Vaudreuil, France). Ultrapure water was supplied from a Milli-Q® ultrapure water system equipped with a Milli-Q® Reference and a Q-POD® element.

5.3.3.2. Instrumental LC–MS/MS Analysis

The LC–MS/MS analysis was performed using a Dionex® Ultimate 3000 System (UHPLC, Thermo Scientific, Germany) coupled to a TSQ QuantisTM triple-stage quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA). The UHPLC was equipped with four modules, a SR–3000 Solvent Rack, an LPG-3400RS pump, an WPS-3000TRS auto sampler with temperature control, and a TCC-3000RS column compartment. The triple-stage quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source. The LC–MS/MS operation and acquisition data system was controlled by the XCaliburTM 4.1 Thermo Scientific SP1 (0388-00CD-7B33, USA) software.

5.3.3.3. Sample Preparation for LC–MS/MS Analysis

All plastic materials and glassware were cleaned carefully to avoid contamination. Organic solvents (LC–MS grade) and distilled water were analyzed before use, to minimize background interferences.

Before injection into the LC–MS/MS system, all extracts were dissolved in 1 mL MeOH, filtered with a 13-mm, 0.22-μm nylon syringe filter (Filter-Lab®, Sant Pere de Riudebitlles, Spain) using a 500 μL syringe (Gastight 1750 Hamilton®, Vernon Hills, IL, USA), and diluted 10 times with MeOH. The liquid extract was transferred using a syringe filter (Filter-Lab®, Sant Pere de Riudebitlles, Spain) into a conical insert into a sterile 2-mL vial (9-425 C0000752) with a screw cap and red PTFE/white silicone septa (Alwsci® Technologies, Shaoxing, China).

5.3.3.4. Chromatographic and Mass Spectrometry Conditions

The separation of compounds was achieved using an Accurore™ RP-MS Column (2.6 μm, 150 × 2.1 mm, Thermo Fisher Scientific), by setting the sample injection volume to 10 μL. The gradient mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). Samples were injected and eluted at a flow of 0.200 mL/min with the following linear gradients: a 1 min re-equilibration phase at 5% B, 0.0–15.0 min at 5–50% of B, 15.0–20.0 min at 50–99% of B, 20.0–29.0 min at 99% of B, and 29.0–30.0 min at 99–5% of B.

Mass spectrometry (MS) analysis was carried out using the triple-stage quadrupole mass spectrometer. The electrospray ionization (ESI) parameters were set as follows: spray voltage, +3500/−3000 V; sheath gas flow, 50 L/min; auxiliary gas flow, 10 L min/L; sweep gas flow, 0 L min/L; ion transfer tube temperature, 320 °C; vaporizer temperature, 30 °C. The cycle time was set to 0.5 s, using a calibrated radio frequency (RF) lens and a collision induced dissociation (CID) gas of 1.5 mTorr. The collision energy was tested to 10, 20, and 40 V. Samples were injected in select reaction monitoring (SRM) mode, using multiple reaction monitoring (MRM). Selective MRM transitions were monitored for each targeted analyte, according to Tables A6 and A9 settings, and are provided, along with the instrumental parameter fragmentation settings used for the mass spectrometry conditions, as appendix. An intensive literature review was performed for each sponge's species under study, on previously reported compounds that have been identified along with their mass ions, adducts and their fragments. Briefly, the identification and classification of detected metabolites was performed by reference to the literature and to public databases (e.g., DrugBank, FoodB, GNPS, HMDB, MoNA, Metabolomics Workbench, and PubChem).

Figure 5.2. The LC-MS/MS equipment used for dereplication of farmed and wild sponge extracts at NOVA University of Lisbon, Portugal.

5.3.4. Antibacterial Assays

The antibacterial activity of the crude sponge extracts was evaluated by performing growth inhibition assays for two strains belonging to the Gram-positive and -negative human opportunistic pathogenic bacteria. The tested strains were the methicillin-resistant *Staphylococcus aureus* strain COL (MRSA) and the *Escherichia coli* strain ATCC 25922, respectively. The protocol followed was based on the one proposed by Pinto-Almeida et al. (2022) (Pinto-Almeida et al., 2022). In detail, *S. aureus* strains

were cultured in tryptic soy broth (TSB; Becton Dickinson, Germany), and *E. coli* cells in Lysogeny broth (NZYtech), at 37 °C. The assays were performed in 96-well polystyrene flat bottom microplates. Bacterial overnight cultures were diluted to an optical density (OD_{600nm}) of 0.005 and were incubated statically in the presence of different concentrations of each crude extract, solubilized in DMSO (1% *w/v*). All cultures were two-fold serially diluted, resulting in final concentrations of the extracts ranging from 250 to 0.4935 μg/mL. After 24 h of incubation at 37 °C, the minimal inhibitory concentration (MIC) value was determined by visual inspection. The latter is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after overnight incubation (Andrews, 2001). The resulting values were compared with a positive control (vancomycin for MRSA, and tetracycline for *E. coli*), a DMSO solvent control, and a negative control (inoculated medium without any extract addition) on the same plate.
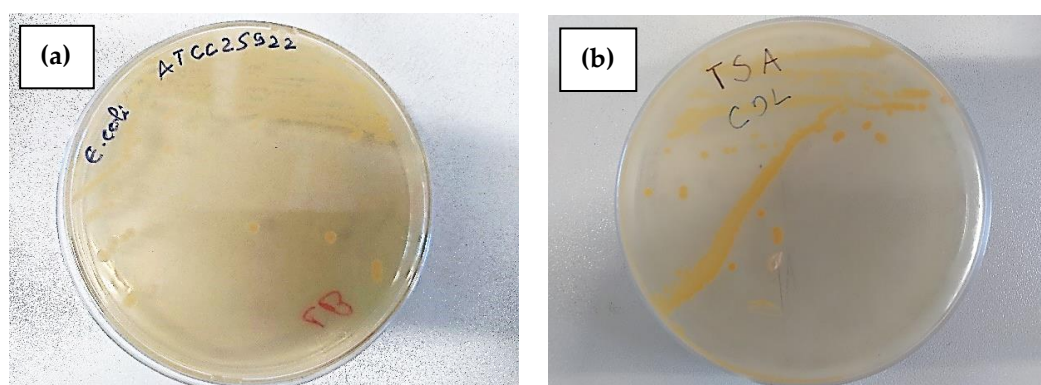
Figure 5.3. Plate cultures of (a) gram-negative (i.e., *E. coli* strain ATCC 25922) and (b) gram-positive (i.e., MRSA strain COL) human pathogenic bacteria used as models for screening the antimicrobial activity of sponge extracts at the Laboratory of Molecular Microbiology of Bacterial Pathogens (UCIBIO, FCT-NOVA, Lisbon, Portugal).

Figure 5.4. Antimicrobial activities of the crude sponge extracts, determined by employing a 96-well MIC assay. Clear, transparent wells indicate no bacterial growth in the presence of the specific sponge extract.

5.3.5. Anticancer Assays

The sponge crude extracts of wild and farmed populations of *A. oroides* and *S. foetidus* were tested *in vitro* against the human colorectal carcinoma cell line HCT-116 (ECACC 91091005, Porton Down, UK), according to Florindo et al. (2016) and Prieto-Davó et al. (2016) (Florindo et al., 2016; Prieto-

[129]

Davó et al., 2016). Cells were cultured in McCoy's 5A medium, supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Gibco, Thermo Fisher Scientific, Paisley, UK) and maintained at 37 °C under a humidified atmosphere of 5% CO_2. For cell viability assays, HCT-116 cells were seeded in 96-well plates (0.5×10^4 cells/well). After 24 h, treatment with the marine sponges' extracts (concentrations ranging from 0.30 to 125 μg/mL), DMSO (vehicle control), or 10 μM 5-fluorouracil (5-Fu, positive control) was followed for 72 h. Cell viability was assessed through MTS metabolism, using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Fitchburg, WI, USA), following the manufacturer's instructions. Absorbance signal (490 nm) was recorded using a Glomax®-MultiDetection System (Promega). All data were expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Data analysis was performed using GraphPad Prism 8.4.2 software (La Jolla, CA, USA). Dose–response curves were established and IC_{50} best-fit values determined using the log-(inhibitor) vs. response–variable slope (four parameters).

5.3.6. Statistical Analysis

Principal component analysis (PCA) was performed using the XLSTAT software (version 2016; Addinsoft Inc., New York, NY, USA) to identify similarities/differences between the extracts obtained from wild and farmed sponge populations, with respect to their chemical composition. The content of each detected metabolite in the extracts was determined by using the peak intensity data generated from the LC–MS/MS analysis. Peak intensity percentages of constituents were used as active variables in PCA. Metabolites that gave zero-peak intensity values were excluded from the analysis. *t*-tests were performed using MetaboAnalyst 5.0. online platform (**http://www.metaboanalyst.ca**; accessed on 13 March 2023) to investigate significant differences in the content and production levels (expressed as weight-normalized intensity values) of the various metabolites present in both wild and farmed sponge populations. The same was applied to the IC_{50} values obtained from the different sponge populations, in order to compare their respective anticancer activities. The level of significance was set to $p = 0.05$.

5.4. Results and Discussion

5.4.1. The Metabolomics Profile of Farmed and Wild *Agelas oroides* Sponges

The crude extracts obtained from wild and farmed *A. oroides* specimens were analyzed by LC–MS/MS to determine their chemical composition with the aid of a comprehensive metabolites/ions/MS fragments library (Table A6). Three chemical superclasses were identified in the *A. oroides* extracts—alkaloids, indoles, and lipids (**Table 5.1**)—with alkaloids being the most predominant constituents in both types of sponge populations (farmed; 97.5%, wild; 98.9%). The majority of the detected alkaloids comprised pyrrole classes, representing 96.2% and 98.3% of the total metabolite content in the farmed

and wild *A. oroides* sponges, respectively. Based on skeletal features of the produced pyrrole alkaloids, these were further categorized into three chemical subclasses: (1) linear pyrrole alkaloids, (2) fused cyclic pyrrole alkaloids, and (3) dimeric pyrrole alkaloids (Chu et al., 2022). The subclass of terpenoid alkaloids was detected in lower percentages among the farmed and wild sponges (1.3% and 0.6%, respectively). An intriguing class of indole metabolites with unexplored potential in myriad research fields (e.g., chemistry, pharmacology, physiology, and medicine) (Gul and Hamann, 2005) was also present in the studied extracts, but in significantly low proportions (farmed; 0.03%, wild; 0.02%).

Table 5.1. Distribution of metabolite superclasses, classes, and subclasses in the extracts of wild and farmed *Agelas oroides* sponges. Numbers in parentheses represent the standard deviation. ND—not detected.

Superclass	Class	Subclass	Wild (%)	Farmed (%)
Alkaloids	Pyrrole alkaloids	Linear pyrrole alkaloids	97.2 (1.4)	95.1 (0.9)
		Fused cyclic pyrrole alkaloids	0.9 (0.1)	0.7 (0.0)
		Dimeric pyrrole alkaloids	0.2 (0.2)	0.4 (0.1)
	Terpenoid alkaloids	ND	0.6 (0.5)	1.3 (0.1)
Indoles	ND	ND	<0.03	<0.03
Lipids	Fatty acyls	ND	1.0 (0.9)	2.4 (0.8)
	Glycerolipids	ND	<0.002	<0.003
	Steroids	ND	<0.002	<0.001

The lipids superclass was present in low abundance and accounted for 2.4% and 1.5% of the total metabolite content in cultured and wild *A. oroides* extracts, respectively. MS^2 analysis indicated that the extracts with lipid molecules belong to three classes: (1) fatty acyls, (2) glycerolipids, and (3) steroids. The fatty acyls compounds were detected in the following content, (farmed sponges; 2.4%, wild sponges; 1.0%) and to a much lower extent, the classes of glycerolipids and steroids (<0.01%).

The individual components detected in *A. oroides* extracts, along with their chemical and mass spectrometric characteristics are summarized in Table A6. In total, 28 metabolites were identified in wild *A. oroides* extracts, with only three of these compounds being completely absent in the farmed sponge samples (i.e., ageliferin; Kobayashi et al., 1990, oxysceptrin; Kobayashi et al., 1991, and trichodermanone C; Neumann et al., 2007). However, it should be highlighted that these specific compounds were detected in only one replicate of the wild sponges, at significantly low levels (i.e., <0.01%).

The content of each metabolite, expressed by its relative abundance (%) in the tested samples, is presented in Table A7. Based on these results, the linear pyrrole–imidazole alkaloid oroidin, which was identified using electrospray ionization in positive ion mode (ESI+), was the most abundant metabolite found in both farmed and wild *A. oroides* populations (70.3% and 80.4%, respectively).

Oroidin constitutes the characteristic metabolite of *A. oroides* sponges (König et al., 1998; Kovalerchik et al., 2020; Sauleau et al., 2017). In fact, it was the first metabolite isolated from this species (Forenza et al., 1971) and possesses broad-spectrum biological activities (e.g., antibacterial, antifouling, antimalarial properties and anti-predatory defenses against reef fish) (Chu et al., 2022).

Apart from oroidin, abundance differences between farmed and wild sponges were observed in other detected metabolites. Regarding cultivated sponges, the following most abundant metabolites were the closely oroidin-related molecules keramadine (Nakamura et al., 1984a) (8.0%) and dispacamide B (Cafieri et al., 1996) (7.8%). Their respective levels in wild extracts were determined to be as high as 3.6% and 2.9%, respectively. Conversely, the second major metabolite of wild sponges (i.e., 6.8%) was the oroidin hydrolysis product 4,5-dibromopyrrole-2-carboxylic acid (Chanas et al., 1997). This metabolite was detected at similar abundance levels in the extracts of farmed *A. oroides* specimens (6.6%) and yet was the fourth major metabolite of these extracts. Additionally, small amounts of the linear pyrrole alkaloids dispacamide A (Cafieri et al., 1996) and hymenidin (Kobayashi et al., 1986) (farmed; 1.6–1.0%, wild; 2.1–1.4%, respectively) were determined in both sponge populations.

The differences observed in the ranking of metabolites between wild and cultivated specimens are partially in contrast with the findings of Rodriguez et al. (1994) (Rodríguez et al., 1994). In the reported study, it was found that *Acanthella cavernosa* sponges, collected from natural habitats and populations held in controlled aquaculture systems, shared common major constituents, but with different contents in their respective chemical profiles. In our case, oroidin was the common dominant metabolite among studied extracts, but the chemical composition thereafter seemed to be population-specific.

In addition to linear pyrrole alkaloids, which determined the overall metabolite composition of both farmed (97.2%) and wild (95.1%) extracts, compounds belonging to other alkaloid subclasses were also present. Dibromophakellin, an analogue derived through oroidin cyclization/oxidation processes (Chu et al., 2022; Jiménez and Crews, 1994), previously described from the sponge *Phakellia flabellata* (Sharma and Magdoff-Fairchild, 1977), was the most abundant fused cyclic pyrrole alkaloid; however, it was detected at a low percentage (i.e., <1.0%) in both types of extracts. This was followed by its relative congeners longamide B methyl ester (Umeyama et al., 1998), longamide B (Cafieri et al., 1998), monobromoisophakellin (Assmann and Köck, 2002), and 3-debromohanishin (Freire et al., 2022). Overall, the percentage of fused cyclic pyrrole alkaloids reached up to 0.7% and 0.9% for cultured and wild *A. oroides* specimens.

Dimeric pyrrole alkaloids, formed by oxidation, cyclization, and dimerization reactions of simple monomers (i.e., oroidin, clathrodin, and hymenidin) (Wang et al., 2014) was the least abundant

alkaloids group within the extracts (farmed; 0.4% and wild; 0.2%). Nakamuric acid (Eder et al., 1999) and debromosceptrin acetate (Keifer et al., 1991) represented the majority of such metabolites, while bromoageliferin (Kobayashi et al., 1990) and sceptrin (Walker et al., 1981) were present in very low percentages (<0.01%).

Terpenoid alkaloids, which involve a nitrogen-based functional group (in the form of an amine or ammonia, etc.) attached to preformed terpenoid moieties (Chu et al., 2022; Funayama and Cordell, 2014), were also present at low levels in *A. oroides* extracts (up to 1.1% for farmed and 0.5% for wild sponges). This was the case for metabolites belonging to the families of agelasines (e.g., agelasine; Cullen and Devlin (1975), agelasine A; Nakamura et al. (1984b) and E; Wu et al. (1984)), as well as agelasidines (e.g., agelasidine A; Nakamura et al., (1985)).

The lipid content of both farmed and wild *A. oroides* was mainly characterized by fatty acyls, which were dominated by the compound 10-methyl-9(Z)-octadecenoic acid (Yu et al., 1996), accounting for 2.4% and 1.0% of the total metabolite composition in the cultured and wild sponge extracts, respectively. Interestingly, this unsaturated fatty acid has previously only been recorded in extracts of the marine fungus *Microsphaeropsis olivacea*, which was isolated by Yu et al. (1996) from a sponge collected in Florida. Along with Tasdemir et al. (2007), who previously reported the presence of the isomer 11-methyloctadecanoic acid in *A. oroides* (Tasdemir et al., 2007), we are the first to discover the existence of 10-methyl-9(Z)-octadecenoic acid in this sponge species. Its glyceride (Yu et al., 1996) was also detected, but at trace concentrations in both types of extracts (i.e., <0.003%). Detected steroids were mainly members of the ecdysteroids class, such as 20-hydroxyecdysone-22-acetate (Costantino et al., 2000), β-ecdysterone (Cafieri et al., 1998) and ponasterone A (Costantino et al., 2000), which collectively accounted for <0.01% of the sponge extracts.

5,6-dihydroxyindole was the only representative of the indole family. However, it was present in very low concentrations for both farmed (0.03%) and wild (0.02%) sponge extracts. This metabolite is an intermediate of the melanin biosynthetic pathway, which has previously demonstrated antibacterial activity against Gram-negative (e.g., *Escherichia coli*) and Gram-positive bacterial pathogens (e.g., *Staphylococcus aureus*), as well as antifungal activity (Zhao et al., 2007).

The metabolic profiling of the crude sponge extracts was further investigated by an unsupervised principal component analysis (PCA), to evaluate the similarities and differences between the extracts of the two populations and access clustering trends, as well as identifying outliers. The results obtained from the PCA (**Figure 5.5**) indicated the high spatial distribution of the extracts produced by wild *A. oroides* fragments, which are highlighted in green. Extracts derived from farmed specimens (highlighted in red) are closely clustered. Based on these findings, it can be assumed that *A.*

oroides sponges are likely to provide extracts with a similar chemical profile when subjected to farm conditions, whereas the composition of the wild individuals can be more diverse. This result presumably reflects the more homogenous environment of the farm conditions, in contrast to the complexity of a natural habitat, where the biodiversity assemblages and interactions between their organisms at micro and macro levels are more complex and intricate, thus inducing diversification to the sponges' chemical phenotype. However, according to the PCA scores plot (**Figure 5.5**a), the extracts derived from the farmed sponges are closely similar to one of the wild specimens, supporting a consistency of the core chemical profile of *A. oroides*, disregarding the prevalent drivers in the natural versus aquaculture environments.

In a similar study, Page et al. (2005) investigated the biosynthesis of metabolites from sponges *Mycale hentscheli*, collected from aquaculture and natural habitats in New Zealand (M. J. Page et al., 2005). Their results showed that the levels of the cytotoxic compounds mycalamide A, pateamine, and peloruside A varied not only within wild specimens, but also among farmed explants. Consequently, differences in the chemical profile were evident at an individual-specific level, rather than among different populations. Similar findings were reported for the bioactive metabolite amphitoxin, produced by cultured and natural species of the Indonesian reef-dwelling sponge *Callyspongia biru* (de Voogd, 2007).

Studies have shown that the variability of secondary metabolite production in sponges can be pronounced at the intraspecific level, among populations or even among individuals (M. Page et al., 2005; Rohde et al., 2012; Turon et al., 1996). A variety of biological traits (e.g., sponge shape; Turon et al. (2009) and size; Becerro et al. (1995)) or environmental factors (e.g., response to predation; Rohde et al. (2004), pollutants; Agell et al. (2001), light; Becerro and Paul (2004); Uriz et al. (1995), and temperature; M. Page et al. (2005)) can induce these marine organisms to modify their levels of secondary metabolites. Considering the fluctuation of the aforementioned parameters in natural habitats, compared to the less complex artificial environment of a fish farm, a higher diversity of secondary metabolites is expected for wild versus farmed sponge specimens.

Overall, the first two principal components (PC1 and PC2) explained 52.5% and 17.8% of the total variance present in the dataset (**Figure 5.5**). Interestingly, the loadings plot (**Figure 5.5**b) shows that the metabolites highly enriched in carbon content (i.e., C_{22}–C_{29}), such as dimeric pyrrole and terpenoid alkaloids, as well as steroids, are grouped together in the bottom left quadrant of the panel. As was expected, the linear pyrrole alkaloids oroidin and 4,5-dibromoprrole-2-carboxylic acid, which constitute the major constituents of the wild specimens, are scattered closely to the wild extracts. The

[134]

same stands for the linear pyrrole alkaloids keramadine and dispacamide B with respect to farmed sponges.

Figure 5.5. Principal component analysis (**a**) scores plot and (**b**) loadings plot of crude *A. oroides* extracts belonging to wild and farmed sponges. Color circles represent the origin of the analyzed sponge extract (i.e., green for wild or red for farmed) and triangles represent the different chemical subclasses of the identified metabolites, according to the legend.

[135]

However, a series of *t*-tests (**Figure 5.6**) revealed that significant differences in the metabolite content were observed only for the bromopyrrole alkaloids oroidin ($p = 0.025$) and dibromophakellin ($p = 0.025$), which were richer in wild *A. oroides* sponges. Furthermore, compounds of the same class, including ageliferin and oxysceptrin, were completely absent in farmed *A. oroides* sponges. Previous studies have highlighted the role of oroidin-like brominated pyrrole alkaloids as chemical defenders against fish predation for the genus *Agelas* (Chanas et al., 1997; Chanas and Pawlik, 1995) and enhanced bromine content with increased feeding deterrent potency (Assmann et al., 2000). Given that sponges occurring in natural habitats are prone to face increased antagonistic interactions compared to their farmed counterparts, a higher content of bromopyrrole alkaloids can thus be expected in wild specimens.

Interestingly, farmed *A. oroides* sponges exhibited a significantly higher content in the pyrrole alkaloid dispacamide B ($p = 0.031$), which was also included in the top three abundant metabolites of the respective extracts (**Figure 5.6**). However, the ecological role of this alkaloid has not yet been well established. It is likely to be related to other biological functions of this species, such as growth or reproduction, but a lack of evidence means its higher biosynthesis in the farmed population cannot be explained. Nevertheless, oroidin, dibromophakellin, and dispacamide B could serve as chemotaxonomic markers for distinguishing farmed from wild *A. oroides* specimens, but further investigation is required to support this statement. Overall, the distribution of the various metabolite classes between the two sponge populations remained constant, but the composition of the three above mentioned compounds varied according to the population.
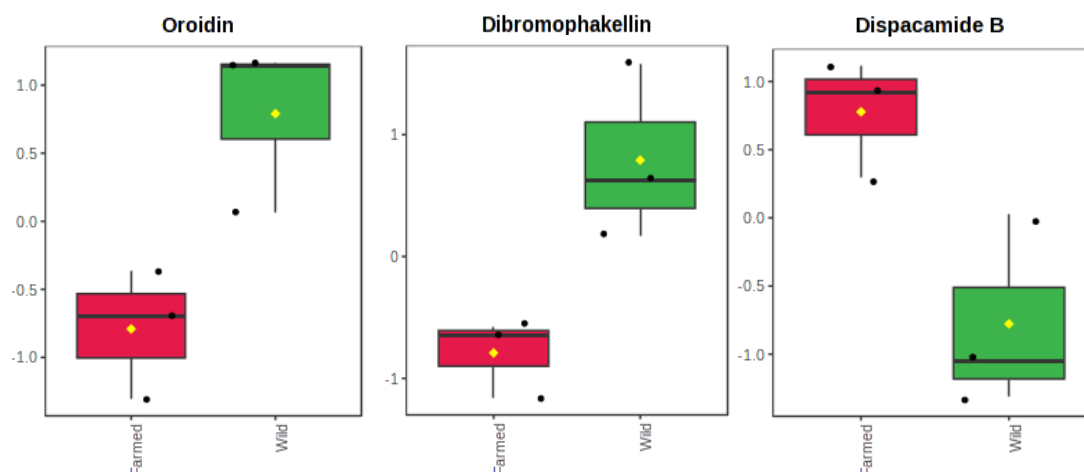
Figure 5.6. Boxplot of relative abundances for metabolites oroidin, dibromophakellin, and dispacamide B, which presented significant abundance differences (*t*-test, $p < 0.05$) between the extracts content of farmed (red) and wild (green) *A. oroides* sponges. Data were normalized to the total spectral area and presented in the auto-scale mode of the MetaboAnalyst 5.0 software. Boxes range from the 25% to 75% percentile; whiskers indicate the 5% and 95% percentiles; black dots show the individual data points; the horizontal line and the yellow diamond within each box indicate the median and mean value, respectively.

Apart from assessing differences in the metabolite content, we further aimed to compare production levels of the respective bioactive compounds in the two sponge populations. For the quantification of the detected metabolites, we introduced the term of the weight-normalized intensity, that considers the dry weight of each sponge fragment subjected to extraction (expressed as intensity units per gram of sponge dry weight). The calculated values are presented in Table A8. Out of the 25 metabolites shared between farmed and wild sponges, only two showed significant variation in their production levels. These were the terpenoid alkaloids agelasine (*t*-test, *p* = 0.012) and agelasine A (*t*-test, *p* = 0.019), which were increasingly produced by farmed explants (**Figure 5.7**).
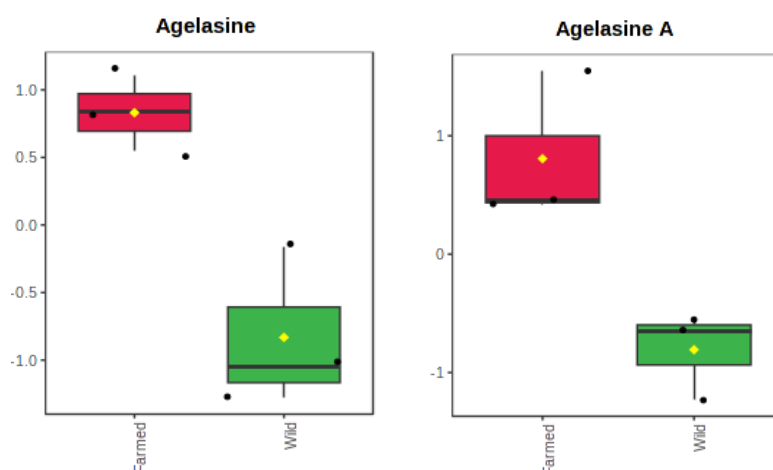
Figure 5.7. Boxplot of weight-normalized intensities for metabolites agelasine and agelasine A, which presented significant differences in their production (*t*-test, *p* < 0.05) between the extracts of farmed (red) and wild (green) *A. oroides* sponges. Data are presented in the auto-scale mode of the MetaboAnalyst 5.0 software. Boxes range from the 25% to 75% percentile; whiskers indicate the 5% and 95% percentiles; black dots show the individual data points; the horizontal line and the yellow diamond within each box indicate the median and mean value, respectively.

A handful of studies have similarly indicated that farming can promote the production of specific bioactive metabolites in sponges. By following a 9-month strategy of harvesting explants from cultures of the New Zealand demosponges *Latrunculia wellingtonensis* and *Polymastia croceus*, Duckworth et al. (2003) measured similar or higher bioactivity in farmed specimens than that exhibited by natural populations (A. Duckworth and Battershill, 2003). However, it should be noted that the overall metabolite production was estimated in terms of a murine leukemia bioassay, and not by using metabolomics approaches.

Furthermore, an array of *ex situ* trials revealed that farmed sponge explants are greater producers than their wild counterparts with respect to specific biomolecules. This was the case for the Mediterranean sponge *Aplysina aerophoba*, and its brominated isoxazoline alkaloids aplysinamisin-1, aerophobin-2, and isofistularin-3, as well as the biotransformation product aeroplysinin-1, when explants of the specific species were kept for 9 months in controlled aquarium systems (Klöppel et al., 2008). Similar to the previous work, Duckworth et al. (2003) reported higher levels of the antitumor

[137]

compound stevensine in cultures of *Axinella corrugata*, by following a multicomponent diet (Duckworth et al., 2003). Contrarily, Munro et al. (1999) found that the overall halichondrin content of the cultured *Lissodendoryx* sponge was not as high as that of the wild specimens, reaching the opposite conclusion (Munro et al., 1999). No effect of farming on metabolite production was indicated by Carballo et al. (2010) and Ternon et al. (2017), who conducted sponge mariculture of the species *Mycale cecilia* and *Crambe crambe*, respectively (Carballo et al., 2010; Ternon et al., 2017).

5.4.2. The Metabolomics Profile of Farmed and Wild *Sarcotragus foetidus* Sponges

The LC–MS/MS analysis of *S. foetidus* crude extracts revealed that, in total, 31 metabolites were shared between farmed and wild sponges, with all the compounds being present in at least one biological replicate of each population (Tables A9 and A10). Detection was supported by building a comprehensive metabolites/ions/MS fragments library (Table A9). The detected *S. foetidus* metabolites were classified into five superclasses, namely benzenoids, dipeptides, indoles, lipids, and polyketides (**Table 5.2**). This species was revealed to be an efficient lipid producer, by presenting extracts with an average lipid content as high as 95.5% and 94.3% among wild and farmed specimens, respectively. The detected lipids classes were: (1) fatty acyls, (2) glycerolipids, (3) prenol lipids, and (4) steroids. This slightly higher predominance of lipids observed in the extracts of farmed sponges was evaluated as statistically significant (t-test, $p = 0.002$), and is attributed to the higher levels of prenol lipids (t-test, $p = 0.011$) in the respective extracts when compared to their wild counterparts. However, this specific class held only a small share in the overall chemical composition of both sponge populations (farmed; 3.1% and wild; 1.5%). Lipids were mainly dominated by the fatty acyls class, which accounted for more than 55% of the overall metabolite abundance, followed by steroids, averaging at approximately 28.7% and 30.1% within farmed and wild sponges, respectively. However, this steroid dominance in wild individuals was regarded as marginally significant (t-test, $p = 0.037$). Glycerolipids were present at similar abundance levels within the sponge populations, collectively accounting for 7.4% and 7.2% of the farmed and wild sponge extracts.

Benzenoids were the second major compound superclass, and, yet, were only detected at small percentages in both types of extracts (i.e., <4.2%). It comprised four classes, (1) anthracenes, (2) benzene and substituted derivatives, (3) benzopyrans, and (4) phenols. Interestingly, benzenoid moieties were more pronounced in wild specimens (t-test, $p = 0.007$), due to their higher content in benzenes and substituted derivatives (t-test, $p = 0.016$). Moreover, metabolites belonging to the class of benzopyrans were identified at similarly low levels within the farmed and wild sponge populations (i.e., 0.4% and 0.5%, respectively). Furthermore, other compound classes contributed to the benzenoid profile of farmed and wild *S. foetidus* extracts, such as anthracenes and phenols, but these were present as trace elements (i.e., <0.01%).

[138]

Table 5.2. Distribution of metabolite superclasses and classes in the extracts of wild and farmed *Sarcotragus foetidus* sponges. Numbers in parentheses represent the standard deviation. ND—not detected.

Superclass	Class	Wild (%)	Farmed (%)
Benzenoids	Anthracenes	<0.01	<0.01
	Benzene and substituted derivatives	4.2 (0.3)	3.2 (0.4)
	Benzopyrans	0.5 (0.2)	0.4 (0.0)
	Phenols	<0.01	<0.01
Dipeptides	ND	<0.02	<0.02
Indoles	ND	0.1 (0.0)	0.1 (0.1)
Lipids	Fatty acyls	55.5 (1.2)	56.2 (0.5)
	Glycerolipids	7.2 (0.4)	7.4 (0.3)
	Prenol lipids	1.5 (0.4)	3.1 (0.5)
	Steroids	30.1 (0.7)	28.7 (0.4)
Polyketides	ND	0.8 (0.1)	0.8 (0.0)

The chemical profile of *S. foetidus* exhibited superclasses of polyketides and indoles that were found in all extracts of this keratose demosponge. The respective contents were similar for sponges of farmed and wild populations. More specifically, polyketides accounted for 0.8% of both farmed and wild sponge extracts. Indoles were present at percentages lower than 0.1% in both *S. foetidus* populations. Additionally, dipeptides were the least abundant metabolite superclass among the sponges' populations (i.e., <0.02%).

The distribution of metabolites within the extracts of *S. foetidus* was determined using the MS^2 peak intensity data, and the calculated abundance values are presented in Table A10. Fatty acyls of farmed and wild sponge extracts were entirely represented by the primary metabolite *N*-hexadecanoyl-*L*-homoserine lactone (Schaefer et al., 2002), which has been previously detected in wild specimens of this species through *in situ* chemical extraction (Bojko et al., 2019) and its existence has been associated with the quorum-sensing activities of its host microbiome (Saurav et al., 2016). The steroid 24-methylcholesta-5,7,22-trien-3β-ol (i.e., ergosterol; Cimino et al., 1972) was the second most abundant metabolite, and it accounted for the majority of steroids detected in *S. foetidus* extracts. Its content was determined to be as high as 28.6% and 30.0% in sponges derived from farmed and wild sponges, respectively.

Monovaccenin (Liu et al., 2006a) and 1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(10-methylhexadecyl)glycerol (1-*O*-(2,3,4,5-4OHCyp-3-*O*-10-MeHG; Gil et al., 2006) were the major compounds of the glycerolipids family, and their respective average percentages were 4.6% and 2.5% within the analyzed extracts. However, the prenol lipid 4-hydroxy-3-tetraprenylbenzoic acid (Cimino et al., 1972) was detected at similar levels as 1-*O*-(2,3,4,5-4OHCyp-3-*O*-10-MeHG), but only in farmed specimens (i.e., 2.6%). Wild extracts exhibited approximately two times lower percentages of this

compound (i.e., 1.3%). The opposite trend was observed for the benzenoid molecule toluate (i.e., 2.1% and 1.5% in wild and farmed sponges, respectively), while the second major benzenoid 8-*O*-4'-dehydrodiferulic acid (Elissawy et al., 2017) accounted for 1.3% and 1.6% of the total metabolite content of farmed and wild sponge populations.

Other compounds belonging to benzenoids (i.e., 3-phenylpropane-1,2-diol; Elissawy et al. (2017), 3-isochromanone; Casellas et al. (1997), and 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone; Kashiwada et al. (1984)), indoles (i.e., indole-3-methylethanoate; Liu et al., (2006b)), lipids (i.e., 1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(11-hexadecenyl)glycerol, 1-*O*-(2,3,4,5)-4OHCyp-3-*O*-HG; Gil et al. (2006), and 1,4-dihydroxy-2-tetraprenylbenzoic acid; Cimino et al. (1972)) and polyketides (i.e., 3,8-dihydroxy-6-methoxy-8-methylxanthone; Kingston et al. (1976), chrysophanol; Danielsen et al. (1992), and griseofulvin; Grove et al. (1952)) exhibited minor abundances (i.e., 0.1-0.4%) in relation to the total metabolite content of *S. foetidus*. Anthracenes, represented by emodin (Wells et al., 1975) and endocrocin (Kurobane et al., 1979), and dipeptides comprised 3-nitropropionic acid (Williams, 1982), along with some benzene derivatives (i.e., 3,4-dimethoxybenzoic acid; Wu et al. (2000), 4-hydroxyphenylacetic acid; Mao et al. (2006), tyrosol; Cross et al. (1963)), prenol lipids (i.e., 7*E*,12*E*,20*Z*-variabilin; Barrow et al. (1988) and (+)-12,15-dihydroxycurcuphenol; Bassaganya-Riera et al. (2010)), steroids (i.e., 24-methylcholest-7-en-3β-ol, 24-methylcholesta-5,7-dien-3β-ol, 24-methylcholesta-7,22-dien-3β-ol, cholest-7-en-3β-yl acetate, and cholesta-5,7-dien-3β-ol; Dini et al. (1984)), and polyketides (i.e., dechlorogriseofulvin; Grove et al. (1952), and norlichexanthone; Yang et al. (2013)) were detected as trace amounts within the tested extracts (i.e., <0.1%).

The compositional differences between the extracts derived from farmed and wild *S. foetidus* sponges were further evaluated by PCA analysis. Similar to the case of *A. oroides*, data points representing the chemical profiles of wild sponges showed a broader distribution, while a clear clustering was observed for the samples obtained from farmed explants (**Figure 5.8**a). The first two principal components (PC1 and PC2) explained 41.6% and 22.8% of the total variance present in the dataset.

Several members of lipids superclass, belonging to the classes of glycerolipids (i.e., 1-*O*-(2,3,4,5)-4OHCyp-3-*O*-HG and monovaccenin), prenol lipids (i.e., (+)-12,15-dihydroxycurcuphenol, 1,4-dihydroxy-2-tetraprenylbenzoic acid, and 4-hydroxy-3-tetraprenylbenzoic acid), and steroids (i.e., Δ⁷-cholesterol and 22,23-dihydroergosterol), together with the majority of detected polyketides (i.e., griseoxanthone C and norlichexanthone), are visually clustered in the bottom left side of the PCA
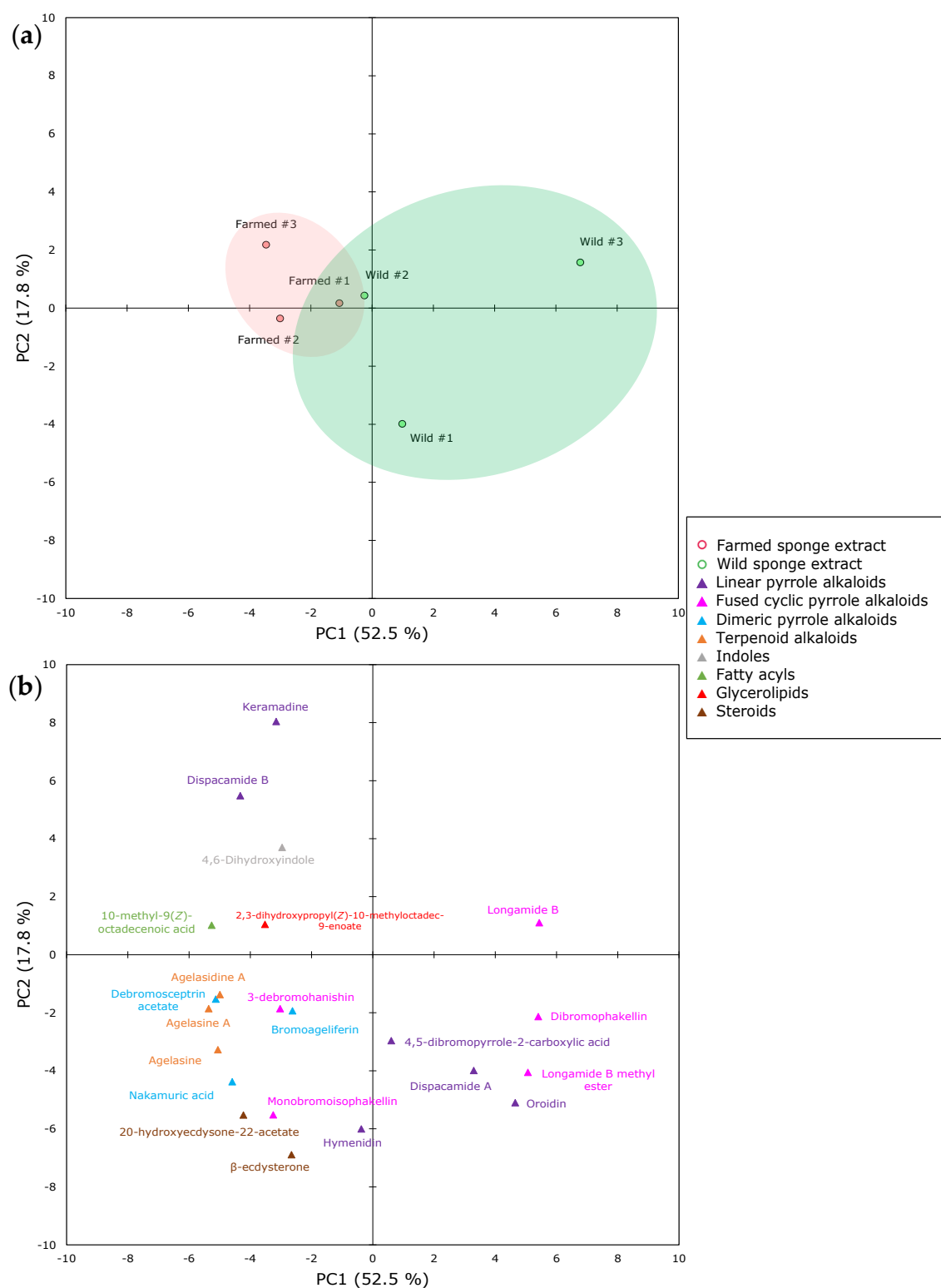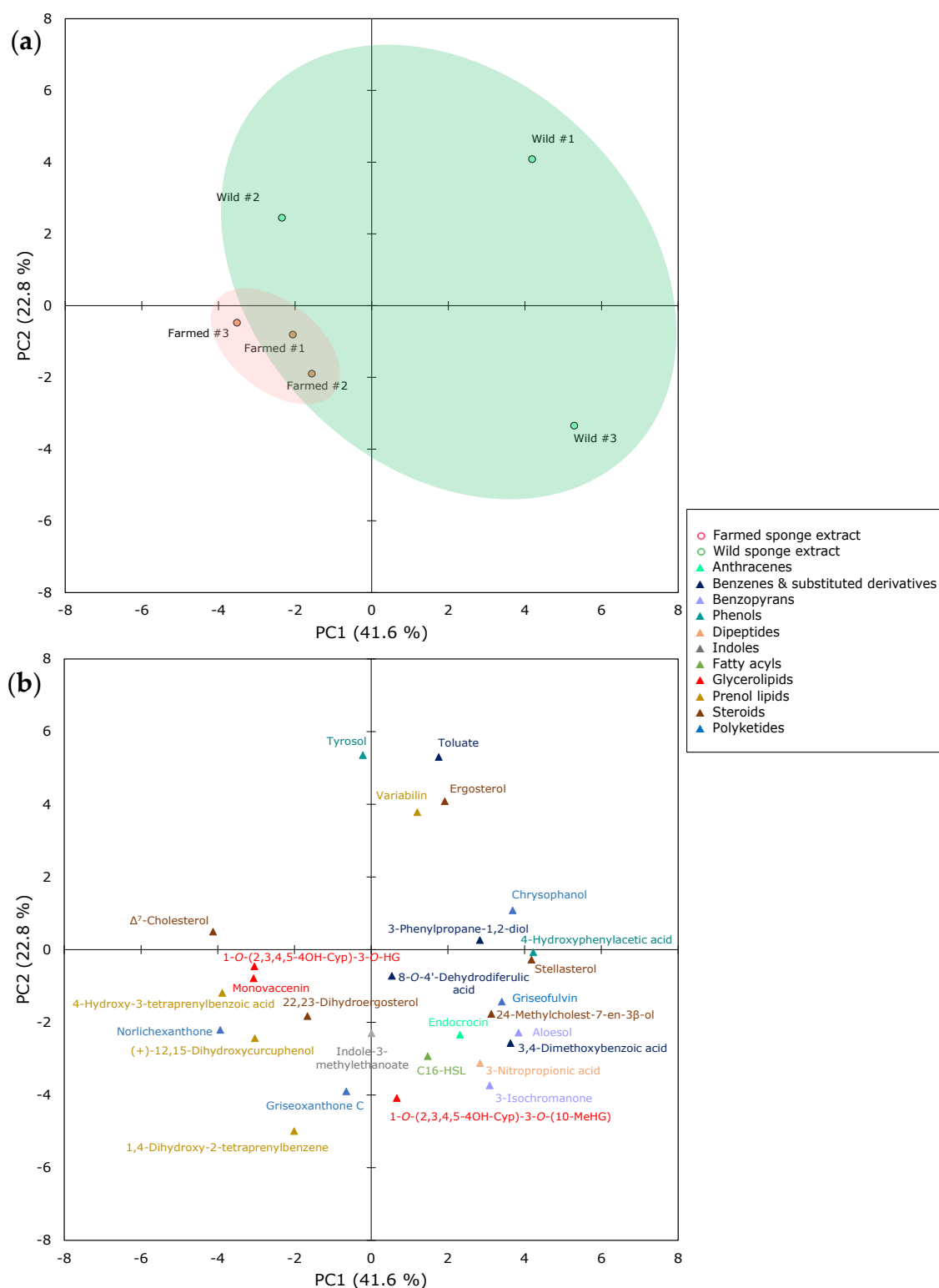
Figure 5.8. Principal component analysis (a) scores plot and (b) loadings plot of crude *S. foetidus* extracts belonging to wild and farmed sponges. Color circles represent the origin of the analyzed sponge extract (i.e., green for wild or red for farmed) and triangles represent the different chemical classes of the identified metabolites, according to the legend: 1-*O*-(2,3,4,5)-4OHCyp-3-*O*-HG, 1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(11-hexadecenyl)glycerol, 1-*O*-(2,3,4,5-4OHCyp-3-*O*-10-MeHG, 1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(10-methylhexadecyl)glycerol); aloesol, 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone; C16-HSL, *N*-hexadecanoyl-*L*-homoserine lactone; Griseoxanthone C, 3,8-dihydroxy-6-methoxy-8-methylxanthone; Δ⁷-Cholesterol, Cholesta-5,7-dien-3β-ol; 22,23-Dihydroergosterol, 24-methylcholesta-5,7-dien-3β-ol; stellasterol, 24-methylcholesta-7,22-dien-3β-ol; ergosterol, 24-methylcholesta-5,7,22-trien-3β-ol.

loadings plot (**Figure 5.8**b), corresponding to the extracts derived from farmed *S. foetidus* sponges in the

[141]

scores plot (**Figure 5.8**a). This supports the notion that these specific compounds might be the key elements accounting for the variation presented in the farmed and wild sponges' chemical profiles. To shed light onto this pattern, we tested the differences in the % abundance of each metabolite between farmed and wild sponges. The results showed that, indeed, some of the abovementioned compounds were significantly higher in abundance in farmed *S. foetidus* explants, namely the prenol lipids (+)-12,15-dihydroxycurcuphenol (p = 0.006) and 4-hydroxy-3-tetraprenylbenzoic acid (p = 0.018) and the polyketide norlichexanthone (p = 0.029) (**Figure 5.9**). Furthermore, wild *S. foetidus* specimens were enriched in ergosterol (p = 0.039), which was illustrated in the upper right side of PCA biplot, close to one of the wild samples.
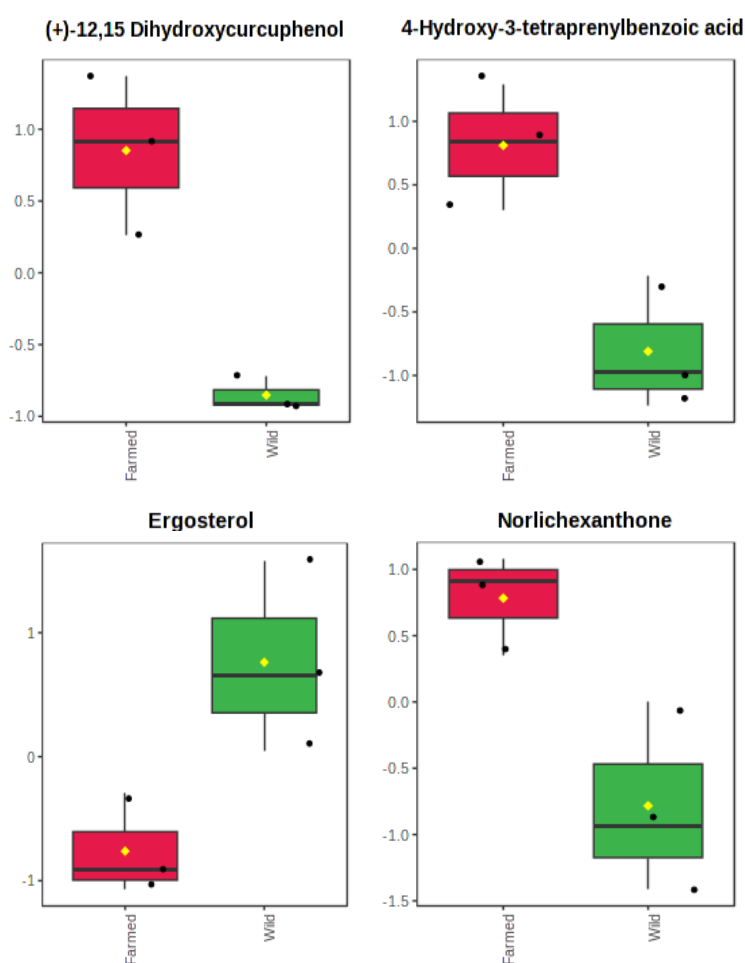
Figure 5.9. Boxplot of relative abundances for metabolites (+)-12,15 dihydroxycurcuphenol, 4-hydroxy-3-tetraprenylbenzoic acid, ergosterol, and norlichexanthone, which presented significant abundance differences (*t*-test, $p < 0.05$) between the extracts content of farmed (red) and wild (green) *S. foetidus* sponges. Data were normalized to the total spectral area and presented in the auto-scale mode of the MetaboAnalyst 5.0 software. Boxes range from the 25% to 75% percentile; whiskers indicate the 5% and 95% percentiles; black dots show the individual data points; the horizontal line and the yellow diamond within each box indicate the median and mean value, respectively.

All the lipids that varied in abundance between farmed and wild *S. foetidus* populations have been associated with sponge defense mechanisms. More specifically, (+)-12,15-dihydroxycurcuphenol constitutes an intermediate metabolite in the synthesis of abscisic acid, which is assumed to be produced

by sponges as a response to heat stress (Bassaganya-Riera et al., 2010). Additionally, the compound 4-hydroxy-3-tetraprenylbenzoic acid has been shown to possess strong pungent activity (Correa et al., 2011), potentially serving as a defensive compound by the sponges to deter predators. Norlichexanthone is a marine-derived fungi metabolite (Yang et al., 2013), which has been previously reported in the fungal extracts of the *Sarcotragus muscarum* symbiont *Arthrinium* sp. (Elissawy et al., 2017). Ahluwalia et al. (2015) included this metabolite in high oxidation state compounds (Ahluwalia et al., 2015), which have been previously reported to exhibit great antimicrobial activity, while boosting symbionts' competitive efficiency and host resistance to other pathogens (Liu et al., 2009). However, all these defensive lipid chemicals were found to be more abundant in farmed populations of *S. foetidus*, which is quite contradictory, given the lesser environmental pressures they face, compared to their wild counterparts. However, it should be mentioned that extracts provided by farmed *S. foetidus* specimens are less rich in ergosterol. This specific compound is the prime sterol in plasma membranes of fungal cells, and like many steroids, has been characterized as a bioactive compound, due to its antibacterial and anti-inflammatory activities (Dos Santos Dias et al., 2019). Higher levels of this fungi steroid in wild sponge extracts indicate a higher pronounced presence of symbionts in the respective specimens and their potential involvement in the sponge-associated defense mechanisms. Taking this into account, we can perceive why the metabolism of farmed *S. foetidus* sponges involved alternative defense pathways, enriched with other minor metabolites, such as prenol lipids.
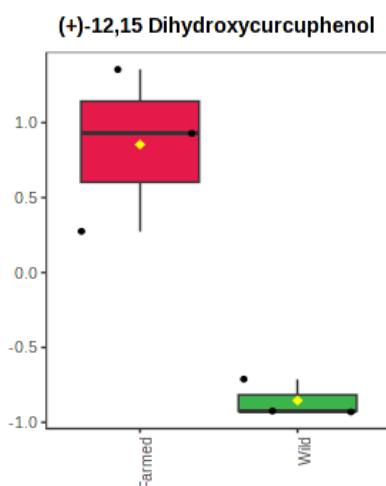
Figure 5.10. Boxplot of weight-normalized intensities for the metabolite (+)-12,15 dihydroxycurcuphenol, which presented significant differences in its production (*t*-test, $p < 0.05$) between the extracts of farmed (red) and wild (green) *S. foetidus* sponges. Data are presented in the auto-scale mode of the MetaboAnalyst 5.0 software. Boxes range from the 25% to 75% percentile; whiskers indicate the 5% and 95% percentiles; black dots show the individual data points; the horizontal line and the yellow diamond within each box indicate the median and mean value, respectively.

In terms of production levels, the weight-normalized intensity values calculated for each metabolite of *S. foetidus* sponges are presented in Table A11. Only the prenol lipid (+)-12,15-dihydroxycurcuphenol was found to vary significantly between farmed and wild *S. foetidus* sponges

(p = 0.006) (**Figure 5.10**). In agreement with the previously reported one-way ANOVA results (i.e., assessing differences in % metabolite abundance among extracts), the production of (+)-12,15-dihydroxycurcuphenol was more intensive in farmed specimens. Consequently, rearing in proximity to a fish farm facility may affect the production efficiency of specific bioactive compounds in *S. foetidus* individuals, but this is an exception to a broader uniformity in metabolite production levels.

5.4.3. Evaluation of the Sponges' Biotechnological Potential

In addition to comparing the chemical profiles of cultivated and wild sponges, we further examined the biological activities of *A. oroides* and *S. foetidus* extracts to evaluate whether farming can affect those properties. Antimicrobial assays were performed against two human pathogens: methicillin-resistant *Staphylococcus aureus* (MRSA, COL), representing Gram-positive bacteria, and *Escherichia coli* (ATCC 25922), representing Gram-negative bacteria. In addition, the anticancer activities and general toxicities of the extracts were assessed against the human colorectal carcinoma cell line HCT-116 (ECACC 91091005).

5.4.3.1. Antimicrobial Activity Evaluation

To assess the antibacterial potential of *A. oroides* and *S. foetidus* marine sponges and compare the bioactivities of wild versus farmed populations, a total of 12 extracts were tested against two strains of Gram-positive and -negative bacteria, representative of clinically relevant human pathogens, as mentioned above: *Staphylococcus aureus* (MRSA strain COL) and *Escherichia coli* (strain ATCC 25922). The studied samples consisted of the crude extracts of three explants collected from both farmed and wild populations of targeted sponge species *A. oroides* and *S. foetidus*, analyzed in triplicate, using the microdilution assay to determine the minimum inhibitory concentration (MIC) (**Table 5.3**). In total, eight crude sponge extracts revealed antibacterial properties, with all showing marginal potency. More specifically, seven extracts were found to be active against the *S. aureus* strain, while only one was proven to be effective against *E. coli*.

Regarding the species *A. oroides*, one out of the three wild specimens (33%) provided extracts with inhibitory activities against the Gram-positive methicillin-resistant bacterium *S. aureus*, while the same stood for two out of the three studied farmed samples (67%). However, both types of *A. oroides* extracts (i.e., farmed vs. wild) exhibited activity against MRSA, albeit only for the highest tested concentration (i.e., 250 μg/mL).

Interestingly, one farmed *A. oroides* extract (i.e., #3), besides revealing *S. aureus* growth inhibition, also showed activity against *E. coli* (MIC; 250 μg/mL). None of the wild sponge extracts belonging to *A. oroides* demonstrated inhibitory effects against *E. coli*. The same results were observed for farmed *A. oroides* extracts, except for replicate #3, which targeted both Gram-positive and -negative

[144]

bacteria, suggesting a mechanism not related to the cell wall. Indeed, among all the tested biological samples, the farmed *A. oroides* replicate #3 extract contained the highest levels of the alkaloids dispacamide B, keramadine, and agelasidine A, as well as the indole compound 4,6-dihydroxyindole and the glycerolipid 2,3-dihydroxypropyl(*Z*)-10-methyloctadec-9-enoate (**Table A7**). Of these metabolites, keramadine and agelasidine A have been previously reported to display partial growth inhibitory effects against *E. coli* strains, with MIC values in the range of 32–100 μg/mL (Kusama et al., 2014; Medeiros et al., 2006), while 4,6-dihydroxyindole was found to have strong antibacterial activity against both Gram-positive and -negative bacteria, including *E. coli* (Zhao et al., 2007). These compounds might be the key metabolites equipping *A. oroides* sponges with broad-spectrum antibacterial properties, by acting either individually or synergistically in the respective extracts.

Table 5.3. Antimicrobial activity of *A. oroides* and *S. foetidus* crude extracts against methicillin-resistant *Staphylococcus aureus* (MRSA, strain COL) and *Escherichia coli* (strain ATCC 25922). MIC values are expressed in μg/mL. NA—not active at the tested concentrations. Vancomycin and tetracycline were used as positive controls for *S. aureus* and *E. coli* growth, respectively. Inoculated medium without any extract addition was used as negative control.

Species	Type of Sponge Population	Sponge Replicate	MIC Values (μg/mL) for *S. aureus* MRSA COL	MIC Values (μg/mL) for *E. coli* ATCC 25922
Agelas oroides	Wild	#1	NA	NA
		#2	NA	NA
		#3	250	NA
	Farmed	#1	NA	NA
		#2	250	NA
		#3	250	250
Sarcotragus foetidus	Wild	#1	125	NA
		#2	125	NA
		#3	250	NA
	Farmed	#1	NA	NA
		#2	250	NA
		#3	NA	NA
Positive control			1.9	3.9

In contrast, extracts of wild *S. foetidus* sponges had a stronger effect on *S. aureus* growth, with one of these being active at the highest tested concentration and the rest of the samples reaching lower MIC values of 125 μg/mL. However, it is worth noticing that this value represented the highest antimicrobial activity exhibited by all the studied extracts. Concerning the cultivated *S. foetidus* fragments, only one out of three (33%) generated extracts that marginally inhibited the growth of the Gram-positive bacterium (MIC; 250 μg/mL), but did not reach as low values as their wild counterparts. On the other hand, all the *S. foetidus* extracts did not show any effect on *E. coli* growth.

Based on these observations, we can assume a higher presence of anti-*S. aureus* compounds in the extracts of wild *S. foetidus* populations, which target the cell wall structure, because only Gram-positive bacteria are inhibited. More specifically, wild extracts #1 and #2, which stood out for their

inhibitory properties, demonstrated the highest contents of the natural products toluate (i.e., >2.0%) and ergosterol (i.e., >29.9%) among all the tested samples. Toluate, or *p*-toluic acid, is a primary metabolite involved in the natural degradation of *p*-xylene (Duan et al., 2019) and can be found in a myriad of organisms (Loots et al., 2007; Mussinan and Walradt, 1975), including the sponge *S. foetidus* (Bojko et al., 2019). To the best of our knowledge, there are no reports examining its antimicrobial effects, except for those focused on its congener, benzoic acid. This compound is a food preservative, and its MIC values against various *S. aureus* strains have been demonstrated in several previous studies (Cruz-Romero et al., 2013; Sullivan et al., 2020). On the other hand, ergosterol, a derivative of cholesterol, plays an important role in the function of eukaryotic cell membranes by maintaining their permeability. However, it is absent in the cytoplasmic membrane of bacteria (Andrade et al., 2014). Tintino et al. (2017) reported that ergosterol causes detrimental effects on bacterial cells (Tintino et al., 2017). This could be a possible explanation for the enhanced antimicrobial activity observed in the majority of crude extracts collected from wild *S. foetidus* specimens. However, there are no data in the literature describing ergosterol-driven effects on bacteria.

Although the reported MIC values of the present study are relatively high, it should be stressed that they concern the activity of crude sponge extracts, which have not been priorly subjected to any fractionation. In agreement with the results obtained from Govinden-Soulange et al. (2014) for crude and fractionated extracts of the Mauritian sponges *Biemna tubulosa* and *Stylissa sp.* (Govinden-Soulange et al., 2014), MIC values of our crude samples were rarely found to be higher than 100 μg/mL. This is attributed to the chemical complexity of the specific extracts, which constitute mixtures of both active and non-active compounds.

5.4.3.2. Anticancer Activity Evaluation

The anticancer properties of crude sponge extracts, derived from farmed and wild populations of *A. oroides* and *S. foetidus* species, were investigated against the human colorectal carcinoma cell line HCT-116, by employing the MTS assay. **Figure 5.11** and **Figure 5.12** illustrate the variation of MTS cell metabolism as a function of the applied extract concentration, with respect to *A. oroides* and *S. foetidus* sponges. The cytotoxicity effects were evaluated according to half-maximal (50%) inhibitory concentration (IC$_{50}$) values, which were obtained from the curve slopes of the abovementioned graphs and are presented in **Table 5.4**. As shown in **Figure 5.11**, none of the extracts belonging to wild and farmed *A. oroides* explants exerted anticancer activity against HTC-116 cells, except for one extract of a farmed specimen (#2, **Figure 5.11**e) that showed approximately 25% inhibition at the highest test concentration (i.e., 125 μg/mL). However, this inhibition rate was considered to be relatively low, hence, the IC$_{50}$ values were not determined.
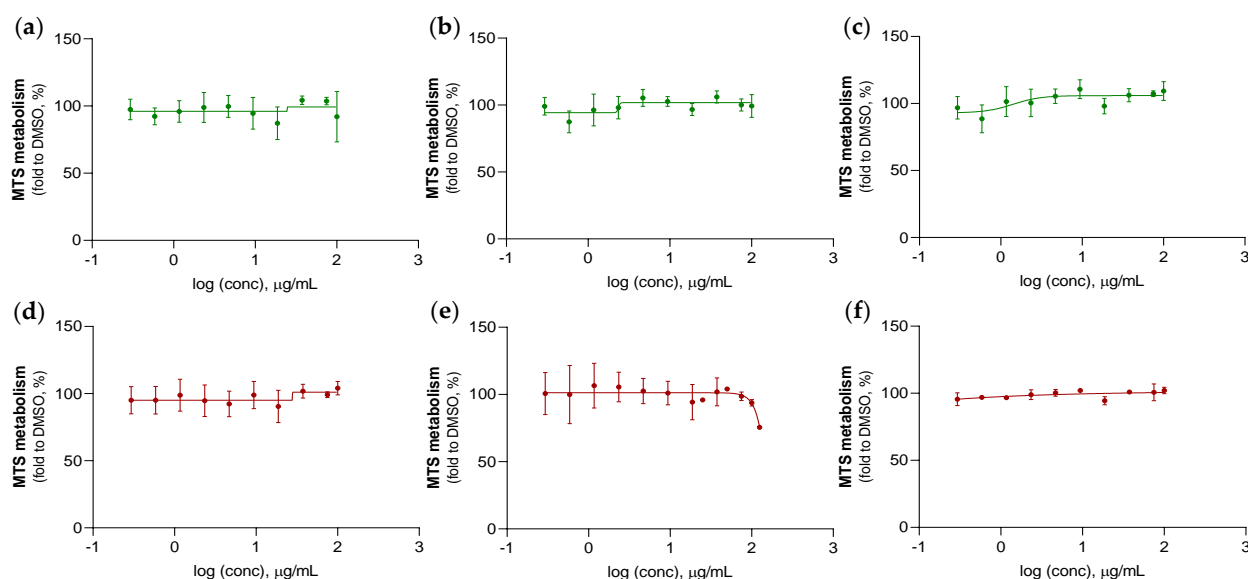
Figure 5.11. HCT-116 cells viability determined by the MTS assay after a 72-h treatment with extracts derived from wild (a–c) and farmed (d–f) *A. oroides* specimens at different concentrations (presented in a logarithmic scale). All data points are expressed as mean ± standard error of the mean from at least three independent experiments. DMSO was used as vehicle control, and 10 μM 5-Fu as positive control.

On the other hand, all *S. foetidus* sponges provided extracts with moderate anticancer activity, whether they derived from wild or farmed populations. More specifically, wild explants exhibited IC$_{50}$ values in the range of 70.8–71.5 μg/mL, while their cultivated counterparts showed a more profound variation, with values starting from 80.5 μg/mL and reaching 41.2 μg/mL. However, the average cytotoxicity against HTC-116 cells, which was derived from the IC$_{50}$ values of the three tested sponge extracts, was evaluated as statistically similar between the two populations (*t*-test, *p* = 0.7). This was found to be as high as 70.9 ± 0.5 μg/mL for wild sponges and 65.3 ± 21.1 μg/mL for the farmed ones.

Table 2.4. Marine sponge crude extracts with anticancer activity against human colorectal carcinoma cell line HCT-116. Results are expressed as mean IC$_{50}$ values, determined by MTS assay. DMSO was used as vehicle control, and 10 μM 5-Fu as positive control.

Species	Type of Population	Replicate	IC$_{50}$ (μg/mL)	95% CI
Sarcotragus foetidus	Wild	#1	70.8	70.3–80.4
		#2	70.4	65.7–74.7
		#3	71.5	68.4–73.8
	Farmed	#1	80.5	74.1–93.3
		#2	41.2	30.1–50.0
		#3	74.2	70.8–79.2

Based on these observations, it can be assumed that *S. foetidus* extracts contain metabolites that are more active against HCT-116 cells than those detected in *A. oroides*. However, previous studies have indicated the cytotoxicity of *Agelas* species against various carcinoma cells, but the associated results concern the activity of isolated metabolites, like oroidin (Dyson et al., 2014) and sceptrin derivatives (Kwon et al., 2018), and not the whole sponge extract. Although these metabolites were present in our

tested *Agelas* extracts, it is likely that the levels at which they were produced, remained relatively low to exhibit bioactivity, or other components might counteract their inhibitory properties. Nevertheless, the highest percentage of HCT-116 cell inhibition, observed for one of our farmed *A. oroides* specimens, was within the range reported by Ang et al. (2023) for various crude extracts of *Agelas* species against the HCT-116 carcinoma cell line (Ang et al., 2023). However, it should be mentioned that the latter study revealed the lowest percent of cell viability (i.e., 75.8%) at 30 μg/mL, which was four times lower than our "active" concentration (i.e., 125 μg/mL) for crude *Agelas* extracts.
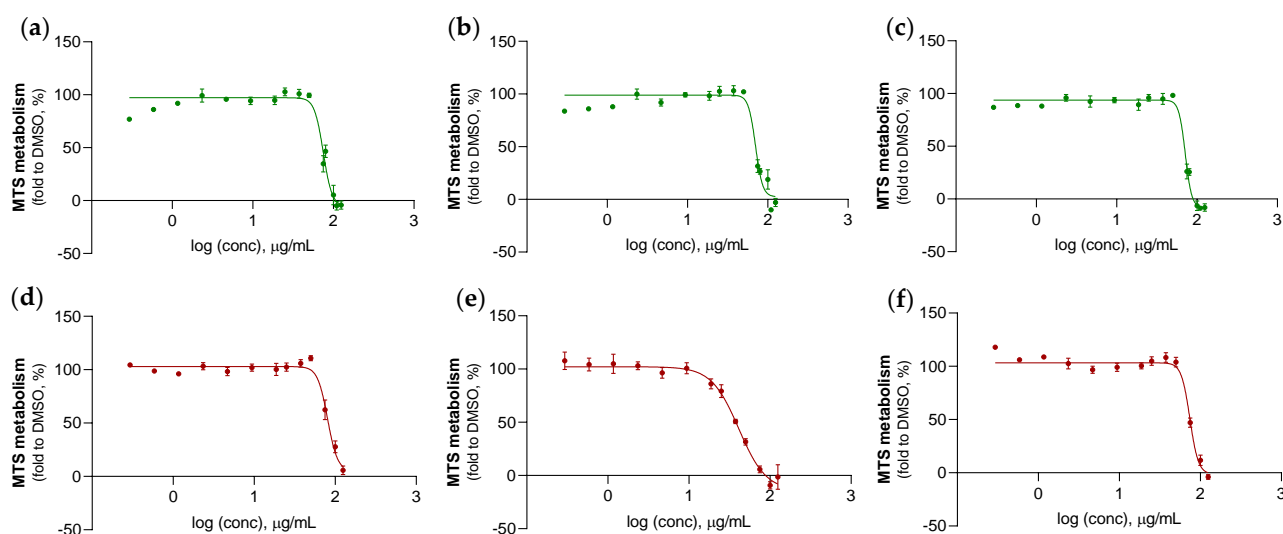
Figure 5.12. HCT-116 cells viability determined by the MTS assay after a 72-h treatment with extracts derived from wild (a–c) and farmed (d–f) *S. foetidus* specimens at different concentrations (presented in a logarithmic scale). All data points are expressed as mean ± standard error of the mean from at least three independent experiments. DMSO was used as vehicle control, and 10 μM 5-Fu as positive control.

Regarding *S. foetidus*, it can be observed that all study extracts, either derived from wild or farmed specimens, showed 50% inhibition against HCT-116 cells. However, according to the American National Cancer Institute guidelines (NCI), crude extracts achieving 50% anti-proliferative activity are regarded as cytotoxic at less than 30 μg/mL after a 72-h exposure (Steenkamp and Gouws, 2006). Based on this, we can consider that only one extract, which belongs to a farmed *S. foetidus* specimen (i.e., #2), is marginally cytotoxic, given its mean IC_{50} value of 41.2 μg/mL. This bioactivity was two times lower than the one demonstrated by the rest of farmed and wild *S. foetidus* counterparts, revealing a higher presence of cytotoxic compounds in its composition. By scrutinizing the % abundance and weight-normalized intensity values of metabolites among extracts, it is observed that indole-3-methylethanoate is present at significantly higher levels in the cytotoxic extract. Specifically, its abundance reaches 0.3% in the farmed extract #2, while it is detected at levels less than 0.1% in the rest of the tested samples. In terms of production levels, the weight-normalized intensity values of indole-3-methylethanoate differ by an order of magnitude between farmed extract #2 and the rest of analyzed *S. foetidus* samples.

[148]

Although there is no report indicating the anticancer potential of this specific metabolite, indole compounds derived from marine sources, including sponges, have long been viewed to possess cytotoxic properties against tumor cell lines (Blunt et al., 2005; Faulkner, 2002).

5.5. Conclusions

Open-sea sponge farming is regarded as a promising source of high added-value natural products, in addition to effective cleanup technology. In this study, cultivations of the sponges *A. oroides* and *S. foetidus* were assessed for their metabolic profiling, as well as their bioactivities, and the results were compared with those obtained from their wild counterparts.

LC–MS/MS analysis revealed an array of natural products present in the studied extracts, with bioactive compounds belonging to alkaloids, benzenoids, indoles, lipids, polyketides, and other chemical classes. However, the biosynthesis of metabolites seemed to be species-specific, with alkaloids being the predominant constituents of *A. oroides* extracts, and lipids representing the major components for *S. foetidus*. In both cases, farming did not impose inhibition or alterations to the compositions and production levels of the sponge-related metabolites, while in some cases it even promoted the production of the bioactive compounds.

In terms of bioactivity, farmed sponge extracts had similar or slightly lower antimicrobial potency against Gram-positive strains than that demonstrated by their wild counterparts. A reverse effect was observed only in the case of *A. oroides* sponges, regarding Gram-negative bacteria. Extracts belonging to *S. foetidus* populations showed consistent, but marginal, anticancer activity against the human colon carcinoma cell line HCT-116, a bioactivity that was not detected in *A. oroides*.

Our findings demonstrate the significance of sponge mariculture in reproducing individuals of similar chemical fingerprints and bioactivities when compared with their wild donors. Although open-sea sponge farming can potentially serve as an additional source of profit for aquaculture-related enterprises, the "sponge-driven bioproduction/bioremediation" concept is still new and unexplored. Altogether, our study emphasizes the significance of sponge mariculture as a promising prospect for diversifying fish farm productivity through the growth of biotechnologically important marine invertebrates, with the possibility for future bioremediation applications and bioactive metabolites supply.

[149]

Chapter 6. A guide to the use of bioassays in exploration of natural resources

Footnote:

6.1. Abstract

Bioassay are the main tool to decipher bioactivities from natural resources thus their selection and quality are critical for optimal bioprospecting. They are used both in the early stages of compounds isolation/purification/identification, and in later stages to evaluate their safety and efficacy. In this review, we provide a comprehensive overview of the most common bioassays used in the discovery and development of new bioactive compounds with a focus on marine bioresources. We provide a comprehensive list of practical considerations for selecting appropriate bioassays and discuss in detail the bioassays typically used to explore antimicrobial, antibiofilm, cytotoxic, antiviral, antioxidant, and anti-ageing potential. The concept of quality control and bioassay validation are introduced, followed by safety considerations, which are critical to advancing bioactive compounds to a higher stage of development. We conclude by providing an application-oriented view focused on the development of pharmaceuticals, food supplements, and cosmetics, the industrial pipelines where currently known marine natural products hold most potential. We highlight the importance of gaining reliable bioassay results, as these serve as a starting point for application-based development and further testing, as well as for consideration by regulatory authorities.

Keywords: bioassay selection; bioactivity; natural products; drug discovery; blue biotechnology; bioactivity-guided purification; validation; preclinical trials; biodiscovery

6.2. Introduction

The most common approach to discovering new bioactive compounds is extensive screening of crude natural extracts using bioassay-guided protocols to determine their activity, followed by isolation and characterization of the active compounds, which are then used in a variety of biotechnological applications, including food, feed, agriculture, cosmetics, and veterinary and human medicine. The discovery of new marine natural products in the last five years has been driven primarily by marine fungi, but also by sponges, tunicates (ascidians), molluscs and cyanobacteria, which are the source of most of the approved drugs in the marine pharmacology pipeline. Bacteria associated or symbiotic with marine invertebrates are recognized as an important source of marine natural products (El-Seedi et al., 2023; Jiménez, 2018; McCauley et al., 2020; Newman and Cragg, 2020; Rotter et al., 2021a). In addition, marine archaea, green algae, thraustochytrids, and dinoflagellates, have long been studied as sources of natural bioactive products. To increase the chemical space and diversity of activities detected in bioassays, modifications of culture conditions or co-cultivation are used in the search for natural products from culturable microorganisms (e.g., Lauritano et al., 2016; Marmann et al., 2014; Oh et al., 2005; Romano et al., 2018). Other sources of marine natural products include actinomycetes, brown, and

red algae, cnidarians, bryozoans, echinoderms, crustaceans, and fish (Barreca et al., 2020; Carroll et al., 2021; Jimenez et al., 2020; Rotter et al., 2021a). The ecological diversity of the marine environment and (micro)organisms in this habitat, combined with the large genetic diversity, represents a unique and rich source of compounds that can be exploited by the pharmaceutical industry and potentially provide solutions to the increasing number of drug-resistant infectious and non-infectious diseases (Bettio et al., 2023; Hughes and Fenical, 2010; Liang et al., 2019; Liu et al., 2019).

The authors of this review are members of COST Action CA18238 Ocean4Biotech, a network of more than 150 blue biotechnology scientists and practitioners from 37 countries (Rotter et al., 2021b, 2020). Our goal is to provide a guide for decision making in the selection and use of bioassays to improve the efficiency of bioprospecting and discovery of bioactive marine compounds. A comprehensive overview of bioassays currently used in the marine bioprospecting community is provided, along with their strengths and weaknesses, followed by considerations for bioassay-guided identification and isolation. We also consider the importance of incorporating *in vitro*, *ex vivo*, and 3D human cell- or tissue-based bioassay protocols as important tools in the preclinical process to avoid drug failure in clinical trials, most often due to lack of clinical efficacy and/or unacceptable toxicity. We then present quality control procedures, including validation, that are required for further safety and efficacy testing, which will then pave the way for eventual regulatory approval for commercialization. The procedures and workflows described are general in nature and can be applied to a wide range of potential applications of bioactive compounds, from industrial enzymes to pharmaceuticals for human consumption. Therefore, we use the term bioactive compounds to refer to all structural variants of natural molecules, from small molecules to large polymers, including, for example, proteins and polysaccharides. Finally, we provide an application-oriented overview of the industrial pipelines most commonly supplied with marine-derived natural products, including those focused on the development of pharmaceuticals, dietary supplements, and cosmetics. By providing insight into the assays used to evaluate bioactivity and best practices in bioassays, this review aims to guide the natural products and blue biotechnology community in decision making for natural product discovery and development.

6.3. Bioassay types and their use in bioactive compound discovery

The biological relevance of natural extracts and pure compounds, whether natural or synthetic, is determined by the bioactivity assays or bioassays used (Weller, 2012). The term "bioactive" is defined as "having or causing an effect on living tissue" (Strömstedt et al., 2014). Different characteristics of bioassays such as throughput, complexity, speed, and cost are relevant to different stages of the biodiscovery process (**Figure 6.1**). In the pre-screening and screening phase, the goal is to detect and potentially quantify bioactivity potential. Therefore, bioassays should be performed in a high-

throughput format screening format (HTS) that allows rapid and cost-effective testing of large number of samples or large libraries of extracts, extract fractions, or pure compounds. In the monitoring phase, bioassays are used to guide purification or fractionation processes to isolate and identify single pure bioactive compounds (bioactivity-guided approach), so they must be designed to have a high throughput capacity, be fast and easy to perform, and be cost-effective. Interestingly, innovative *in silico* approaches have recently been developed that do not require extract fractionation and are known as compound activity mapping (CAM) and are freely available (www.npanalyst.org) (Gaudêncio et al., 2023; Kurita et al., 2015; Lee et al., 2022; O'Rourke et al., 2020). Finally, in the secondary phase, bioassays are used to identify and characterize the biological mode of action of the bioactive compound, which typically requires a series of bioassays that must be highly specific and accurate and are usually time-consuming and expensive (Claeson and Bohlin, 1997; Strömstedt et al., 2014; Suffness and Pezzuto, 1990).
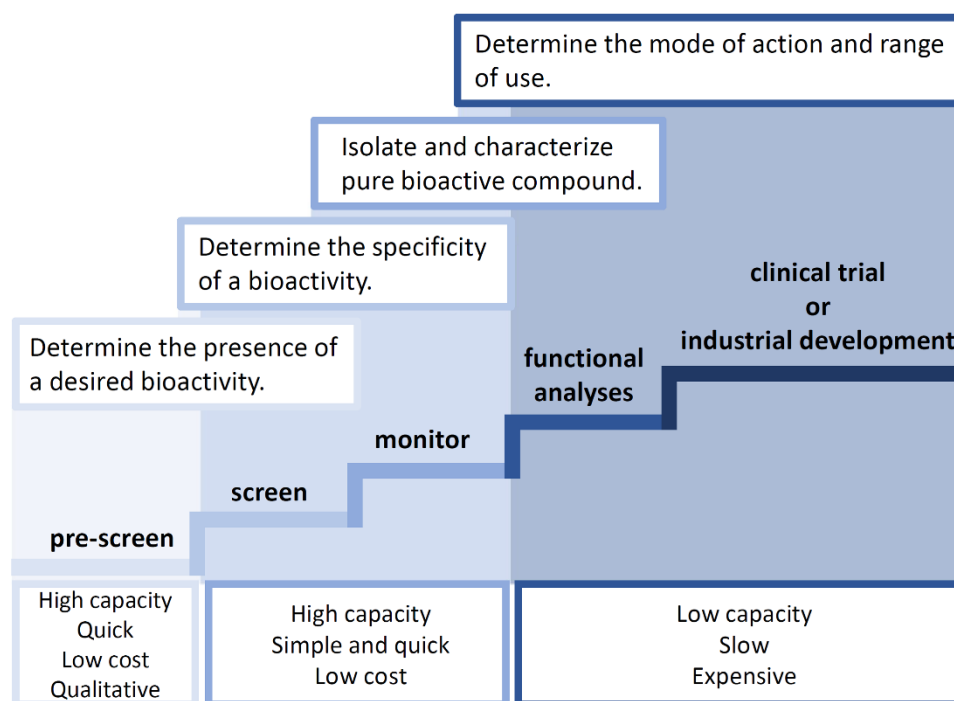
Figure 6.1. Characteristics of bioassays used at different stages of biodiscovery. The biodiscovery process consists of several stages (centre), which place different demands on bioassays' characteristics (bottom) in order to achieve the progressive goals of biodiscovery (top).

Bioassays can be performed *in silico*, *in vitro*, *ex vivo*, or *in vivo* at any of the levels described, and usually a combination of these methods is used to characterise a new compound or the bioactivity potential of a natural resource. When screening an extract for medicinal activity, *in silico* and *in vitro* assays are typically used to identify the bioactive compound and its mode of action, while *in silico* and *in vivo* assays (e.g., animal studies) provide information on pharmacological activity and toxicity (Mbah et al., 2012; Strömstedt et al., 2014).

[155]

6.4. Practical considerations in choosing bioassays to detect target bioactivity

The following paragraphs provide a list of questions and considerations, the answers to which provide information on what to consider when selecting or designing a bioassay (Table A12, **Figure 6.2**).
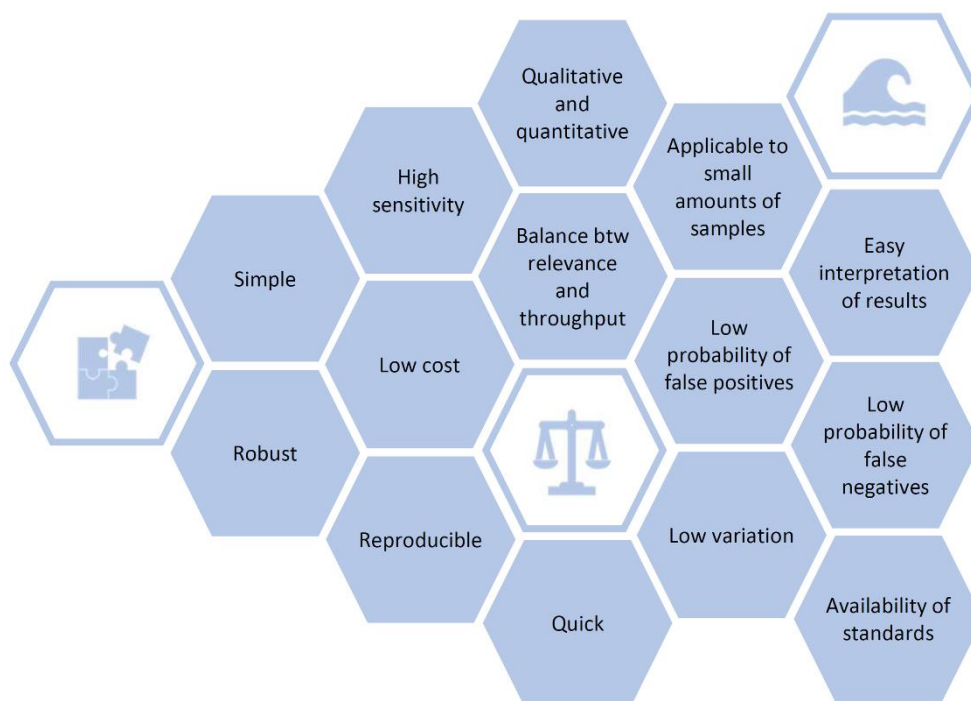
Figure 6.2. Characteristics of a good bioassay. For each bioassay, different characteristics must be balanced (indicated by scales) in order to arrive at a complete description (indicated by a puzzle) of a good bioassay. The blue biotechnology theme of the review is indicated by the wave symbol, but these characteristics apply to every bioassay.

At what stage of the discovery process and for what purpose will the bioassay be performed? Considering the target bioactivity of interest, appropriate bioassays can be selected and used to screen crude or fractionated extracts, to guide subsequent purification, or to explain underlying mechanisms of action, as described in the previous section. First and foremost, the target bioactivity should be selected. An overview of the most commonly used bioassays can be found in Table A13.

Is there an interest in a specific or general activity? In general, bioassays can be divided into two distinct categories: "single-target bioassays" and "functional multi-target bioassays". Single-target bioassays are generally designed to detect the effect of the tested compounds on a particular target with a high degree of specificity and based on a distinct mechanism of action (Claeson and Bohlin, 1997). Examples include the analysis of specific enzymatic activities, such as the degradation of proteins or breakdown of plastics, or the inhibition of enzymatic activities, such as the inhibition of proteases and the blocking of target receptors. Another variation of single-target bioassays is "chemical-genetic profiling" in yeast. A panel of yeast strains with selective mutations that highlight sensitivity to specific drugs is used to screen known compounds with unknown modes of action or mixtures of compounds

[156]

such as natural product extracts (Harvey et al., 2015). The second category, "functional multi-target bioassays", includes bioassays that use whole animals, organs or cells. These bioassays are non-specific in their outcome and measure phenotype change or a general biological effect, such as an antimicrobial or cytotoxic effect. The response to the bioactive compound tested cannot necessarily be attributed to a specific mode of action. These are often referred to as the "phenotype-based approach" (Claeson and Bohlin, 1997; Swinney, 2013).

Which are the most common bioassays for determining target activity? The target bioactivity can be assessed using a variety of bioassays, but the scientific community may prefer certain assays for which troubleshooting, appropriate controls, and interpretation support are available (Table A13).

Are resources available to perform bioassays (in terms of ease of execution or technical complexity)? Specialized equipment and/or trained personnel are required to perform certain bioassays. In terms of safety, it is also important to consider whether the bioassay uses hazardous chemicals or organisms that must be handled in safety chambers and comply with local regulations (e.g., consider the biosafety level (BSL) of the target organisms, the use of genetically modified organisms (GMOs), and waste management).

What are the associated costs for personnel, equipment, and materials? Will the bioassay be used as a routine method? A bioassay may be simple (e.g., an enzymatic reaction detected by a colour change) and performed by a technician, whereas some types of bioassays (e.g., bioassays using cell culture) require extensive training. Similarly, bioassays may be more or less labour-intensive and require specialised equipment or expensive consumables.

Is high throughput and full automation of the analytical process required? Bioassays often use a 96- or 384-well plate format, whereas a higher density layout of 1536-wells is also available but less popular. Performing a manual 384-well plate assay is challenging, especially for assays where precise time intervals between stages are critical. Nevertheless, it is feasible for selected bioassays. A common plate-related phenomenon is the so-called "edge effect", in which the response in peripheral wells differs from the response observed in the inner wells of a microplate. There are several approaches to avoid this problem, such as using only the inner wells, randomization in plate design, or replication (White et al., 2019). Recently, some manufacturers offer plates with a built-in moat surrounding the outer wells (or even both inner and outer wells), that is filled with water, and serving as an evaporation buffer during prolonged incubation. Depending on the desired throughput, robotic liquid handling systems can be used to fully automate almost any bioassay workflow, but the initial cost of such systems can be prohibitive for small laboratories.

Are standardized forms of bioassay available? Although standardization of bioassays facilitates interpretation and comparison of data between laboratories and allows better monitoring of bioassay performance, standardized bioassay protocols are available for only a limited number of bioassays. Inter-laboratory reproducibility or precision under the same operating conditions becomes more and more valuable in stages of higher levels of technology readiness (TRL).

What is required to interpret the results of the bioassay? What are the appropriate controls to distinguish true results from false positives or false negatives? Before beginning to interpret the results, it is assumed that the test performance was appropriate. This can be verified by including an external positive or negative control (or sometimes an internal standard) in the assays, such as organisms with a known phenotype, to ensure that the bioassay performance was optimal. The measurements obtained can be compared to positive and/or negative controls, as well as to blank measurements, to evaluate the effects of medium/buffer/background. Although method validation at the discovery level is not essential, evaluation of precision, i.e., the degree of scatter between a series of replicate measurements obtained from multiple samplings of the same homogeneous sample under the same conditions – expressed as coefficient of variation (CV) - makes the data more robust and reliable.

How are the results to be interpreted in a meaningful way? Is the extract/compound bioactive? Benchmarks and thresholds for bioactivity must be considered, as there are common thresholds below which an extract is considered very active or moderately active, while above these thresholds it is considered of little interest for further development. Meaningful evaluation of the results in combination with chemical dereplication strategies (i.e., evaluating the presence of known compounds in the crude extracts) (Gaudêncio and Pereira, 2015) plays a very important role in prioritizing samples for further development and deciding which samples are worthwhile for further development investment.

What is the expected content of bioactive compounds in the extract? How complex is the crude extract and what is the level of background substances that would interfere with the measurement of bioactivity? Advanced dereplication methods are used for natural product profiling/fingerprinting of complex extracts (Gaudêncio and Pereira, 2015). An estimate of the expected content of bioactive target compounds helps in the selection of the bioassay to avoid false positives in terms of required sensitivity (high sensitivity for low-content compounds), selectivity (the extent to which the bioassay can differentiate and detect a target analyte without interference from concurrently present irrelevant compounds), and specificity, which is a measure of high selectivity (the ability to unambiguously detect the target analyte in the presence of other substances, including those with similar chemical structures). It also helps in the selection of appropriate controls and thus in the interpretation of data. For some

compounds, spiking samples with a reference standard can be a solution for detection and quantitation, but a suitable standard must be available.

What is the desired level of quantitative response (qualitative, semi-quantitative, quantitative results)? Does the potency need to be accurately assessed? Measurements can be binary (activity present or absent), or quantitative information can be obtained by comparison with appropriate controls. Although only quantitative bioassays are suitable for unambiguous determination of potency, the need for such accurate information may be more important at later stages of discovery, purification, safety, and efficacy testing. Potency is usually expressed as a percentage of the extract volume or as a unit of mass in the screening stages if the bioactive compound is not known; later, molar concentrations are used for pure compounds with known molecular and functional properties. Quantitative assays often use standard compounds (spiking, calibration curves), and it is worthwhile to check the availability of appropriate standards. In the context of interpretation of results, determination of the limit of detection (LOD) and limit of quantification (LOQ) provides better reliability of data. In addition, selection of bioassays with lower limits of detection and quantitation usually results in a higher degree of confidence in the final data.

It is useful to know what may affect the precision or repeatability of bioassays. Some metabolites show synergistic effects and bioactivity is lost after fractionation, or metabolites may act antagonistically and activity is detected only after fractionation. Fractionation may also lead to an apparent loss of compounds due to their dilution or binding to discarded material (e.g., with pelleted debris in clarification steps). In addition, physical parameters of the extract (viscosity, pH, colour, etc.) can lead to false-positive and false-negative results. Potential interferences can arise from the material of the sample containers (usually polypropylene and polystyrene, treated or untreated, or glass), and these should be carefully selected based on the charge and polarity of the molecules to be tested, if known (Strömstedt et al., 2014).

What is the solubility and stability of the compound of interest? Is it a small molecule or a complex molecule? The solvent used for extraction must not be toxic or should not be used at a concentration that is toxic to the microorganisms, cells, tissues, organs, or organisms. When aqueous solutions are not used for extraction, extractions are usually performed with dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), methanol, or ethanol, which can be tolerated in microbial or cell-based assays only at low concentrations (e.g., up to 1 % DMSO) and whose presence may affect final results (Dyrda et al., 2019; Hipsher et al., 2021; Rekha et al., 2006). Compounds extracted with organic solvents can be vacuum dried to mitigate this issue. Nevertheless, the effect of extraction solvents can be evaluated by performing the bioassay with the solvent as a control. In addition, poor water solubility

[159]

can lead to misleading results. Bioassay optimization strategies are recommended to improve bioassay performance for poorly soluble compounds (Di and Kerns, 2006). As mentioned earlier, the effect of extraction medium is evaluated by performing the bioassay with the extraction solution alone. If necessary, this control is performed each time the bioassay is conducted. Characteristics of the extraction medium such as thermostability, volatility, and complexity (sedimentation properties and migration) can also affect the design of the bioassay, while characteristics of the target substance such as thermostability, susceptibility to proteolytic degradation, and complexity that affect the temperature and timing of extraction can also affect the desired bioactivity. For example, enzymes are typically isolated at low temperatures because they can be sensitive to proteolytic degradation or thermal denaturation, which can lead to loss of bioactivity. In addition, natural products should be handled at temperatures below 40 °C to avoid degradation and loss of bioactivity. In general, it is preferable to work with compounds that are stable under various conditions, especially with regard to further development and for practical reasons with regard to the application and marketing of the final products.

Do seasonal and geographic differences or legal aspects of sampling affect samples used for bioactivity screening and thus affect biodiscovery? For many types of natural samples, re-sampling is limited due to large seasonal or geographic variations. In addition, issues of safety and sustainability should be considered. Legal issues can also limit transnational access to (marine) biological resources, but this obstacle can be effectively addressed under the Nagoya Protocol, and the conventions CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and CBD (Convention on Biological Diversity), and CMS (Convention on the Conservation of Migratory Species of Wild Animals) by following well-regulated procedures (Kuunal et al., 2020; Schneider et al., 2023).

Is there a need and possibility to validate the bioassay? Validation of bioassays in the discovery phase is useful for evaluating efficacy of candidate bioactivities with high precision and accuracy. This is also important for planning safety and efficacy testing and clinical trials, establishing the basis for discussions with regulatory authorities during planning. At later stages, at the quality control level, bioassays should also reliably assess the quality across different product batches.

What are the relevant target organisms? In bioassays involving living organisms, e.g., microorganisms, cell lines, or animals, it is important to select appropriate target organisms with respect to their relevance and the particular requirements for handling these organisms. An important aspect to consider is the growth conditions, as different growth conditions may affect the outcome of the bioassay.

Do we have a clear idea of the intended application? If there is a clear idea of an application/use, the local regulatory authority should be approached early in biodiscovery, as it is beneficial to use those bioassays that are congruent with product development, as this can be very useful to expedite the process.

6.4.1. Specifics of marine samples

When working with marine extracts or marine microorganisms in bioassays, special considerations should be made and methods adapted to account for the unique challenges posed by the presence of salt, poorly hydrophilic, often highly coloured or auto-fluorescent, and chemically complex materials. These features characteristic of the marine environment require customized protocols for working with samples that may exhibit increased background interference, altered solubility properties, and greater chemical diversity. Moreover, when working with higher organisms as a source of bioactivity, it should be verified whether the bioactivity originates from the macroorganism or from the associated microbiota (Beutler, 2009; de la Calle, 2017; Macedo et al., 2021). Geographic or seasonal variations in the production of bioactive metabolites, which have been demonstrated for different marine organisms (El-Wahidi et al., 2011; Heavisides et al., 2018; Hellio et al., 2004; Henrikson and Pawlik, 1998), are another important issue.

6.5. Prevalent bioassays in marine biodiscovery

Using a keyword search of the PubMed database, we analysed research efforts on marine natural product discovery between 2000 and 2022 (**Figure 6.3**). There is a panoply of bioassays that can be used to screen natural resources for their bioactive properties. We have compiled the most common of these in Table A14 and provided a critical overview of their advantages and disadvantages. Here, we provide an overview of antimicrobial, antifungal, antiviral, and cytotoxicity bioassays, as well as those that investigate the antioxidant and anti-ageing potential of marine extracts. These include both phenotype-based and single-target bioassays to varying degrees, e.g., antimicrobial assays are mostly phenotype-based, whereas both phenotype-based and single-target bioassays can be used to assess cytotoxicity.

6.5.1. Antimicrobial bioassays

The most research efforts in the field of bioactivity of natural marine sources have been dedicated to the detection of antimicrobial activities using phenotypic assays (**Figure 6.3**). The increased efforts are mainly due to the worldwide decline in the development of antibiotics, while the increasing emergence of microorganisms resistant to antimicrobials is becoming a global health threat (Dadgostar, 2019). The problem is of particular concern for the Gram-positive and Gram-negative bacterial

pathogens that belong to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp.)*, and some fungal pathogens (*Candida auris, Candida glabrata, Aspergillus fumigatus, Cryptococcus neoformans*), for which an increasing number of multidrug-resistant strains have been identified worldwide (Arendrup and Patterson, 2017; Liu et al., 2019; Minarini et al., 2020). The term antimicrobial activity is used in studies investigating compounds that kill or inhibit the growth of bacteria and fungi, and therefore includes both antibacterial and antifungal activities. However, the term antimicrobial activity is also often used in studies that focus solely on bacteria, which should lead to us to use this term with caution. In addition, there are studies that focus on one group of organisms and investigate either antibacterial or antifungal bioactivity.
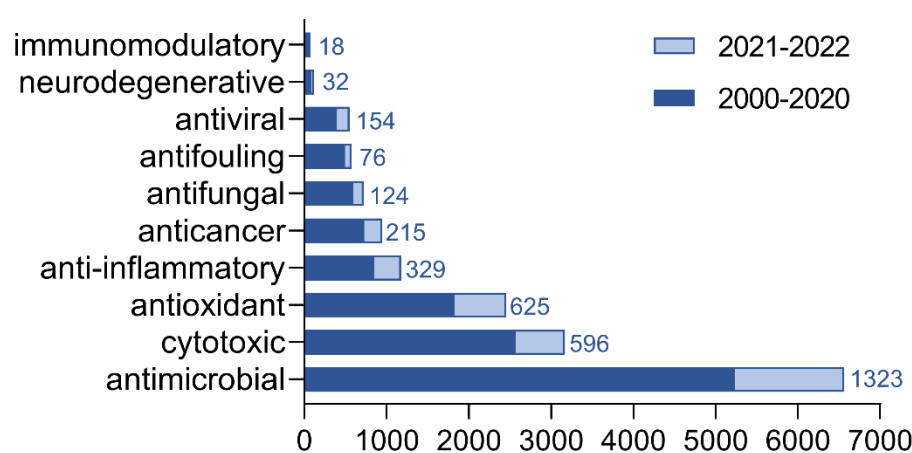
Figure 6.3. Distribution of research efforts to assess the bioactivity of marine natural products from 2000 to 2022 based on the PubMed database. For each bioactivity, a keyword search (together with keyword marine compound) was performed for all publications and only for reviews in the two specified time periods (2000 to 2020 and 2021 to 2022). The number of publications found for each keyword, excluding reviews, is shown here. The last two years are highlighted with the number of publications (excluding reviews) shown next to the columns. The greatest increase in research efforts has been in antioxidant, anti-inflammatory, antiviral and neurodegenerative bioactivities, with more than 25% of publications in the last two-year period compared to the entire 2020-2022 period.

The most commonly used bioassay to investigate the antimicrobial activity of marine natural products is the determination of minimum inhibitory concentration (MIC) in the form of broth microdilution, macrodilution, and agar dilution, followed by the disc diffusion/Kirby–Bauer method (**Figure 6.4**, Table A13). These bioassays determine the lowest concentration of an antimicrobial agent that prevents visible or measurable growth of a microorganism.

The main advantages of dilution methods are cost-effectiveness, practicability, accuracy, reproducibility, versatility, availability of standard protocols, low sample volume requirements, and the ability to obtain quantitative MIC values (minimum concentration that inhibits microbial growth) and MBC values (minimum bactericidal concentration, lowest concentration at which 99.9% of bacteria are killed). Published MIC values for marine extracts vary from µg/mL to even mg/mL and are generally

[162]

below 100 μg/mL for pure compounds (Choudhary et al., 2017). There are common thresholds at which the extract is considered very active (<10 μg/mL), moderately active (10-250 μg/mL), and with little or no activity (> 250 μg/mL) (Fajarningsih et al., 2018; Nweze et al., 2020; Pech-Puch et al., 2020). The optimal MIC and IC$_{50}$ (concentration at which 50 % of growth inhibition is achieved) for a pure substance should be below 1 μg/mL, while concentrations above 10 μg/mL are considered of little interest for further research (Cushnie et al., 2020). Following a detailed structural characterization of the bioactive compound, potency can be defined in molar units, which may require consideration of the characteristics of the active site (e.g., the oligomeric state required for bioactivity). In drug discovery, compounds are often considered highly bioactive if they are active at micromolar (μM) or nanomolar (nM) concentrations. In the diffusion-based method, there is no quantitative result or only a limited one. However, both types of bioassays can be useful to analyse the difference in antimicrobial activity of individual natural products observed in different strains of a given species (e.g., resistant and non-resistant mutants). *In vitro* assays are characterised by simplicity of design and performance. They are traditionally time-consuming but can be automated. However, the results are usually not available within a day and do not provide information on the mechanism of action. To ensure the quality of the bioassay performed, a positive control of a standard antibiotic should be tested against authenticated microbial strains, preferably from a type culture collection such as national type cultures collections (e.g., National Collection of Type Cultures (NCTC) in the United Kingdom; German Collection of Microorganisms and Cell Cultures DSMZ; American Type Culture Collection - ATCC). Reagent sterility controls and negative controls (e.g., influence of solvents) should also be included in each bioassay. When working with complex samples such as natural extracts, the presence of other metabolites in the extract can potentially serve as a carbon source for the microorganism used, which can mask the effect. Both technical and biological replicates should be performed to increase measurement accuracy.

Gram-positive bacteria are more sensitive to the effects of many known agents than Gram-negative ones, which increases the likelihood of hits in screening studies (Cos et al., 2006). For this reason, microorganisms from different groups should be included in the screening process. For each microorganism tested, the optimal growth medium and inoculum size should be determined to avoid underestimation or masking of antimicrobial activity (Wiegand et al., 2008). In most cases, rich complex media (e.g., Mueller-Hinton broth - MHB, tryptic soy broth - TSB, nutrient broth - NB) are used without supplements for non-fastidious organisms and with supplements (e.g., salts, dyes, vitamins, minerals) for fastidious organisms. Many published studies have used Lysogeny Broth (LB) media for antibacterial testing, but their use should be avoided due to the imbalanced composition of carbohydrates, low availability of divalent cations, and occasional contamination with bile salts (Nikaido, 2009; Sezonov et al., 2007).

[163]

When choosing methods for antimicrobial bioassays, the type of solvent used to prepare the extracts should be taken into account. For example, lipophilic compounds do not diffuse well into solid culture media, whereas strongly charged molecules may undergo ion exchange processes in agar. Therefore, the agar diffusion method is more suitable for the analysis of single metabolites with known polarity and not for complex extracts.
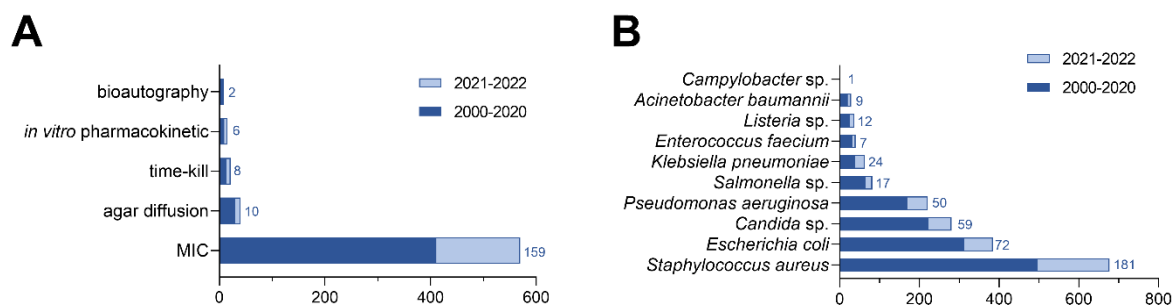
Figure 6.4. Distribution of research methods and target microorganisms for the antimicrobial bioactivity of marine natural products from 2000 to 2022 based on the PubMed database. For each category, a keyword search (together with the keyword marine compound) was performed for all publications and only for reviews in the two specified time periods (2000 to 2020 and 2021 to 2022). The number of publications found for each keyword, excluding reviews, is shown for each method or microorganism. (A) Research effort by bioassay method. The greatest increase in research effort was in the use of time-kill and in vitro pharmacokinetic methods, with more than 35% of publications in the last two-year period compared to the entire 2020-2022 period, while the number of publications for all these methods increased by more than 25 % in the same period; (B) Research efforts by microbial species, the greatest increase in research efforts was for *Klebsiella pneumoniae*, *Listeria* sp., *Acinetobacter baumanii*, *Staphylococcus aureus* and *Campylobacter* sp., with more than 25% of publications in the last two-year period compared to the entire 2020-2022 period. MIC, minimum inhibitory concentration assay determines the lowest concentration of a substance that inhibits the visible growth of a microorganism.

Two organizations develop standardized reference methods for antimicrobial susceptibility testing: the Clinical & Laboratory Standards Institute (CLSI) (https://clsi.org/) and the European Committee on Antimicrobial Susceptibility Testing (https://www.eucast.org/). Although some guidelines from standardized protocols should also apply to bioassays performed on marine samples, noncompliance with these guidelines is relatively common. Items whose standardization has a critical impact on the repeatability and reliability of results include the selection of microbial species and strains, the size and age of the *inoculum*, the type of culture medium, and the duration of incubation.

To further investigate the antimicrobial activity of natural molecules, time-kill assays and flow cytometry methods can be used to provide information on the nature of the inhibitory effect and the cellular damage inflicted on the test microorganism (Balouiri et al., 2016). This bioassay is used in a second phase of testing to determine the dynamics of microbial inhibition kinetics (Dinarvand et al., 2020). Most antimicrobial bioassays are performed *in vitro*, but secondary screening for highly potent compounds may also include *in vivo* assays, (e.g., in murine models), to gain better insight into their preclinical potential (Martín et al., 2013). *In vivo* bioassays are generally not performed with extracts because of the difficulty of interpreting effects based on an unknown mixture of compounds. However,

in some examples, *in vivo* testing is recommended early in the development timeline because potential systemic side effects may be antagonistic or synergistic (Sabotič et al., 2020).

6.5.2. Antibiofilm assays

In recent years, the control of microbial biofilms has gained significant attention as it is increasingly recognized that biofilms are responsible for microbial persistence. Antibiofilm agents are therefore considered as an alternative to fight microbial resistance to antibiotics, since microorganisms do not need to develop resistance to adapt, as their population is not decimated, but merely prevented from persisting in the selected environment. However, the tested compound may have antimicrobial activity, which then also has an effect on biofilm development by inhibiting growth, but not on the biofilm properties themselves. Therefore, determination of both antibiofilm (i.e., inhibition of biofilm formation or promotion of biofilm dispersion) and antimicrobial (i.e., inhibition of growth and/or survival) activity is important to understand whether the compounds tested affect biofilm formation directly or indirectly. Antibiofilm strategies for combating microorganisms focus on the one hand on preventing biofilm formation by inhibiting adhesion or bacterial cell to cell communication (quorum sensing) and on the other hand on eliminating biofilms by dispersion.

Biofilms can be grown using various conditions and formats, but commonly they are grown in a microplate format that can be adapted for high-throughput screening evaluation of antibiofilm efficacy under laboratory conditions. Alternative methods have been developed that provide a better approximation of real biofilm conditions but require specialized equipment, such as delicate microfluidic systems (Goeres et al., 2005; Millar et al., 2001; Tremblay et al., 2015), the Calgary Biofilm Device (Ceri et al., 1999) or the BioFilm Ring Test (Olivares et al., 2016). Biofilm formation is usually monitored by crystal violet staining, which is used to stain the biomass of the biofilm. Other commonly used methods include measuring the metabolic activities of biofilm cells with tetrazolium salts, culturing biofilm cells after sonication to determine the number of CFUs (colony forming units) in the biofilm, or microscopy, which can be either scanning electron microscopy or confocal laser scanning microscopy (Bridier et al., 2010; Haney et al., 2021; Kırmusaoğlu, 2019; Klančnik et al., 2017; Peeters et al., 2008). Quorum sensing reporter strains are typically used to detect interference in quorum sensing. However, this approach has some limitations, including negative effects on reporter strain growth, so appropriate control experiments are essential to obtain reliable results (Defoirdt, 2018; Defoirdt et al., 2013; Taga and Xavier, 2011; Zhao et al., 2020). Simultaneous detection of antimicrobial and antibiofilm activity against important pathogenic bacteria is also possible by studying their growth kinetics with a microplate reader and using a growth curve analysis (Sterniša et al., 2022). Antibiofilm activity is often expressed as minimum biofilm inhibitory concentration (MBIC) or CFU log reduction. In antibiofilm assays, typically screening of individual compounds at concentrations of up to 100 μM is used and

[165]

identifying active hits as those that inhibit biofilm formation by ≥ 80% while simultaneously inhibiting bacterial growth by ≤ 40% (Kwasny and Opperman, 2010). Inhibiting biofilm formation without affecting bacterial growth is preferable because there is less pressure on survival and consequently on the development of resistance (Sterniša et al., 2022).

To date, there is only one standardized assay for antibiofilm activity, namely the single-tube method (ASTM E2871), which is supported by a standard practice for biofilm growth in a CDC biofilm reactor (ASTM E3161) optimized for biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (ASTM E2871-21, 2021; ASTM E3161-21, 2021; Lozano et al., 2020).

6.5.2.1. Special consideration for antifungal bioassays

The prevalence of fungal infections (both invasive and opportunistic fungal infections) is rising due to the increase in the ageing population and immunocompromised patients (Webb et al., 2018). In addition, acquired resistance has emerged in clinically relevant fungi such as *Candida* spp. and *Aspergillus* spp. Therefore, antifungal susceptibility testing (AFST) is of increasing importance in clinical microbiology laboratories, both for selection of appropriate therapy and to provide information on resistance rates at local and global levels in epidemiological studies. The same tests are also used for screening natural products and guiding the discovery of new antifungal agents. Many factors can influence the outcome of *in vitro* AFST tests, including the definition of the endpoint, the inoculum size of the studied fungus, the incubation period, the temperature, and the culture media used for the test (Berkow et al., 2020). For this reason, AFST is not recommended for every fungal pathogen detected in a sample and is performed in clinical microbiology laboratories primarily for yeasts.

The nature of filamentous fungal growth requires the use of adapted antimicrobial bioassays described above to test the antifungal activities of metabolites and molecules. Broth microdilution bioassays are routinely used for fungi, and there are two standard methods for broth microdilution testing of yeasts in clinical laboratories (Clinical and Laboratory Standards Institute, 2017a; Rodriguez-Tudela et al., 2008) and two others for molds (Arendrup et al., 2008; Clinical and Laboratory Standards Institute, 2017b): those established by the Clinical and Laboratory Standards Institute (CLSI) and those established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The four standards use the same criteria to define the test endpoint and use similar criteria to develop clinical breakpoints and thus interpret antifungal resistance and/or susceptibility. However, they differ in several aspects regarding media composition, test microorganism preparation (including inoculum size), measurement methods, and positive controls. Standardized protocols based on disk diffusion are available for both yeasts (Clinical and Laboratory Standards Institute, 2009) and filamentous fungi (Clinical and Laboratory Standards Institute, 2010). Although the qualitative results of the disk diffusion method are suitable for routine use in the clinical laboratory, the quantitative MIC data are more

[166]

relevant for the treatment of invasive infections. Agar-based antifungal screening or "poisoned food assays", in which fungal growth on a standard agar containing antifungal agents is evaluated.

Alternative methods for determining antifungal activity using specialized equipment have also been developed. These techniques include flow cytometry, in which changes in fluorescence are interpreted as changes in cell viability and fungal damage (Chaturvedi et al., 2004). With MALDI-TOF, changes in the proteome compared to a drug-free control are interpreted as indicators of antifungal activity (Sanguinetti and Posteraro, 2016). Isothermal microcalorimetry is used to determine changes in metabolic heat flow of cultured fungi in response to an antifungal agent and indirectly assess its activity (Furustrand Tafin et al., 2013).

6.5.3. Cytotoxicity bioassays

Cytotoxic activity is the second most studied bioactivity for marine natural products in the last twenty years (**Figures 6.3**, **6.5**, **6.9**). Cytotoxicity is often studied in terms of possible anticancer activity. There are several types of bioassays to analyse the cytotoxic properties of natural products, which include phenotypic and single-target bioassays. They are based either on the selective penetration of dyes into dead and living cells or on the detection of markers leaking from the cytoplasm of dead cells. Cytotoxicity bioassays based on selective dye penetration can be divided according to the nature of their endpoints into colorimetric assays (e.g., tetrazolium salts such as MTT, MTS, XTT, or WST, trypan blue, sulforhodamine B (SRB), neutral red uptake (NRU), crystal violet), fluorometric assays (Alamar Blue (AB), 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM), carboxyfluorescein succinimidyl ester (CFSE), propidium iodide (PI), Hoechst-33342, protease viability using glycylphenylalanyl-aminofluorumarin (GF-AFC) as substrate), and luminometric assays (ATP-based and real-time viability) as reviewed elsewhere (Aslantürk, 2017; Riss et al., 2019). The most commonly used bioassays based on markers leaking from dead cells measure the activity of lactate dehydrogenase (LDH), adenylate kinase (AK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or aminopeptidase. Similarly, the commonly used Annexin-V detects phosphatidylserine, which is normally located in the inner membrane but is exposed during apoptosis. Another option is to preload cells with a measurable marker such as calcein-AM or radioactive ^{51}Cr, which is typically used for mixed cell assays in immunology (Aslantürk, 2017; Riss et al., 2019). Assays are usually performed either in microplate format or flow cytometrically. Regarding the evaluation criteria for cytotoxic activity, it was suggested that crude extracts showing 50 % growth inhibition (GI$_{50}$) at concentrations below 100 μg/ml should be considered cytotoxic, while those holding promise for further investigation should have a GI$_{50}$ below 30 μg/ml (Suffness and Pezzuto, 1990). For pure compounds, GI$_{50}$ values in the nanomolar (nM) or low micromolar (below 10 μM) range are considered potentially effective. The accuracy of cytotoxic bioassays is strongly influenced by cell type, seeding density, and medium composition.

Therefore, it is important to include appropriate controls such as background control (no cells), negative control (untreated cells), and positive control (all cells dead) and to test different cell types (Aslantürk, 2017; Carlsen et al., 2020; Cox et al., 2021; Riss and Moravec, 2004). In addition to cancer cell lines, non-malignant cells, preferably first derived from the same tissue and then also using more normal cell types, should be used to evaluate the selectivity of anticancer bioactivity. Based on the cytotoxic activity against cancer cells compared to normal cells, the selectivity index (SI) is calculated (SI = GI_{50} in normal cells/GI_{50} in cancer cells.). A higher SI value (at least above 2) reflects better cytotoxic selectivity (Lopez-Lazaro, 2015; Nguyen and Ho-Huynh, 2016).

Testing different cell types is essential, especially in the context of cancer research, as each cell type may respond differently to treatment (Niepel et al., 2017). The screening of 60 human tumour cell lines for anticancer drugs (NCI60) by the US National Cancer Institute (NCI) was developed in the late 1980s as a tool for *in vitro* drug discovery and then expanded into a service screening to support cancer research. In 2018, the NCI established a Program for Natural Product Development (NPNPD) to develop a publicly accessible HTS-amenable library of more than 1,000,000 fractions from 125,000 marine, microbial, and plant extracts gathered from around the world to advance HTS efforts and accelerate drug development. By 2019, 384-well plates containing over 326,000 fractions were made available for free screening against any disease target (Gaudêncio et al., 2023; Thornburg et al., 2018).

Although cytotoxicity screening aims to identify compounds with growth inhibitory or toxic effects on specific tumour types (disease-oriented approach), the patterns of relative drug sensitivity and resistance generated with standard anticancer drugs can also help to determine the mechanisms of action of the compounds tested. The information-rich nature of the screening data thus provides additional insight into cytotoxic effects (Shoemaker, 2006). The pattern recognition algorithm COMPARE assigns a biological response pattern to the 60-cell line dose-response data for a compound and evaluates whether the response is unique or resembles a known or prototypical compound to assign a putative mechanism of action to a tested compound. As more data are collected on the characterization of different cellular molecular targets of the compounds tested, the compounds most likely to interact with a particular molecular target can be selected (Park et al., 2010; Zaharevitz et al., 2002).

An important aspect to consider when selecting an appropriate bioassay is understanding the mechanism of cell death and the resulting kinetics. In this context, apoptosis-specific (e.g., Annexin-V binding or addition of a caspase inhibitor) or necrosis-specific assays (e.g., detection of the released High mobility group box 1 (HMGB1) protein or addition of specific inhibitors) can be used (Raucci et al., 2007; Riss and Moravec, 2004; Shounan et al., 1998). Preferably, cytotoxicity assays should be performed to cover multiple endpoints and determine multiple parameters from the same cell sample that can reveal the actual cause of cell death (Aslantürk, 2017; Santacroce et al., 2015). Another aspect to

consider is whether the effect is cytotoxic or cytostatic (Anttila et al., 2019; Mervin et al., 2016). Understanding the mode of action and molecular mechanisms targeted by cytotoxic compounds is important for rational decision making about their use in specific cancer types, and for assessing the risk of potential cross-reactivity with other treatments, and side effects.
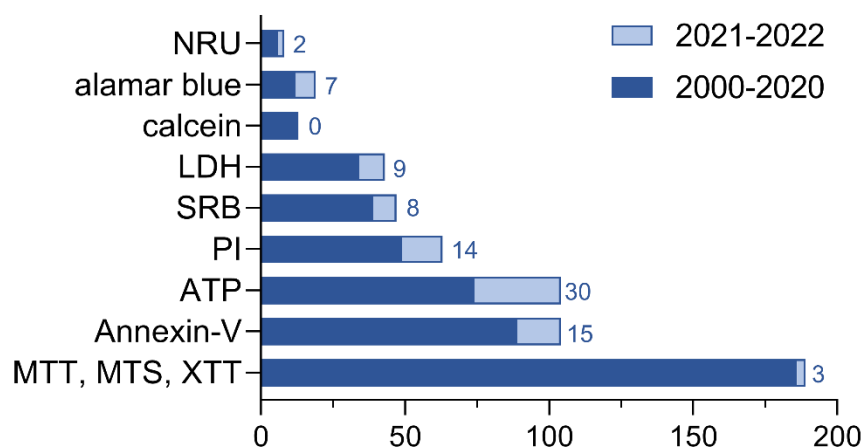
Figure 6.5. Distribution of research methods used between 2000 and 2022 to assess the cytotoxic activity of marine natural products (based on the PubMed database). For each category, a keyword search (together with the keyword marine compound) was performed for all publications and only for reviews in the two specified time periods (2000 to 2020 and 2021 to 2022). The total number of publications found for each keyword, excluding reviews, is shown for each method used, with the last two years highlighted in light blue and the number next to each column. The greatest increase in research effort was seen in the use of NRU, ATP, and alamar blue methods, with more than 25% of publications in the last two-year period compared to the entire 2020-2022 period. NRU, neutral red uptake cytotoxicity assay; alamar blue is a metabolic dye used to quantify proliferation; calcein assay measures cell viability by following conversion of calcein-AM to fluorescent calcein in living cells; LDH measures the activity of lactate dehydrogenase released from damaged cells; SRB, sulforhodamine B is a fluorescent dye used to quantify cellular proteins; PI, propidium iodide is a fluorescent dye that can pass freely through the cell membranes of dead cells and is excluded from viable cells; ATP, adenosine triphosphate assay measures cell viability based on the presence of ATP; Annexin-V is a protein that binds to phosphatidylserine on the plasma membrane and is used to detect apoptosis; MTT, MTS, XTT are tetrazolium salts that are reduced to formazan in living cells, with MTS and XTT yielding a water-soluble formazan dye that is detected spectrophotometrically.

6.5.4. Antiviral bioassays

Viral infections are a major cause of disease in the world because of their complexity, diversity, and rapid spread, which is often accelerated by urbanization, increased migration, and globalization (Drexler, 2010). The 21st century is characterized by major viral epidemics and pandemics, such as influenza A (H1N1) pdm/09, Ebola, Zika, severe acute respiratory syndrome (SARS), Middle Eastern respiratory syndrome (MERS) and SARS-CoV-2 (Ong et al., 2020). In light of these emerging viruses, as well as endemic viruses and the emergence of viral resistance, attention has focused on natural products as sources of new antiviral drugs, including those from the marine environment (Bhadury et al., 2006; Dias et al., 2019; Linnakoski et al., 2018; Silva et al., 2006; Tziveleka et al., 2003). The very first step before an antiviral assay is to determine the potential toxicity of the compounds or extracts to host cells (**Figure 6.6**) followed by a selected antiviral assay. Several different assays can be used to determine antiviral

activity, which can be divided into direct and indirect methods. Direct methods detect the presence of the virus itself, while indirect methods observe the effects of the virus on cell lines used *in vitro* (Table A13) (Declercq et al., 2013; Louten, 2016; Sauer et al., 1984; Sidwell, 1986; WHO Scientific Group, 1987). In general, all the assays described below allow the detection of infectious viruses, with the exception of some that will be highlighted later, which allow to determine the presence of the virus but not to distinguish whether the virus is viable or non-infectious.

Prior to the antiviral assay, it is essential to rule out the possibility that the antiviral properties observed *in vitro* are not due to cytotoxicity. For cytotoxicity screening, any of the methods described in the previous section can be used. Although the MTT assay has been widely used in the past, the ATP-based assay has proven to be the gold standard for measuring cell viability to date. It is more sensitive than conventional biochemical methods because it detects cell death by a general rather than a specific biological mechanism (Herzog et al., 2007; Ponti et al., 2006). However, assays based on cell metabolism are not suitable for metabolically inactive cells, for which the fluorometric microculture cytotoxicity assay (FMCA) is becoming increasingly popular. The FMCA assay is based on the hydrolysis of the fluorescein diacetate (FDA) probe by the cytosolic esterases of intact cells (Burman et al., 2011; Lindhagen et al., 2008; Strömstedt et al., 2014), and cell survival is reported as an index of survival after treatment. Usually, the concentration of the compounds to be tested is between 400 μM and 1.5 μM. According to ISO 10993-5, a cell viability of more than 80 % indicates no cytotoxicity, 80-60 % indicates weak cytotoxicity, 60-40 % indicates moderate cytotoxicity and less than 40 % indicates strong cytotoxicity (International Organization for Standardization - ISO, 2022), so that compounds with a viability between 74 % and 100 % are used for the subsequent antiviral tests. If the results of the cytotoxicity assays indicate no effect on cell line fitness, the compounds can then be tested with primary antiviral assays (Table A13, **Figure 6.6**) (Gomes et al., 2016).

In cytotoxicity evaluation, the value of the 50% cytotoxicity concentration (CC_{50}), defined as the concentration of a compound that produces a 50% cytotoxic effect (Hu and Hsiung, 1989), is determined and used together with the value of the 50% effective concentration (EC_{50}, i.e., the concentration of a compound that produces a 50% inhibition of viral replication) to evaluate the efficacy of an antiviral candidate. This relative efficacy of a compound in inhibiting viral replication with respect to inducing cell death is defined as the therapeutic or selectivity index (SI) and calculated as $SI = CC_{50}/EC_{50}$. Theoretically, a high SI ratio corresponds to a safer and more effective compound that is cytotoxic only at very high concentrations and exhibits antiviral activity at very low concentrations (Naesens et al., 2006; Reymen et al., 1995). The antiviral activity is considered effective/useful when the CC_{50} value is 20 times higher than the EC_{50} value (Cao et al., 2015). Since the CC_{50} and EC_{50} values for a given compound depend on the assays used, the SI value varies from laboratory to laboratory. Nevertheless, the SI value

is a widely accepted parameter of a compound that expresses its *in vitro* efficacy in inhibiting viral replication (Naesens et al., 2006; Reymen et al., 1995).
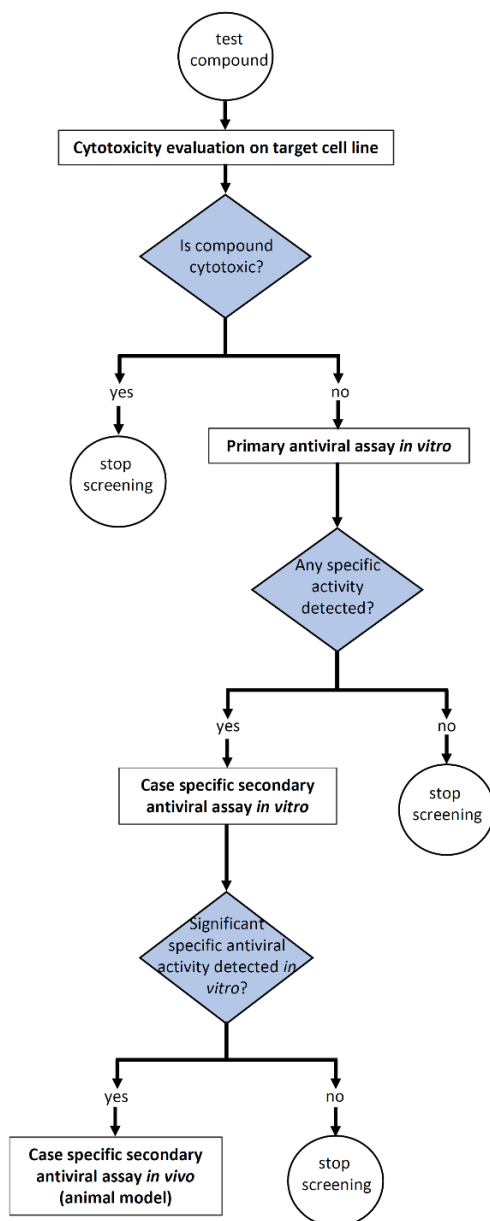
Figure 6.6. Screening for antiviral activity begins with determining the potential toxicity of the compounds or extracts to cell lines that allow viral replication using bioassays such as tetrazolium salts or ATP-based assays or fluorometric microculture cytotoxicity assays (FMCA). It must then be determined which cell system(s) is best suited for virus replication to test for antiviral activity. The ability of the cell line to support viral replication varies and can be measured by cytopathic effect (CPE), focus-forming assay (FFA), plaque quantification (PRA, VRA), or hemagglutination inhibition (HI). Once specific antiviral activity has been established, it needs to be verified in more complex systems and using *in vivo* models.

At this point, it is necessary to determine the cell system(s) best suited for virus replication on which to test new antiviral agents. Depending on the cell type used, the replication capacity of the virus and its actual effect on cells varies considerably (i.e., some viruses may cause a cytopathic effect (CPE), while others may form plaques or induce specific functions such as hemagglutination (e.g., orthomixyxovirus and paramixovirus) or hemadsorption. Biosafety issues must be considered when

working with viruses and other microorganisms. Therefore, when performing antiviral bioassays, specialized equipment and trained personnel should be considered with regard to biosafety level (BSL) requirements. These requirements depend on various factors, such as the pathogenicity of the virus strain under investigation, its biological stability, its transmission potential, the nature of the procedures and manipulations with the pathogen, and the availability of effective vaccines or therapeutic interventions (CDC and NIH, 2020). In general, bioassays and relevant research activities with viral strains that are unlikely to cause disease in humans should be conducted under BSL-1 (e.g., canine adenoviruses). Strains that can cause disease but for which immunization or antiviral/antibiotic treatment is available should be handled in BSL-2 (e.g., hepatitis A and E), while in the case of severe or potentially fatal disease due to inhalation of pathogens, BSL-3 facilities should be used (e.g., highly pathogenic avian influenza). In addition, viral pathogens that pose a high individual risk of aerosol-borne laboratory infections and life-threatening diseases should be handled in BSL-4 facilities (e.g., Ebola and Marburg virus).

A cytopathic effect (CPE) test is based on the observation of morphological changes that occur in a confluent monolayer of host cells as a result of viral infection and replication, and therefore requires experienced personnel. The CPE-based assay was the first assay developed to evaluate whether a compound is antivirally effective, and it can also be scaled up for high-throughput screening (Maddox et al., 2008; Severson et al., 2007). Because viral replication leads to cell death, cell viability assays can be considered a substitute for CPE assessment because they are more accurate, automatable, and objective compared to visual assessment by an operator. Although the CPE assay was one of the first antiviral assays developed, commercial kits (e.g., Viral ToxGlo Assay) that measure cellular ATP as an indicator of host cell survival have enabled standardization of the procedure in many laboratories, and ATP depletion can be correlated with viral load. Since CPE is an indirect measure of viral load, the result regarding the protective effect of drugs against a virus may also vary and be lower than other tests that measure viral load directly (PRA, VRA, see below) (Gorshkov et al., 2021).

Plaque reduction assay (PRA) is widely used direct viral detection method for viruses that produce plaques on target cell lines. It is based on counting plaques formed by lysis of infected cells in a monolayer. The plaques are visible to the naked eye or under a light microscope after staining with neutral red or crystal violet. The plaque assay is the preferred method of viral titration because it is economical and technically simple, but it can be tedious because visible viral plaques can take from 24 hours to several weeks to form (El Sayed, 2000). Conflicting results may be obtained due to various limitations (see Table A13). Therefore, in addition to PRA, the virus yield reduction assay (VRA) is recommended to determine the EC_{50} value by assessing viral progeny production in a growth experiment performed on a confluent monolayer of cells permissive to infection. The assay conditions

must be optimized, especially the multiplicity of infection (MOI, i.e., the ratio of virus to cell number), because this single parameter can significantly affect the evaluation of antiviral activity and a high MOI can reduce the sensitivity of the virus to an antiviral agent (Collins and Bauer, 1977; Sauer et al., 1984). Therefore, it is advisable to perform VRA at both low MOI (multicycle viral replication, e.g., MOI of 0.0001 to 0.1) and at high MOI (single-cycle replication, e.g., MOI of 1 to 5), to compare the resulting EC_{50} values, and to evaluate the range of action of the antiviral molecule as accurately as possible (Yang et al., 1989).

For viruses that do not cause cytopathic effects, the focus-forming assay (FFA), a direct method for virus measurement, can be used. This is a variant of the plaque assay that relies on immunohistochemical techniques, as it uses chemically or fluorescently labelled antibodies specific for a viral antigen to detect infected cells (Flint et al., 2008). If the antibody used recognises a viral antigen that is expressed early in the replication cycle, this assay may not detect non-infectious viruses as there may be an arrest of the replication cycle that prevents the formation of complete infectious virions. For example, quantification of infectious viral particles for α- (hCoV229-E) and β- (hCoV-OC43) coronaviruses relies on an enzymatic antigen detection method that uses horseradish peroxidase (HRP) to label antigen-antibody complexes (Lambert et al., 2008).

For the viruses expressing hemagglutinin (HA), an envelope glycoprotein (e.g., influenza virus, respiratory syncytial virus), the hemagglutination inhibition assay (HIA) can be used. This indirect method is based on measuring the ability of virions to adsorb to and agglutinate red blood cells (RBCs) by binding to glycans (e.g., sialic acid) on the surface of red blood cells (usually from rabbits, horses, chickens or guinea pigs). In practice, the hemagglutination assay is used to determine the viral concentration that agglutinates an exact (standard) number of erythrocytes, making it extremely accurate, although it is only applicable to certain viruses (Joklik, 1988). Standardization of the HIA assay has been described (Kaufmann et al., 2017). In particular, before performing the assay, the following should be considered: (i) although HIA assays provide consistent results across multiple plates, the same amount of virus particles must be used in each plate; (ii) according to WHO, the standard amount of HA used in the HIA assay is 4 units per 25 μL [HA unit is the amount of virus required to agglutinate an equal volume of standardized RBC suspension]; (iii) the RBCs used depend on the type of influenza virus in the assay; and (iv) for different types of 96-well microtiter plates (V- or U-bottom), the incubation time and the occurrence of non-agglutinated cells are different (Kaufmann et al., 2017).

An example of direct detection method is the use recombinant viruses, in particular, fluorescent protein-expressing viruses or viruses expressing reporters fused to viral proteins can be used, as they are rapidly detectable and even quantifiable, making these recombinant viruses suitable for high-throughput applications, e.g., large-scale screening of antiviral drugs (Falzarano et al., 2014). Indeed,

[173]

some *in vivo* applications of GFP/Cherry/reporter viruses have also been established, such as monitoring the efficacy of antiviral therapies and more detailed pathogenesis studies. Unfortunately, a foreign gene or an alteration of existing viral genes can change the biological properties of "modified" viruses, which may, for example, result in reduced virulence of these viruses. In addition, such alterations can put pressure on the virus to eliminate the genetic information encoding the reporter protein, resulting in attenuation/loss of expression of the reporter gene.

Modern assays such as flow cytometry, tunable resistive pulse sensing (TRPS), and quantitative real-time PCR (qPCR) are also increasingly being developed to determine antiviral activity. In particular, qPCR was widely used as direct method to detect SARS-CoV-2 virus during the SARS-CoV-2 pandemic because it allowed testing of antiviral activity of many molecules against this pathogen in a short time. However, it is important to emphasize that during viral replication, the ratio of whole virions to nucleic acid copies is rarely 1:1 and that the viral assembly process can produce complete virions, empty capsids, and/or an excess of free viral genomes. Therefore, positive qPCR results may also be due to the presence of residual viral nucleic acid (i.e., non-infectious virus) rather than infectious virus (Tandon and Mocarski, 2012). For this reason, many molecules with true antiviral activity might be rejected *a priori* simply because they are unable to reduce viral genome copy number in a solution, even if the viruses present are no longer active or infectious. Therefore, it is better to use qPCR-based methods for routine laboratory testing and to confirm the results obtained with the classical methods described above when necessary.

After a certain type of antiviral activity is detected, it is necessary to further investigate this activity using several specialized secondary bioassays for screening and/or monitoring purposes. These *in vitro* or *in vivo* assays are time-consuming, more expensive, and more challenging than the primary screening bioassays and require the expertise of biochemists or pharmacologists. Therefore, they can only be performed by a multidisciplinary team. Secondary testing of compounds that interact with the target, for example, examines whether this interaction occurs in biological systems and attempts to determine the structure-activity relationship between the compounds and the target. Secondary tests also include *in vitro* enzyme activity tests with mechanistic relevance or resonance energy transfer (FRET), as well as pharmacokinetic and pharmacodynamic experiments performed *in vitro* or *in vivo* in an animal model. Human viruses adapted to infect animal models (S. I. Ruiz et al., 2013) or humanized animal models (Crawford et al., 2015; Lai and Chen, 2018) can be used at this stage. Such secondary assays are necessary/mandatory to select potential candidates to be tested in human clinical trials (Gomes et al., 2016; Öberg and Vrang, 1990).

6.5.5. Bioassays for cosmetics and cosmeceuticals with a focus on antioxidant and anti-ageing effects

A variety of specialized bioassays have been developed and routinely used to evaluate the overall cosmetic activity of a marine extract (**Figure 6.7**). The majority of these bioassays are single-target bioassays, but phenotypic bioassays are also available. In the primary screening and secondary testing phases for potential cosmetics and cosmeceuticals, bioassays are mostly based on *in vitro* assays for cytotoxicity, antioxidant and anti-inflammatory activities, using either biochemical cell-free assays or immortalized cell lines (e.g., THP-1 and HaCaT cells). These bioassays are leading in terms of their simplicity, speed, throughput, and cost-effectiveness, even though they may not adequately reflect the actual biological processes in skin cells. Therefore, in later stages, the active extracts or compounds are tested for safety, activity, and mode of action in preclinical assays using primary cells (e.g., keratinocytes) and/or *ex vivo* skin tissue models (Brancaccio et al., 2022), with the option to perform final testing in clinical trials.
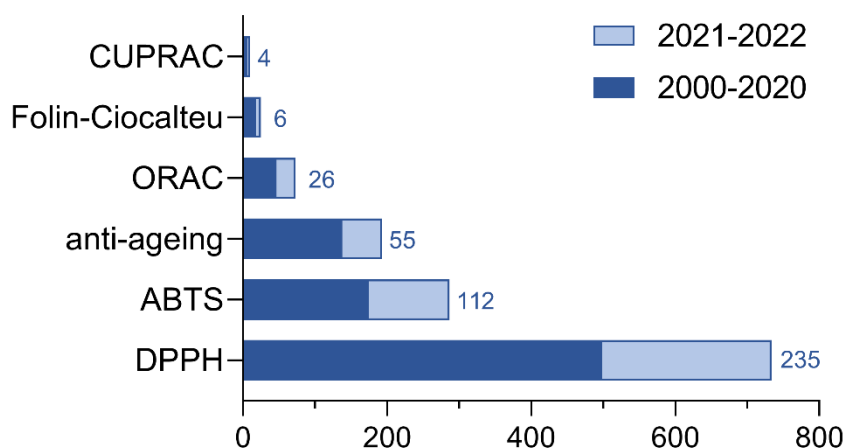
Figure 6.7. Distribution of research methods for antioxidant and anti-ageing activities of marine natural products from 2000 to 2022 based on the PubMed database. For each category, a keyword search (together with the keyword marine compound) was performed for all publications and only for reviews in the two specified time periods (2000 to 2020 and 2021 to 2022). The number of publications, excluding reviews, is shown for each method. The last two years are highlighted, with the number of publications (excluding reviews) shown next to the columns. The greatest increase in research effort was seen in the use of ABTS, ORAC and CUPRAC methods, with more than 35% of publications in the last two-year period compared to the entire 2020-2022 period, while the number of publications for all these methods increased by more than 25 % in the same period. CUPRAC, CUPric Reducing Antioxidant Capacity; ORAC, oxygen radical absorbance capacity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox®-equivalent Antioxidant Capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

In vitro bioassays are used to investigate the antioxidant capacity of extracts by mimicking the damage caused by radicals in the skin and by assessing the efficacy of natural extracts in combating this damage (Thring et al., 2009). Depending on the mechanism by which radicals are scavenged, antioxidant capacity assays are broadly divided into two categories: electron transfer (ET) and hydrogen atom transfer (HAT) based assays (Apak et al., 2007). Compared to HAT-based assays, the ET reaction is

[175]

relatively slow, and its actual rate depends greatly on laboratory conditions, such as solvent and pH (Apak et al., 2007; Huang et al., 2005). ET assays widely used in cosmetics include the DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS/TEAC (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox®-Equivalent Antioxidant Capacity), CUPRAC (CUPric Reducing Antioxidant Capacity), and Folin-Ciocalteu methods, each of which uses a different chromogenic reagent with different redox potential (Ratz-Lyko et al., 2012), as shown in Table A13. Although the actual reducing capacity of an extract or compound is not directly related to its ability to scavenge radicals, these biochemical assays are useful for initial screening procedures (Amorati and Valgimigli, 2015; Apak et al., 2007). Most HAT−based assays are kinetic and rely on a competitive reaction scheme in which the antioxidants of a natural extract and an oxidizable probe compete for peroxyl radicals, the latter being thermally generated in a solution by the decomposition of azo compounds (Apak et al., 2007; Huang et al., 2005). This is the case with the oxygen radical absorbance capacity (ORAC) assay, which is widely used to measure the antioxidant capacity of natural products with anti-ageing and cosmetic potential (Baldisserotto et al., 2012; Dávalos et al., 2004; Dudonné et al., 2011; Ky and Teissedre, 2015; Le Lann et al., 2016). However, it must be emphasized that most HAT and ET assays are sensitive to either hydrophilic or hydrophobic antioxidants and therefore may underestimate the total activity of an extract (Fraga et al., 2013; Ratz-Lyko et al., 2012). Thus, a combination of these biochemical methods may be required to obtain reliable results (Ratz-Lyko et al., 2012).

The anti-ageing effect of the extracts is usually investigated in the screening of cosmetics and cosmeceuticals, including antioxidant (described above) and anti-inflammatory activities (Brancaccio et al., 2022). The anti-inflammatory activity of extracts or pure compounds can be assessed by TNF-α or IL-1β production measured in LPS-stimulated THP-1 activated human macrophage cells (Lauritano et al., 2016). The anti-ageing activity may also be related to their specific ability to block enzymes involved in the breakdown of skin firmness (Thring et al., 2009). These include matrix metalloproteinases (e.g., collagenase), serine proteases (e.g., elastase), and endoglycosidases (e.g., mucopolysaccharide hyaluronidase), which degrade the major components of the extracellular matrix (ECM) of the skin: collagen, elastin, and hyaluronic acid (Li et al., 2019; Rittié and Fisher, 2002). Maintaining high levels of these components is critical for skin elasticity, firmness, and hydration, and thus inhibitors of these hydrolytic enzymes are being sought (Madan and Nanda, 2018). In addition, there is particular interest in the regulation of melanin levels in the skin (i.e., changes in skin pigmentation), the overproduction of which leads to aesthetic problems such as pigmentation spots (Lall and Kishore, 2014; Saghaie et al., 2013), as well as other skin conditions such as discoloration, freckles, and skin cancer (An et al., 2005). Specific assays are available to study the inhibitory properties of extracts on the activity of the enzyme tyrosinase, which catalyses the first rate-limiting steps of the melanin biosynthetic pathway in

melanocytes (Parvez et al., 2006). Typically, L-DOPA (an intermediate in melanogenesis) is used as a substrate and its enzymatic oxidation to the red-colored dopachrome is monitored spectrophotometrically to assess inhibition of tyrosinase. Despite the widespread use of (bio)chemical antioxidant assays, they are usually performed under non-physiological conditions without taking into account the cellular uptake of compounds and their mode of action at the subcellular level, which inherently limits their ability to predict the true antioxidant effect in living systems.

To investigate the regenerative properties of extracts on specific skin cell lines (e.g., fibroblasts), *in vitro* phenotypic assays based on the monitoring of stimulatory effects on the production of ECM components are used (Adil et al., 2010; Boonpisuttinant et al., 2014; Pastorino et al., 2017; Roh et al., 2013; Yodkeeree et al., 2018), as well as their photoprotective effects in terms of cell viability (Moon et al., 2008). The protective role of extracts against photooxidative skin damage can also be evaluated by *ex vivo* approaches. Specifically, a cosmetic formulation is applied to the skin of human volunteers and after a short period of time, strips of the outermost skin layers are removed, exposed to UV radiation, and lipid peroxidation is assessed by measuring the losses of unsaturated fatty acids and the amounts of primary, secondary, or end products of the reaction (Alonso et al., 2009). Cell line-based bioassays are also used to estimate safety parameters by assessing skin irritation by evaluating direct cytotoxicity or other types of damage to the epithelial barrier of the skin by measuring the permeability of fluorescein through epithelial cell monolayers (OECD test no. 460). In addition, mutagenicity and carcinogenicity (OECD test no. 451) are assessed using cell cultures, e.g., the *in vitro* micronucleus test (OECD test no. 487) to detect chromosomal aberrations and the bacterial reverse mutation test (OECD test no. 471) to detect gene mutations. An alternative to animal models for carcinogenicity testing is cell transformation assays (CTA), which are used in combination with other approaches to evaluate carcinogenic potential (Creton et al., 2012; Mascolo et al., 2018; OECD, 2023; Scientific Committee on Consumer Safety - SCCS, 2021).

Ex vivo bioassays using skin tissues have been developed for toxicological studies, such as the reconstructed human epidermis (RhE) test methods (OECD test no. 439, 431), using four validated commercial human skin models, viz. i.e., EpiSkin™, EpiDerm™, SkinEthic™, and EpiCS®, which use reconstructed human epidermis equivalents to evaluate cell viability and are used to assess skin corrosion or irritation potential. Bioassays for the assessment of ocular damage include organotypic assay methods using tissues from slaughterhouses, such as bovine corneas (OECD test no. 437) or chicken eyes (OECD test no. 438), or *in vitro* assays using corneal epithelial cell lines to assess irritation by measuring direct cytotoxicity on rabbit corneal cell lines (OECD test no. 491) or human cornea-like epithelium (OECD test no. 492) (e.g., EpiOcular™). For assessment of genotoxicity or reproductive toxicity, new alternative approach methodologies to animal testing are being implemented worldwide,

[177]

including *in vitro* methods using the whole embryo culture test (WEC) to evaluate developmental toxicity in rodent embryos maintained in culture during the early stages of organ formation, the MicroMass Test (MM), which uses embryonic limb mesenchyme or central nervous system cells from chickens, mice, or rats to evaluate effects on cell differentiation into chondrocytes and neurons as an indication of potential teratogenicity, and the embryonic stem cell assay (EST), which is based on permanent cell lines to predict embryotoxicity by evaluating effects on cell differentiation (OECD, 2023; Scientific Committee on Consumer Safety - SCCS, 2021; Seiler and Spielmann, 2011).

6.6. Quality control and bioassay validation

6.6.1. The concept of validation

The concept of validation can be defined as a systematic approach to collecting and analyzing a sufficient amount of data under specified conditions and based on documented evidence (validation report) and scientific judgment, to provide reasonable assurance that the process of interest will reliably and consistently reproduce results within predetermined specifications when operated within specified parameters (Haider, 2006).

The main objective of the validation process is to produce reliable and consistent data (quality data). In addition, four critical components of data quality are identified, including analytical instrument qualification, analytical method validation, system stability testing, and quality control sampling (United States Pharmacopeial Convention, 2018), with each of these components contributing to overall quality:

- Analytical instrument qualification (AIQ) is the collection of documented evidence that an instrument is fit for its intended purpose and that its use provides confidence in the validity of the data produced. It includes (i) design qualification (DQ), which is performed by the manufacturer prior to purchase to ensure the technical characteristics required by the user; (ii) installation qualification (IQ), which is performed prior to and at the time of installation; (iii) operational qualification (OQ), which is performed after installation and major repairs; and (iv) performance qualification (PQ), which is performed periodically to ensure continued satisfactory performance during routine operation and includes preventive maintenance, recalibration, and performance testing (Bansal et al., 2009; Kaminski et al., 2010; Valigra, 2010).

- Analytical method validation is the collection of documented evidence that demonstrates that an analytical method is fit for its intended purpose and provides assurance that its use with qualified analytical instruments will generate accurate data of acceptable quality (Haider, 2006).

[178]

- System suitability tests (SSTs) are used to verify that the system meets predefined criteria. They are performed in conjunction with sample analyses to ensure that the system is functioning properly at the time of testing.

- Quality control (QC) samples help to ensure the quality of analytical results by being included immediately prior to or during sample analysis.

6.6.2. Validation of the analytical method

The concept of bioassay validation is often associated with compounds that are classified as drugs by regulatory authorities, because the development, production and testing of these products are strictly regulated. Consequently, bioassay validation is an integral part of the quality control system. This may not be the case for cosmetic preparations or dietary supplements, where product characteristics and claims dictate testing or trial requirements, however, in practice many cosmetic preparations claiming bioactivity are also subject to rigorous testing. For biodiscovery and research, it is not usually necessary to meet quality control requirements, but it is good to keep the concepts of validation in mind and apply them wherever possible. This can facilitate the transition from research to industrial development, as well as communication with regulatory agencies, regardless of the type of application.

It is important that the operator performing the validation of the analytical procedure has the scientific and technical understanding, process knowledge, and/or risk assessment capability to adequately perform the quality functions of analytical method validation (Chan, 2011). The parameters to be evaluated for validation depend on the type of method, and the measures used to describe the performance of the analytical method are typically: accuracy (trueness), precision (repeatability), limit of detection (LOD), limit of quantitation (LOQ), linearity (calibration curve), range, selectivity, specificity, and robustness. All of these parameters must be determined for validation of a quantitative analytical method, whereas specificity and limit of detection may be sufficient for a qualitative method. There are numerous guidelines (more than 30) published by regulatory organizations; some of them are summarized in Table A14. These guidelines can be used as a frame of reference for the validation process. Unlike instrument qualification, the type of analytical method (e.g., sample matrix, analytical equipment) determines the parameters to be evaluated, so it is important to select an appropriate guidance document as a frame of reference. It is important to note that the terminology used in different guidelines varies. For example, selectivity, specificity, or diagnostic specificity are defined differently in different guidelines (Borman and Elder, 2017; Chan, 2011; Kadian et al., 2016).

Validation of analytical methods is a progressive, dynamic, and time-consuming process, so it is recommended that a validation schedule (or protocol) be established (EURL, 2022; Shabir, 2003). In addition, there are fundamental differences in validation parameters between different types of assays

[179]

(e.g., chromatography-based or ligand-binding assays), and this issue is addressed differently by different regulatory agencies, either by providing separate validation guidelines (e.g., ICH, EMA) or by specifying certain aspects in a guideline (e.g., FDA) (Borman and Elder, 2017; EMA, 2018; USFDA, 2018).

6.6.3. Data integrity and documentation

The term data integrity refers to the degree of a data-generating system in which the acquisition and storage of data is undivided, coherent, reliable, and accurate. This does not depend on whether the data are in paper or electronic form (Wingate, 2004). The critical issue in ensuring the quality of analytical procedures and data integrity is the documentation of all steps. Good documentation practices (GDocP) is a term used in the pharmaceutical industry to describe the guidelines, standards, and regulations for creating, maintaining, and archiving documents. These apply to all parties involved in a process and to all activities. GDocP-based records have the following characteristics: they are complete, truthful, clear, permanent, accurate, consistent, legible, and concise (Davani, 2017).

6.6.4. Good Laboratory Practice (GLP)

It is recommended that the principles of good laboratory practice (GLP) are followed at all times when performing bioassays. GLP is a quality assurance system that addresses the organizational process and conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived, and reported (OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring, https://www.oecd.org/chemicalsafety/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm, accessed 4 May 2022).

6.7. Bioactivity-guided fractionation and/or purification

With the desired bioactivity in mind, a series of fractionation and analytical steps can be applied to natural resources to isolate and/or purify specific compounds that exhibit the bioactivity of interest. The path from a natural extract exhibiting a specific bioactivity to a dereplicated, purified, identified, and characterised compound exhibiting that bioactivity is often quite long and labour intensive.

A significant portion of the labour and operating costs in a biochemical and analytical laboratory is devoted to the preparation (extraction) of samples for subsequent analytical separation. During the extraction process, the target compound is pre-concentrated and converted into a form suitable for subsequent instrumental analysis and chromatographic or electrophoretic separations, and the complexity of the matrix is reduced. Depending on the solvents and procedures used for extraction, we expect to isolate either small molecules such as polyketides, alkaloids, and terpenoids or complex polymers such as proteins and polysaccharides, and the purification steps are then designed

[180]

accordingly (**Figure 6.8**). The solvents used for the extraction of small molecules usually consist of either a single solvent (e.g., methanol, ethanol, trichloromethane, acetone or water) or a mixture of solvents with a wide range of polarity (e.g., mixtures of ethanol and acetone or ethanol and water) (Varijakzhan et al., 2021). Complex biopolymers are usually extracted using water or buffer solutions (Kazir et al., 2019). The biomass remining after the primary extraction step can be subjected to further extraction with different solvent(s) to extract components with different properties (Izanlou et al., 2023).
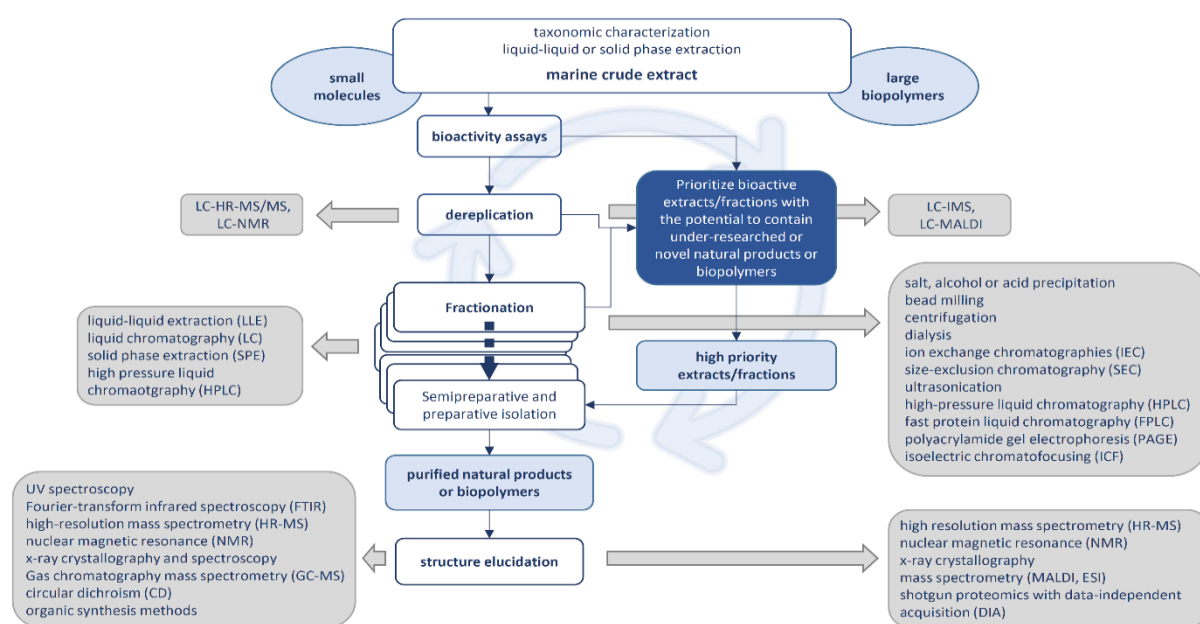
Figure 6.8. The approach for the discovery of new bioactive compounds from marine extracts, with the methodology indicated separately for small (left) and large (right) biomolecules. After extraction, bioassays are performed to determine the potential bioactivities of the extract, and several purification steps are performed to fractionate the extract for analysis and prioritise the purified compounds according to their novelty, for which the dereplication step is crucial. Several purification and analysis runs are required to narrow down the selection of bioactive compounds. Finally, a purification procedure is applied to obtain larger amounts of bioactive compounds that can be further used for compound identification and structure elucidation. The general approach for the discovery of new bioactive compounds is the same for each type of molecule, but the analysis and separation methodology differs depending on the properties.

Extraction and subsequent removal of solid particles is the first important step in the screening process, and the selection of extraction method and solvent(s) is critical for successful downstream processing. For example, bioactive compounds may be present in both a highly polar/aqueous extract and a moderately nonpolar/organic extract. In addition, the physicochemical properties of the starting material determine the steps in the extraction process. For example, microalgae have a rigid cell wall that acts as a natural barrier to prevent solvent molecules from diffusing into the cells and must be broken by mechanical and/or physical techniques such as high-pressure homogenization, shear mixing (high-speed homogenization), ultrasound-assisted extraction (UAE), or microwave-assisted extraction (MAE) prior to or simultaneously with chemical extraction (Benbelkhir and Medjekal, 2022; Tian et al., 2022). It is important to consider all available alternatives of the extraction procedure, including sequential extraction using various solvents (Zhang et al., 2018) to optimize the extraction process and

avoid possible structural or conformational changes of the extracted molecules that can alter their bioactivity. Such changes are more likely to occur in large molecules (e.g., polysaccharides, oligosaccharides). Switching from slow extraction methods (e.g., hydrothermal extraction), which require longer processing time, to faster technologies such as UAE, MAE, or UMAE can help shorten the extraction process and increase the likelihood that the molecule will remain intact (Guo et al., 2022; Qiu et al., 2022). However, chemical reactions can also occur when microwaves and/or ultrasound are used for extraction.

Since a natural extract contains a mixture of molecules, the concept of bioactivity-guided purification is based on the sequential application of different types of fractionations that separate molecules from a mixture and the concurrent application of the selected bioassay to identify fractions containing the bioactive compounds until a satisfactory level of purity is achieved. In each purification step, the individual fractions are tested with the bioassay to select the fractions with the highest bioactivity for further purification. Since numerous fractions usually need to be tested, it is optimal to use a rapid and inexpensive bioassay with low volume requirements. A quantitative bioassay is sufficient to guide the purification.

Purification is usually performed by either liquid-liquid phase separation (LLPS) or the currently predominant solid phase extraction (SPE). SPE has become a standard analytical procedure for the enrichment of target analytes by partitioning and/or adsorption onto a solid stationary phase. SPE is currently the most widely used method for the extraction, concentration, purification, and fractionation of organic compounds from a variety of samples, as well as for solvent exchange; in addition, SPE is also used efficiently for the desalting of proteins and glycan samples. SPE offers several advantages over liquid-liquid extraction, including higher recoveries, avoidance of emulsion formation, lower organic solvent consumption, simpler operation and automation capability, improved selectivity and reproducibility, and shorter sample preparation time. The standard SPE procedure begins with the application of an analysed solution to a solid phase (sorbent), usually in a cartridge, in which the target analytes are eluted with a suitable solvent and collected (Andrade-Eiroa et al., 2016; Faraji et al., 2019).

There are numerous adsorbents for the extraction of different types of molecules. Various SPE mechanisms can be applied to separate target molecules using specific sorbent materials, such as adsorption (e.g., using silica gel, alumina, florisil, or graphitic carbon-based packing), normal separation (e.g., cyanogen-, diol-, or amino-based silica), reversed phase separation (e.g., octadecyl-, octyl-, butyl-, or phenyl-bonded silica), ion exchange (various cation or anion exchangers), size exclusion (e.g., macropore silica or organic gels), affinity separation (carriers with immobilized affinity ligands), and immunoaffinity separation (carriers with immobilized specific antibodies); often two separation

[182]

mechanisms can be used simultaneously (e.g., ion exchange and reverse phase separation) (Andrade-Eiroa et al., 2016).

Efficient SPE can also be performed with magnetically responsive adsorbents. Magnetic SPE (MSPE) is becoming increasingly popular due to its ease of use, high extraction efficiency, and straightforward automation (Jiang et al., 2019; Pena-Pereira et al., 2021; Šafaříková and Šafařík, 1999; Vasconcelos and Fernandes, 2017). MSPE uses various types of magnetically responsive adsorbents based on ferrimagnetic iron oxides (magnetite, maghemite) or ferrites to which specific affinity ligands are immobilized. A popular variation of MSPE is immunomagnetic separation (IMS), which uses magnetic nano/microbeads with immobilized specific antibodies (monoclonal, polyclonal, or engineered) to capture target analytes or cells via antigen-antibody interactions (De Meyer et al., 2014; He et al., 2018; Safarik et al., 2012; Šafařík and Šafaříková, 1999). Magnetically responsive materials can also be used to separate and purify various biologically active compounds on a larger scale (Franzreb et al., 2006; Safarik and Safarikova, 2014, 2004). Stir-bar sorptive extraction (SBSE) is based on the use of a magnetic stir bar covered with a suitable sorbent (usually polydimethylsiloxane or ethylene glycol-modified silicone material) into which the analytes are extracted. The technique has been successfully used for the analysis of samples of varying complexity and for the detection, concentration or removal of marine toxins in crude extracts (Chen et al., 2019; González-Jartín et al., 2020; Pena-Pereira et al., 2021; X. Wang et al., 2017).

Various SPE mechanisms are used to separate compounds from the extracts, which can be performed in a column chromatography format. These are used to fractionate either by size (e.g., size exclusion chromatography), charge (e.g., ion exchange chromatography), hydrophobicity (e.g., hydrophobic interaction chromatography), polarity (e.g., reversed-phase vs. normal phase chromatography), or other specific binding interactions (e.g., affinity chromatography). These chromatographic stationary phases can be used in a variety of platforms/equipments, such as fast protein liquid chromatography (FPLC), generally used for proteins or nucleic acids, or high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC), used for both proteins and small molecules. In addition to column mode, other SPE formats can be used such as extraction disks and membranes, which are usually composed of glass fibers forming a matrix on which particles of pure or modified silica gel are anchored (Andrade-Eiroa et al., 2016). Supercritical fluid adsorption (SFA) or supercritical fluid chromatography (SFC) are another option, especially for nonpolar volatile compounds. SFA can also be used for polar compounds that are poorly soluble in supercritical CO_2 by using a suitable co-solvent such as ethanol (Dinarvand et al., 2020). Various types of chromatography used for isolation, purification, and characterization of natural products have been reviewed (e.g., (Bucar et al., 2013; Nehete et al., 2013; Saini et al., 2021; Sarker and Nahar, 2012; Yang et

al., 2020)). Alternatively, variants of preparative polyacrylamide gel electrophoresis (PAGE) (e.g., native PAGE, isoelectric focusing, 2D PAGE) can be used to separate mixtures of compounds from extracts. Miniaturized analytical techniques can also be used for sample processing. Pipette tip or in-syringe SPE is a miniaturized version of standard SPE in which the absorbent material is packed in plastic micropipette tips or in the needle of syringes; analytes are extracted by repeated aspiration and desorption of the sample. Solid phase microextraction (SPME) can also be used for *in vivo* analyses, such as fish tissue sampling, due to its low invasiveness. Headspace SPME allows selective extraction of volatile and semi-volatile compounds from samples. Thin film microextraction (TFME) increases the volume of the extraction phase and the surface-to-volume ratio, allowing higher extraction efficiency and rapid analysis (Faraji et al., 2019; Pena-Pereira et al., 2021).

An important step in the isolation process is dereplication (Gaudêncio and Pereira, 2015), which is usually performed using tandem mass spectrometry (MS/MS), which determines the presence of known compounds. The bioactive extracts containing unknown compounds are usually selected for further fractionation. Alternatively, known compounds can be tested for new types of bioactivities using other types of bioassays, a process known as repurposing (Dinarvand et al., 2020; Houssen and Jaspars, 2012; Nothias et al., 2018; Pereira et al., 2020; Pushpakom et al., 2019; Veerapandian et al., 2020).

Information about the properties of the bioactive compound can be derived from the purification process, and separation into specific fractions provides information about their characteristics. The number of purification steps required to purify compounds varies from case to case and usually ranges from two to eight. Finally, the structures of compounds are elucidated using 1D and 2D nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HR-MS), X-ray diffraction (for crystalline compounds), and other techniques to determine the absolute configuration (for non-crystalline compounds) (Gaudêncio et al., 2023). It is important to note that the use of low-resolution tandem mass spectrometers (e.g., triple quadrupole mass spectrometers) may be sufficient for targeted analysis of known compounds, but for untargeted analysis of unknown compounds, the use of a high-resolution mass spectrometer (HR-MS) in tandem mode (e.g., quadrupole time-of-flight, Orbitrap) is essential for accurate measurement of both molecular and fragment ions (Berlinck et al., 2022; Guo et al., 2022).

6.8. Application-oriented development

Given the enormous richness of the marine environment in terms of global biodiversity, almost unlimited resources of bioactive compounds are available for various applications (Atanasov et al., 2021; Newman and Cragg, 2020; Rotter et al., 2021a). Over 38 000 compounds of marine origin are listed in the Dictionary of Marine Natural Products (https://dmnp.chemnetbase.com), the MarinLit database

(http://pubs.rsc.org/marinlit/), and the Comprehensive Marine Natural Products Database CMNPD (https://www.cmnpd.org/) (Lyu et al., 2021). Currently, around 1500 new marine compounds are reported annually (Carroll et al., 2021), a substantial increase from the annual average of 1200 compounds reported nearly a decade ago (Kiuru et al., 2014). However, marine natural product discovery faces several challenges. Despite support from research funding organisations in the EU and worldwide, access to the marine environment and sampling of aquatic organisms remain very challenging, while several technical issues, including supply of active compounds and sustainable production, can hinder the biodiscovery process (Schneider et al., 2023, 2022). Furthermore, extracts derived from marine organisms are very complex, and the potentially bioactive components are usually present at low concentrations or are characterised by high structural novelty/complexity, making their identification and isolation in sufficient quantities for extensive biological testing difficult.

By overcoming the above-mentioned challenges, a limited number of promising bioactive compounds are eventually isolated in quantities large enough to enable bioactivity studies and to support the different stages of natural product development. There are no universal sets of bioassays that should be used for specific research applications, while different types of bioassays are important for different phases of biodiscovery and product development. Much practical information on selecting a bioassay has been discussed in **Section 6.4**, but it is prudent to keep in mind the potential uses and regulatory requirements associated with the various intended applications from early discovery on. To illustrate this point, we consider the development pipeline (**Figure 6.9**) of a general natural source value chain and focus on marine products intended for specific target markets, namely the pharmaceutical industry (medicines), the cosmetics industry, and the food industry (dietary supplements and/or ingredients for food or feed).
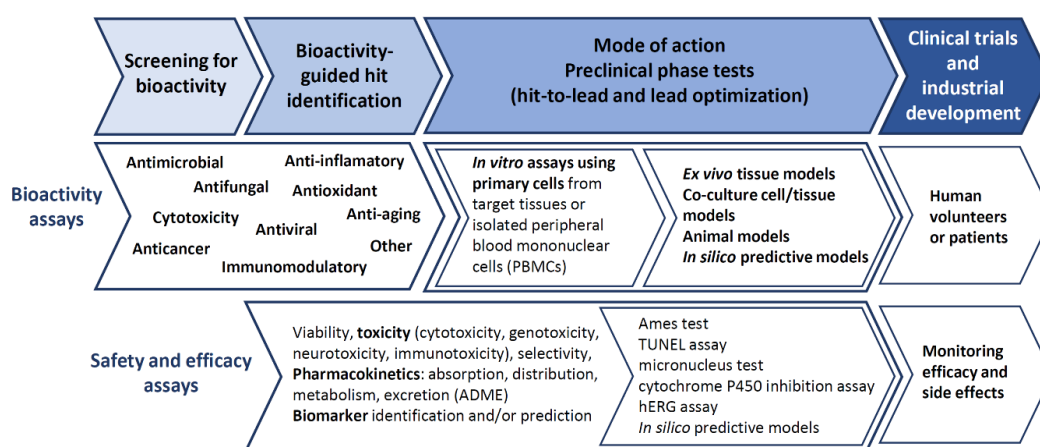
Figure 6.9. Overview of the different stages of drug discovery in the early discovery and preclinical phases of natural product development. Examples are shown of various bioactivity and safety assays that can be used specifically at each stage.

6.8.1. Pharmaceutical drug discovery

The entire process to approval of a new drug can take 12-15 years for the pharmaceutical industry and costs up to $2.8 billion (Wouters et al., 2020). In particular, drug discovery based on natural products has proven to be an extraordinary laborious, costly, and time-consuming process. Nevertheless, this is the most effective approach to new drug development, and the number of natural-product-inspired drugs is much higher than synthetic drugs, as over 69% of modern drugs are based on natural products or their derivatives. Many pharmaceutical companies have turned to combinatorial chemistry for drug structure discovery and optimization; however, only three new chemical drugs have been approved based on this methodology (Jimenez et al., 2020; Newman and Cragg, 2020). To date, 15 approved marine drugs are in clinical use, including 10 anticancer drugs, and 43 marine natural products are in clinical trials (20 in Phase I, 18 in Phase II, and 5 in Phase III). The vast majority of the latter (i.e., 37 of 43) are being tested as anticancer drugs, whereas two are being investigated for viral diseases, one for Alzheimer's disease, one for chronic pain and one for relapsed or refractory systemic amyloidosis (https://www.marinepharmacology.org/approved). The drug development process involves five major steps: (i) discovery and development; (ii) preclinical research; (iii) clinical development; (iv) review by a health authority (e.g., FDA or EMA); and (v) post-marketing surveillance, including numerous phases and stages within each of these steps. Bioassays are primarily used during the first two steps of (i) discovery, including screening and bioactivity-guided purification, and during (ii) preclinical research, which serves as the decision-making basis for the next step of clinical trials (**Figure 6.9**). For pharmaceutical and nutraceutical products, both of which promise health benefits and are subject to the same regulatory requirements, preclinical testing is followed by (iii) the clinical development phase, which includes a sequence of clinical trial phases. Phase I clinical trials focus on testing safety, dose and side effects in a small group of healthy volunteers. Phase II then enrols a medium-sized group of patients with the target disease or condition and treats them for several months to two years, comparing them to a placebo control group or an approved standard drug to obtain efficacy and additional safety data. Phase III studies are larger and of longer duration (1-4 years) and include approximately 300-3000 patients who are treated and compared to a control group. Data collected in phase III provide information on long-term and rare side effects compared to the last two phases. After the drug has been approved (iv) by the regulatory authorities, i.e., the European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) in Europe and the Food and Drug Administration (FDA) in the U.S.A., (iv) post-marketing surveillance (Phase IV) is conducted to obtain additional information on the benefits and risks of using a particular drug.

The screening phase relies on *in silico* and *in vitro* biochemical assays to identify bioactive extracts, fractions, or lead compounds, with high-throughput screening playing a central role. However,

in recent decades, interest from the pharmaceutical industry in conducting HTS programmes, particularly for natural products, has tended to decline (Harvey et al., 2015). This is primarily due to a number of bottlenecks associated with the complexity of biological extracts that can affect the accuracy of targeted molecular screening (e.g., the effects of active compounds can be masked by other components in the crude extract), associated costly efforts to reduce matrix complexity, and the limited success of large HTS campaigns previously conducted by companies. Nonetheless, interest in HTS natural products for drug discovery remains a hot research topic in academia. Laboratory-scale studies have reported the application of HTS techniques to a repertoire of natural products to identify potential therapeutic agents for tumour metastasis (Gallardo et al., 2015), cancer and necroptosis (Li et al., 2016), cell stress and cytotoxicity (Judson et al., 2016), metabolic and age-related disorders (C. Wang et al., 2017), and, more recently, COVID-19 (Chen et al., 2021; Coelho et al., 2020; Gaudêncio et al., 2023). Other studies have investigated natural product-like small molecules for their antimalarial activity (Kato et al., 2016) and their suitability for genome engineering technologies (e.g., inhibition of CRISPR-Cas9 (Maji et al., 2019)).

Over the past decade, High Content Screening (HCS) has made significant technological advances and evolved into a robust cell-based approach that is gaining increasing interest in biological testing and drug discovery. HCS enables automated confocal fluorescence imaging of living cells and is increasingly used to determine whether a natural product or drug candidate elicits a specific bioactivity by monitoring the changes induced in specific cellular pathways (Artusa et al., 2022; Romerio et al., 2023). This is a phenotypic screening approach that considers the final effect on the phenotype of the cells without examining specific molecular targets. By applying a multiparametric HCS approach, the phenotypic function of metabolites from *Jaspis splendens* sponges against Parkinson's disease was recently investigated (Wang et al., 2016). In a similar study, natural products purified from soft corals were screened using an HCS assay to identify potent inhibitors of the ubiquitin-proteasome system (Ling et al., 2018). Currently available HCS platforms can provide rich descriptive quantitative phenotypic data for various cellular markers and parameters (e.g., cell viability, specific protein expression, cell size, etc.), which can be used to detect different types of bioactivities. By considering the entire cellular mechanisms, including compensatory mechanisms, HCS enables the assessment of the biological effect of a molecule as a whole and not just on a specific target. This is particularly valuable for the discovery of bioactivities against complex and multifactorial diseases such as neurodegenerative diseases or cancer.

In addition to experimental efforts, complementary dry-lab approaches (e.g., virtual screening) have emerged under increasing pressure to reduce costs and improve the speed and simplicity of the biodiscovery process (David et al., 2015). These efforts primarily involve the use of structure-assisted

drug design in conjunction with virtual HTS. With respect to natural products, this approach has been applied in a substantial number of studies to accelerate the discovery of antiviral agents against coronaviruses (Jin et al., 2020; Naik et al., 2020), while others have focused on identifying molecular entities with inhibitory activity against typical disease-related enzymes (e.g., cancer, diabetes, and neurodegenerative disorders) (Jhong et al., 2015; Khan et al., 2019; Mohammad et al., 2019).

Natural products that have been evaluated for pharmacological or biological activity and have the potential to be therapeutically useful can be considered drug hits. However, in the early stages of drug development, a hit-to-lead (H2L) process is used that includes mechanism-of-action studies to identify the pharmacological targets of potent hits and a limited optimization of their chemical structure to reduce potential side effects, increase affinity and selectivity, improve efficacy, potency, metabolic stability (half-life) and oral bioavailability. A lead-optimization (LO) process is then performed to synthesize, evaluate, and modify the bioactive compounds using medicinal chemistry approaches to form new chemical entities (NCEs) that improve efficacy and reduce side effects. Lead optimization also involves experimental *in vitro* and *in vivo* testing in a variety of efficacy studies, pharmacokinetic studies, and toxicological assessments, as well as ADMET (absorption, distribution, metabolism, excretion, toxicity) assessments through the use of *in silico* models and animal testing to develop therapeutically effective drugs. For this reason, the preclinical phase is typically more time-consuming, more expensive, and requires less testing capacity than the preceding screening phases, and may require more qualified personnel working according to the principles of good laboratory practice (Andrade et al., 2016; Claeson and Bohlin, 1997; Collins et al., 2020).

Before the bioactive compound (lead structure) enters a new phase of development for a specific application, its toxicity to humans, animals, and the environment must be determined. The conclusions drawn from the safety and toxicity tests are highly dependent on the results of the bioassays used. Bioactivity must be quantified at this stage to determine dose (exposure) and derive potency. Different types of bioassays may be required for these steps, but often only validated versions of the quantitative bioassays already used in the discovery phase are used. The pure compounds (lead compounds) are tested *in vitro* on primary cell lines or *ex vivo* tissue models, or combinations thereof, specifically designed for the application of interest. The lack of adequate human disease models has been described as a major limitation in preclinical drug development (Khanna, 2012). Recently, however, several preclinical human disease models have been developed for several common chronic inflammatory diseases (e.g., osteoarthritis, cardiovascular disease, chronic lung disease, psoriasis, atopic dermatitis) and various cancer types, using two-dimensional (2D) cell culture methods, *ex vivo* and co-culture models and three-dimensional (3D) organoid structures. These disease models serve as immediate *in vivo* testing platforms to evaluate the efficacy and safety of drug candidates prior to entering clinical

phases (Araújo et al., 2020; Ho et al., 2018; Jessica E Neil et al., 2022; Muenzebrock et al., 2022; Veldhuizen et al., 2019). Results from disease models form the basis for designing and planning potential clinical trials or conducting other safety and efficacy testing required by regulatory authorities for a particular application (e.g., pharmaceutical, nutraceutical or cosmetic). It should be emphasized that the safety evaluation of pharmaceutical, food, and cosmetic ingredients is more stringent than that of well-characterised non-food substances, such as industrial chemicals or pesticides (Śliwka et al., 2016). Moreover, cosmetics and dietary supplements are not required to be approved for sale by the FDA or EMA. Nevertheless, the cosmetics industry has recently become interested in incorporating marine bioactive compounds into cosmetic products (e.g., creams and lotions) that have medicinal or drug-like effects. In this context, the term "cosmeceuticals" has been coined to describe the combination of cosmetics and pharmaceuticals, but it does not yet have any legal meaning under current regulations.

The potential toxicity of compounds is determined based on their chemical structure and mechanism of action to characterize concentration-dependent effects, long-term effects, and effects of exposure at low concentrations. Animal testing can provide valuable information on toxicity and pharmacological activity, including pharmacokinetics (ADME) and pharmacodynamics (interaction with the organism), but interspecies differences in drug toxicity and efficacy can become an important issue. Despite the recognized limitations and benefits, there are ongoing efforts to reduce the use of animals for testing. Indeed, *in vivo* testing in animals and humans is subject to strict ethical constraints, is costly, and therefore is generally performed only in the final stages of development (Ferdowsian and Beck, 2011). Current regulatory approaches to toxicity testing and evaluation continue to rely primarily on a checklist of *in vivo* tests that follow standardized test guidelines or protocols. The Interagency Coordinating Committee on the Validation of Alternative Methods ICCVAM, along with other organizations, is promoting the development of non-animal alternatives to current *in vivo* acute systemic toxicity tests (Clippinger et al., 2018; Hamm et al., 2017; Kleinstreuer et al., 2018). There is a trend toward increased use of new technologies such as high-throughput screening (HTS), tissue chips, and computational modelling to better predict human, animal, and environmental responses to a wide range of substances relevant to new product development. The International Cooperation on Alternative Test Methods (ICATM) partnership was created to establish international cooperation in validation studies and the development of harmonized recommendations to ensure global acceptance of alternative methods and strategies (https://ntp.niehs.nih.gov/whatwestudy/niceatm/iccvam/international-partnerships/icatm/index.html).

Significant efforts are being made to develop *in vitro* tests that cover endpoints and target organs/tissues that are most relevant to humans (Bal-Price et al., 2015). However, in some cases, animal models may still be needed to address specific developmental toxicity questions (Clippinger et al., 2018;

Leist et al., 2013; Wambaugh et al., 2018). In this context, zebrafish-based bioassays offer an interesting combination of an *in vivo* model and the possibility of high-throughput screening with low compound consumption. For example, zebrafish embryos have been established as an *in vivo* model for the analysis of angiogenesis and vascular development and can be further developed for other specific high-throughput screening (Crawford et al., 2011). Another alternative to these assays is the use of the whole-animal *Caenorhabditis elegans* (e.g., (Durai et al., 2013; Palacios-Gorba et al., 2020)). In addition, phenotype-based bioassays are also used to retarget known compounds to unknown and novel targets (Pushpakom et al., 2019).

In recent years, computer-assisted methods have been used to predict or model the ADMET properties of lead compounds, enabling drug design and identification of potentially problematic structures in the early stages of drug discovery to avoid late-stage failures (Ortega et al., 2012). Computer-aided drug design (CADD) is increasingly being used in drug discovery. Existing tools for predicting and visualising ADME/toxicity data include: i) predictors of ADME parameters, ii) predictors of metabolic fate, iii) predictors of metabolic stability, iv) predictors of cytochrome P450 substrates, and v) software for physiology-based pharmacokinetic (PBPK) modelling (Romano and Tatonetti, 2019; Wishart, 2009, 2007). These enable pharmacophore modelling (PM), molecular docking (MD), inverse docking, chemical similarity search (CS), development of quantitative structure-activity relationships (QSAR) (Pereira et al., 2015, 2014), virtual screening (VS) (Cruz et al., 2018; Dias et al., 2019; Gaudêncio and Pereira, 2020) and molecular dynamics simulations (MDS), which effectively predict the therapeutic outcome of lead structures and drug candidates and accelerate the discovery process. The importance of predictive models for clinical pharmacology is recognized by regulatory agencies, and this approach is being used for various applications. These models combine different types of data and parameters to estimate pharmacological activities and are commonly referred to as physiologically based pharmacokinetic (PBPK) models. By linking the properties of individual lead molecules to physiological properties, PBPK models also provide a rational approach to predicting drug similarity (Benjamin et al., 2010; Deepika and Kumar, 2023; Karnati et al., 2023; Mbah et al., 2012; Strömstedt et al., 2014).

By exploring structural and other data about the target (enzyme/receptor) and ligands, CADD approaches have identified compounds that can treat disease. Examples of approved drugs that have been supported by CADD include dorzolamide, saquinavir, ritonavir, indinavir, captopril, and tirofiban (Dar et al., 2018). Given the success of this approach, the development of "go/no-go" selection criteria and optimization strategies for drug candidate development should include the use of advanced CADD for drug metabolism and pharmacokinetics (DMPK) profiling in the development of safe and effective drugs.

[190]

6.8.2. Cosmetics

Cosmetic products are intended to be applied to the external parts of the human body, including the teeth and oral mucous membranes, to cleanse, protect, change their appearance, improve their odour or keep them in good condition. Their use is regulated in the EU by the EU Cosmetics Directive (Directive 1223/2009) and in the US by the Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Fair Packaging and Labelling Act (FPLA). In the EU, all cosmetic products are registered with the EU Cosmetic Products Notification Portal (CPNP) and must undergo a safety assessment, have a product information file, and report serious undesirable effects. Manufacturing must be in accordance with good manufacturing practice (GMP), must not involve animal testing, and labelling is subject to strict rules (Regulation EC 1233/2009). In the U.S., registration under the FDA's Voluntary Cosmetic Registration Program (VCRP) is not required but it is encouraged, the use of animals for testing is not prohibited, and truthful labelling is also regulated. It is also important to distinguish between pharmaceuticals and cosmetics, as pharmaceuticals require FDA approval and include products that claim, for example, hair restoration, pain relief, anti-ageing effects, relief of eczema, dandruff or acne, sun protection, etc. Therefore, the path of regulation may vary depending on the product's intended use. Similarly, if a product corrects or alters physiological functions by exerting a pharmacological, immunological or metabolic effect, it should be classified as a medicinal product in the EU (Regulation EC 1233/2009, FDA, 2023).

The ingredients of cosmetic products must not be harmful or toxic and must comply with the lists of prohibited and restricted substances. Only approved colorants, preservatives, and UV filters may be included in cosmetic products. The International Nomenclature Committee (INC) manages internationally recognized systematic names for cosmetic ingredients such as plant extracts, oils and chemicals with the abbreviation INCI (International Nomenclature Cosmetic Ingredient), which are used in the European Commission's database for information on cosmetic substances and ingredients CosIng (https://ec.europa.eu/growth/tools-databases/cosing/index.cfm, accessed May 6, 2023), but inclusion in the database does not imply approval for use. INCI names are primarily used for cosmetic product labelling to avoid confusion, as an ingredient may have different chemical names (e.g., common names, CAS or IUPAC names) in different countries.

Typical safety assessment procedures for cosmetic ingredients include the following elements: (i) hazard identification to identify the intrinsic toxicological properties of the substance using New Approach Methodology; (ii) exposure assessment calculated based on the declared functions and uses of a substance as a cosmetic ingredient, the amount present in each cosmetic product category, and the frequency of its use; (iii) dose-response assessment; and (iv) risk characterization, which usually focuses on systemic effects. The ban on animal testing and the requirement to use only validated replacement

[191]

alternative methods in Europe ensure that the New Approach Methodology (NAM) is followed, which includes *in vitro*, *ex vivo*, *in chemico*, and *in silico* approaches, read-across, and combinations thereof, to support regulatory decision-making by providing information for hazard and risk assessment (Scientific Committee on Consumer Safety - SCCS, 2021).

Marine resources offer an interesting repertoire of bioactive ingredients with cosmetic potential. Extracts from seaweed, algae, soft corals, or other marine life are rich in proteins, amino acids, exopolysaccharides, carbohydrates, vitamins (A, B and C), fatty acids, and trace elements that contribute to hydration, firming, slimming, shine, and protection of human skin, as well as bioactive compounds with, for example, antioxidant and anti-inflammatory properties that protect the skin from ageing and photooxidation (Guillerme et al., 2017). Therefore, beauty products with marine ingredients are becoming increasingly widespread.

6.8.3. Food and feed supplements

Food supplements are foods whose purpose is to supplement the normal diet and consist of concentrated sources of nutrients (e.g., vitamins, amino acids, and minerals) or other substances with nutritional or physiological effects. Their use is regulated by the establishment of substance lists that are positively evaluated by a food safety authority, such as the European Food Safety Authority (EFSA) or United States Food and Drug Administration (FDA) for safety of ingestion and bioavailability (i.e., the effectiveness with which the substance is released into the body). These agencies also provide guidance on the type and extent of information that should be submitted to demonstrate bioavailability and toxicological data. Special regulations apply to foods for infants and young children and to foods for special medical purposes (Younes et al., 2021) (https://www.fda.gov/food/guidance-regulation-food-and-dietary-supplements, accessed May 6, 2023).

Safety testing evaluates safety based on biological, physical, and chemical parameters. Physical tests check for the presence of foreign objects. Biological safety tests ensure the absence of pathogens and toxins, and chemical tests detect trace elements or contaminants such as food additives, flavourings, contaminants such as heavy metals, nitrates, disinfectants, pesticides, dioxins, residues of veterinary drugs including antibiotics, and components of food contact materials (EU Food safety 2022, https://ec.europa.eu/food/safety_en, accessed May 6, 2023).

There is a growing interest in functional food ingredients and dietary supplements for which the marine environment is an important resource. Numerous compounds such as enzymes, proteins, peptides, polysaccharides, polyunsaturated ω-3 fatty acids (PUFA), phenols, pigments, and other secondary metabolites have already found use in the food industry (Boziaris, 2014; Šimat et al., 2020). In addition to routine identification of known toxins or contaminants using analytical chemistry

[192]

methods, bioassays for detection of potentially unknown or unexpected toxic components are important for food and feed safety. Apart from animal testing, bioassays are the only way to identify novel risks in food or feed ingredients, especially when new and alternative resources are introduced. This will become especially important with the advent of the circular economy and green waste plans, which will increase the input of waste streams into the food chain (Gerssen et al., 2019).

6.9. Conclusions

Many new and repurposed biologically active natural products from microorganisms and macroorganisms from the marine environment have been detected and characterized using *in vivo*, *in vitro*, and *in silico* bioassays. The choice of bioassays used in biodiscovery is critical to the successful path from extract to marketed product. Therefore, it is important to realise that each extract contains many bioactivities and that when pursuing a bioactive compound using a series of bioassays to isolate and purify the targeted bioactive compound, the other components of the extract should not be discarded as inactive. Additional valuable bioactivities may be revealed by other bioassays. Conversely, a bioactive compound targeted for a particular application can be reassessed for other types of bioactivities as part of the repurposing process. Only when all these aspects are taken into account is it possible to optimize the potential and make the best use of the various natural resources and, in particular, the marine environment, which is now being increasingly explored.

A careful inspection of the literature reveals many questions regarding the performance of bioassays used for screening and identification of bioactivity. Some of these issues relate to possible artefacts in assay results, variations in activity within different methods, differences in solubility, synergy of compounds in the tested extract, proper use of controls, storage conditions of extracts, etc. For many bioassays routinely used in research laboratories, there are no standardized assay procedures, so it is often very difficult to compare results reported by different laboratories. To improve the potential for standardization of bioassays, fundamental properties such as robustness, reproducibility, relevance, sensitivity, cost-effectiveness, automation, accuracy, and selectivity should be considered in the development and selection of bioassays to be used. A practical aspect is the use of validated protocols, appropriate controls, and biologically relevant concentrations in bioassays. In this way, it can be assessed at an early stage of biodiscovery whether the selected bioactivity has realistic potential, for example, for pharmacological or cosmetic applications, or whether it is merely an interesting but descriptive discovery.

It is important to note that computational approaches should be widely incorporated into biodiscovery screenings for two reasons: (i) these approaches are data-driven, so their inclusion in

screening protocols will provide large amounts of data that can be examined for valuable patterns for further discovery; and (ii) large amounts of data are already available for analysis, so systematic analysis of data should become routine, including genome sequences, gene expression, chemical structures analytical data, genotype or proteome data, human microbiome, or electronic health records. These analyses, performed using computational tools, can save time through dereplication, prediction of new targets for already known compounds, and information on modes of action. Understanding the molecular mode of action of bioactive compounds is particularly important because this knowledge helps in the development of new ways to elicit the same effect when the original bioactive compound proves toxic or immunogenic, cannot be synthesised, and/or is not available in sufficient quantity or is lost from natural resources.

Finally, scientific research must be supported by innovation. The search for products for human and environmental health and well-being, including the development of new bioassays, must consider the principles of ethics, responsible research and innovation (RRI) (Schneider et al., 2022), good laboratory practices, and respect for natural ecosystems and habitats.

Chapter 7. General conclusions and future work

In this dissertation, the two principal aspects required for integrating Greek marine sponges in fish farms were investigated; (a) their ability to minimize the aquaculture-related environmental impact through remediation applications and (b) the valorization potential of their cultivated biomass for delivering added-value products. The overall conclusions drawn by the present research are discussed below;

- All four candidate sponges were capable of mitigating biological and chemical pollution typically experienced in intensive aquaculture systems, over a series of controlled *in vitro* experiments.

- Uptake of particulate and dissolved organic pollutants by sponges can be fairly translated into exponential mathematical expressions, by following first-order kinetics.

- All four examined species were capable of maintaining their filtering performance for at least four or five consecutive days of a 7-h exposure to microalgae.

- Different marine sponge species exhibit distinct retention properties when feeding on microalgae with particular morphological and motility traits. This is of paramount importance for aquaculture applications, since the best-performing bioremediators could be selected and applied in IMTA systems (or other impacted environments with high microalgae loadings), by taking into account the characteristics of the indigenous phytoplankton communities.

- Most of the studied species could efficiently respond to diverse nutrient inputs, from oligotrophic to highly eutrophic environments, by retaining their optimal filtering performance.

- Illumination did not exert a pronounced effect on the bioremediation capacity of most candidate sponges, implying the adaptability of even sciaphilic species to light exposure conditions, which are expected in an open-sea integrated aquaculture.

- The species *A. oroides* exhibited the highest cleanup capacity across an array of microalgal cells, with an overall feeding efficiency of 70 ± 21%, followed by the values of *S. foetidus* sponges (44 ± 19%).

- In addition, all sponge species were able to assimilate dissolved organic substances encountered in aquaculture effluents, from individual chemical pollutants to complex organic mixtures.

- The speed of pollutant uptake was determined by its overall lipophilic character and strong positive correlations were found between sponges' cleanup performance and pollutant hydrophobicity.

- DOM feeding by sponges is facilitated by active water pumping, rather than passive adsorption of pollutants onto the sponge exopinacoderm.

- Uptaken organic pollutants can hardly be released back to the surrounding environment via desorption or sponge excretory mechanisms after a sufficient time period, highlighting the non-reversibility of the DOM feeding process.

- In view of the overall feeding efficiency, the species *A. oroides* exhibited the greatest filtering performance across an array of dissolved organic substances, with the maximum rates reported for the highly lipophilic pollutants.

- In addition to effective cleanup technology, marine sponge farming can serve as a promising source of bioactive compounds belonging to classes of alkaloids, benzenoids, indoles, lipids and polyketides.

- Sponge mariculture can deliver individuals of similar chemical composition and bioactivity to their wild counterparts. The majority of targeted metabolites showed modest differences in their content and on a sponge mass-normalized basis, and in some cases, biomolecules production was even triggered through farming techniques.

- Metabolomic analysis of crude sponge extracts revealed species-specific chemical patterns, with *A. oroides* and *S. foetidus* dominated by alkaloids and lipids, respectively.

- The most potent biofilters offered crude extracts of varying, but marginal antimicrobial activity against Gram-positive human pathogens. This was partially true for the farmed *A. oroides* explants with respect to Gram-negative bacteria.

- Cultivations of *S. foetidus* sponges provided raw extracts of consistent, but marginal anticancer activities against the human colorectal carcinoma cell line HCT-116, highlighting the potential presence of cytotoxic compounds in the respective sponges.

- A spectrum of supplementary biological activities can be further explored in sponge crude extracts, by employing the guide-review presented in Chapter 6, for future drug development applications.

Combining all the results derived for a variety of sponges during the course of this dissertation, we propose the species *A. oroides* and *S. foetidus* as suitable candidates for future large-scale farming and development of a "sponge-driven bioremediation/bioproduction" concept in fish aquacultures. It is remained, however, to augment our results with supplementary research, which can be summarized into the following points:

- Examination of the *in vitro* sponge bioremediation capacity for a wider range of biological pollutants, such as viruses, bacteria, protists, parasites and other pico- and nanoplanktonic organisms, potentially serving as sponge diet components.

[197]

- Implementation of cleanup experiments involving dissolved organic pollutants within a broader spectrum of hydrophobicity, so that the correlation between substrate lipophilicity and sponge's filtering performance to be better understood.

- *In situ* assessment of sponges bioremediation capacity in proximity to fish farms, by analyzing seawater samples collected from the exhalant pores of farmed sponges, as compared to the surrounding aquaculture environment to estimate clearance rates at an operational scale.

- Comparative chemical profiling and evaluation of the antimicrobial/anticancer activity of the extracts derived from wild versus farmed *Axinella cannabina* and *Chondrosia reniformis* sponges.

- Extensive screening of crude sponge extracts using bioassay-guided protocols to determine their antibiofilm, cytotoxic, antiviral, antioxidant, and anti-ageing potential.

- Fractionation of crude sponge extracts to isolate and identify/characterize the bioactive compounds of pharmaceutical and cosmeceutical interest.

In a nutshell, the research presented in the current thesis offers preliminary estimates about the actual benefits that sponge cultivation could bring to; (a) fish farming enterprises, by improving productivity, quality and environmental sustainability in local aquaculture; (b) drugs/cosmetics companies, by unraveling a plethora of intriguing natural products and bioactivities from Cretan sponges and; (c) the general public, by delivering "cleaner" seas to the people. In addition, such an innovative, eco-friendly and cost-effective cleanup technology may have a much broader applicability for other types of aquatic pollution, including pollution inside ports or close to discharge pipes from wastewater treatment plants.

About the author

Despoina Varamogianni-Mamatsi received her Diploma and her Integrated Master's Degree in Chemical Engineering with a specialty in Biotechnology and Food Engineering from National Technical University of Athens (NTUA) in 2019. In 2020, she began her doctoral studies at the School of Chemical and Environmental Engineering (Technical University of Crete, Greece) in collaboration with the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of Hellenic Centre for Marine Research (Heraklion, Crete) under the framework of the multidisciplinary research project "*SPINAQUA: Sponges in integrated aquaculture systems: Towards the delivery of better seawater quality and marine products of high added-value*". Her doctorate research was supported by national funds from the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT). She has extensive experience in laboratory experimentation, gained by working for the nationally funded projects SPINAQUA, LagoMeal and "NoWasteBioTech: Novel Conversion Technologies of Waste Biomass to Food additives and Fine Chemicals", as well as research projects funded by Portuguese grants in FCT—Fundação para a Ciência e a Tecnologia, such as UIDP/04378/2020, LA/P/0140/2020, UIDB/50006/2020 and UIDP/50006/2020. In 2022, she was awarded with a Short-Term Scientific Mission (STSM) grant by the European Cooperation in Science and Technology (COST) to implement part of her doctoral research in the faculties of NOVA School of Science and Technology in Lisbon, Portugal. She has authored 3 publications in peer reviewed journals, and co-authored 2, resulting in 54 citations (ResearchGate). She has presented in numerous international and national conferences and attended in international events, such as hackathons (e.g., the first hackathon for Marine Biotechnology; 2021), workshops (e.g., "Circular bioeconomy and sustainable development"; 2022) and training courses (e.g., "Life-cycle assessment"; 2023). Since 2021, she is an active member of the European COST Action "Ocean4Biotech" (CA18238). Her research interests involve the valorization of marine sponges for bioremediation and bioproduction purposes in integrated aquaculture systems.

Publications

PhD Journal Publications

Sabotič, J., Bayram, E., Ezra, D., Gaudêncio, S.P., Haznedaroğlu, B.Z., Janež, N., Ktari, L., Luganini, A., Mandalakis, M., Safarik, I., Simes, D., Strode, E., Toruńska-Sitarz, A., **Varamogianni – Mamatsi, D.**, Varese, G.C., Vasquez, M.I., 2024. A guide to the use of bioassays in exploration of natural resources. *Biotechnology Advances*, 108307. https://doi.org/10.1016/j.biotechadv.2024.108307

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Other Journal Publications

Karnaouri, A., Chalima, A., Kalogiannis, K., **Varamogianni-Mamatsi, D.**, Lappas, A., Topakas, E., 2020. Utilization of lignocellulosic biomass towards the production of omega-3 fatty acids by the heterotrophic marine microalga *Crypthecodinium cohnii*. *Bioresource Technology* 303, 122899. https://doi.org/10.1016/j.biortech.2020.122899

International Conference Presentations

Unraveling insights through targeted tandem mass spectrometry with a triple quadrupole mass spectrometer, 13º Encontro Nacional de Cromatografia (13ENC), Lisbon, 17-19 December 2023 (poster presentation)

Sponges: Potential biofilters for the remediation of impacted aquaculture settings?, 8th European Bioremediation Conference, Chania, 12-17 June 2022 (oral presentation)

Sponges: Potential biofilters for the remediation of impacted aquaculture settings?, MARIKAT Workshop "Circular BioEconomy and Sustainable Development", Hellenic Centre for Marine Research, Heraklion, 9-11 June 2022 (oral presentation)

Variolization of marine organisms for cosmetics, Hybrid meeting of the European COST Action (CA18238) "Ocean4Biotech", Institute of Marine Biology of Kotor, Montenegro, 22 October 2021 (oral presentation)

National Conference Presentations

Μελέτη βιοαποκατάστασης/βιοπαραγωγής με χρήση θαλάσσιων σπόγγων στην ιχθυοκαλλιέργεια, 4th Conference for Doctoral Students, TUC, Chania, 6-7 April 2023 (oral presentation)

Σπόγγοι: Δυνητικά βιοφίλτρα για την αποκατάσταση ρυπασμένων ιχθυοκαλλιεργητικών μονάδων; 3rd Conference for Doctoral Students, TUC, Chania 1 April 2022 (oral presentation)

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Appendix I: Supplementary figures
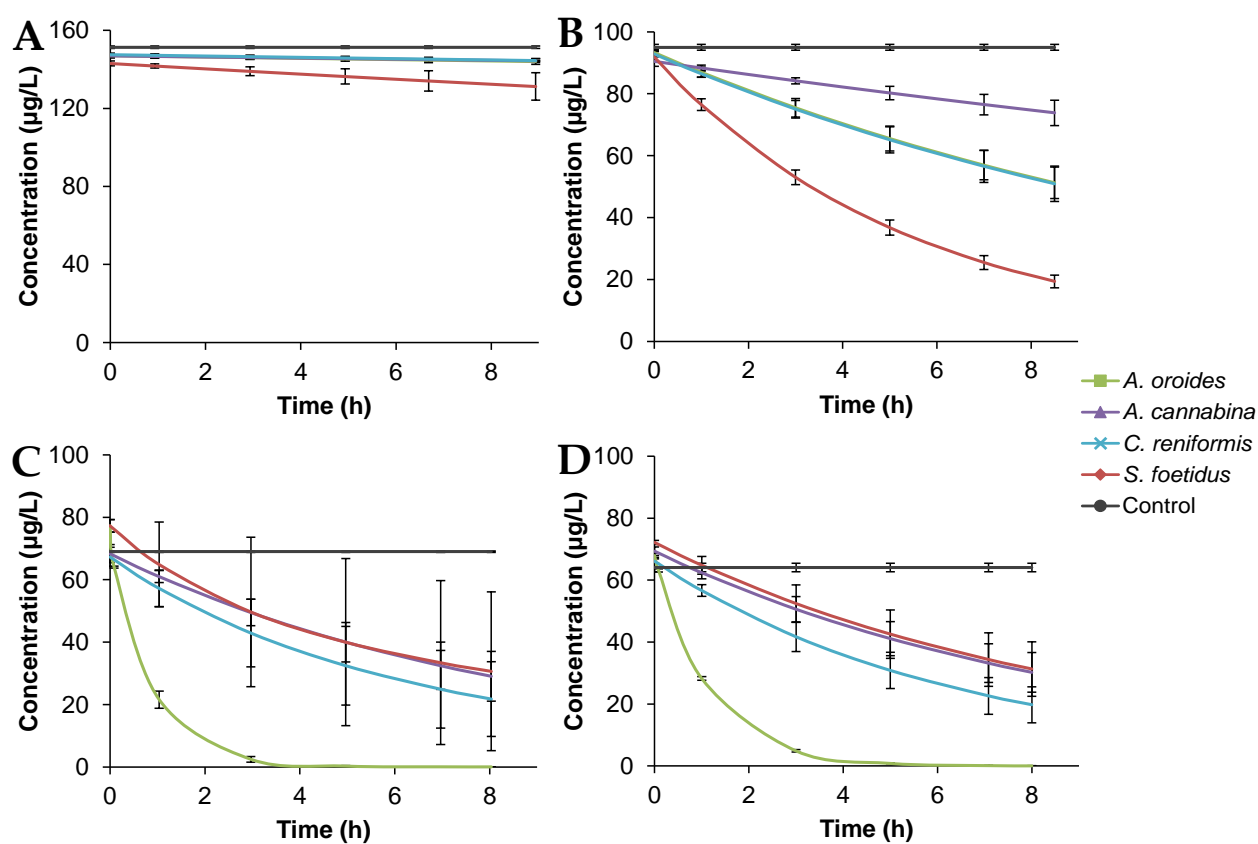
Figure A1. Concentration-time profiles (mean values ± SD) simulating the decrease of (A) OTC, (B) Irgarol 1051, (C) 2,6-DMN and (D) phenanthrene in the treatment (i.e., under the presence of the species *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus*) and control experiments (i.e., without sponges) solely driven by the filtering activity of sponges over the course of 8 h.
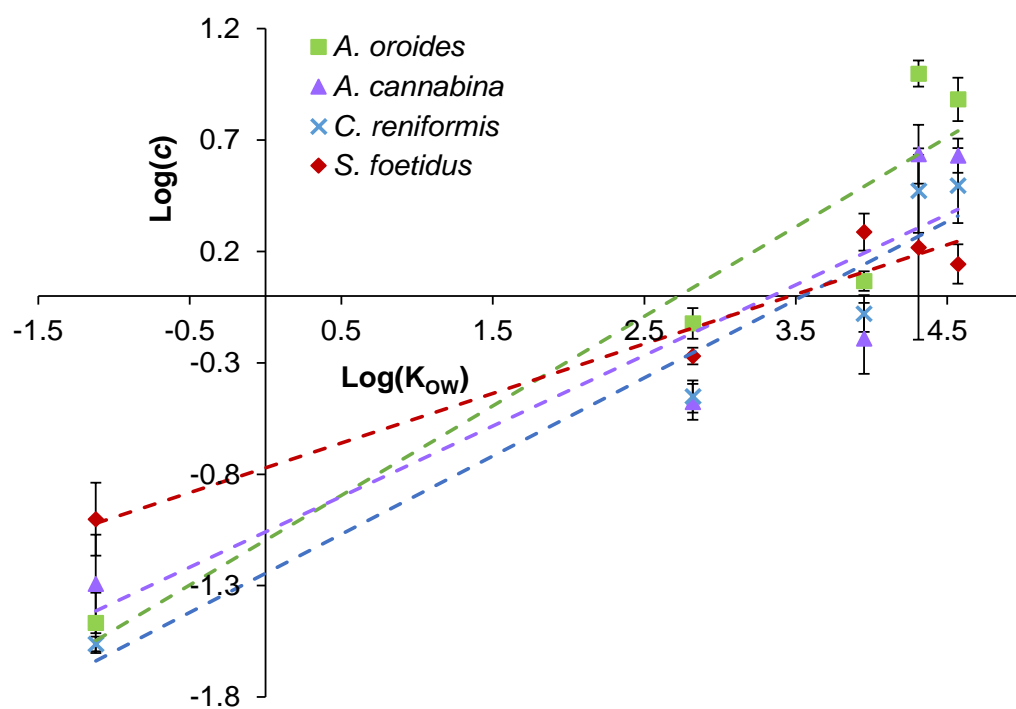
Figure A2. Correlation between pollutant hydrophobicity and sponge's clearance rate.
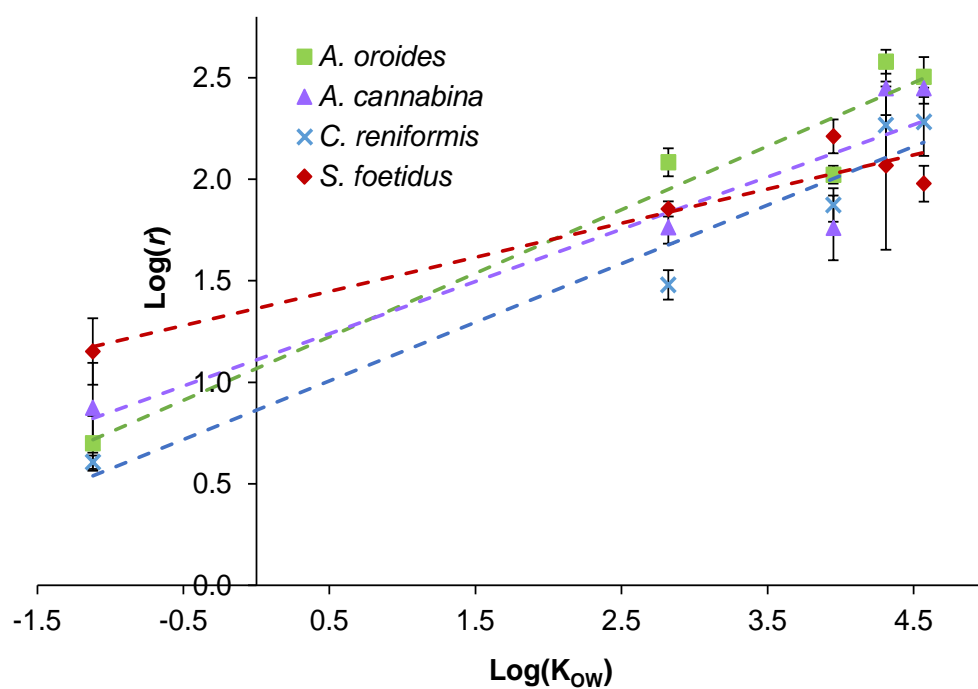
Figure A3. Correlation between pollutant hydrophobicity and sponge's retention rate.
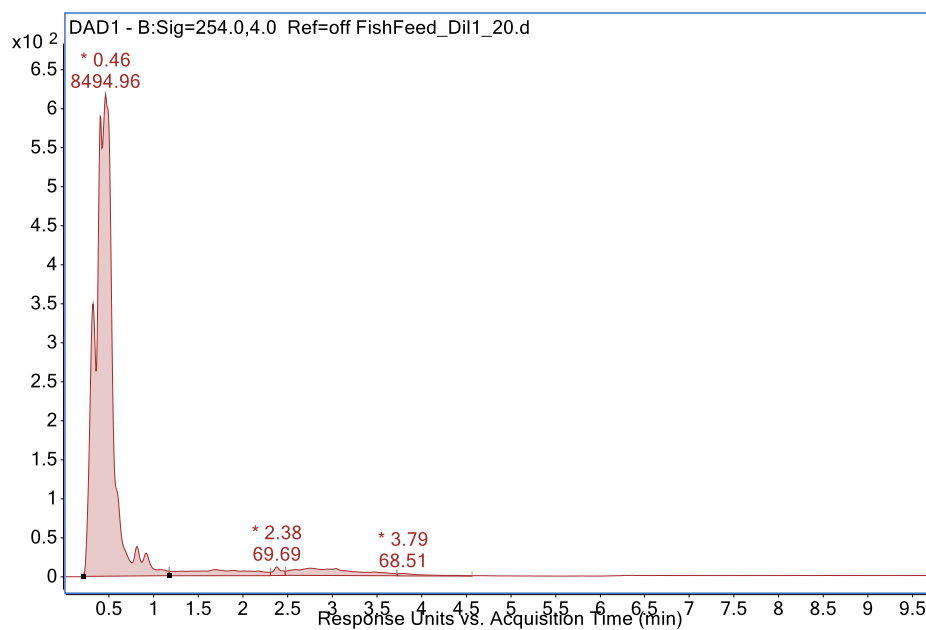
Figure A4. Integrated chromatographic peaks corresponding to the organic substances present in fish feed filtrate.
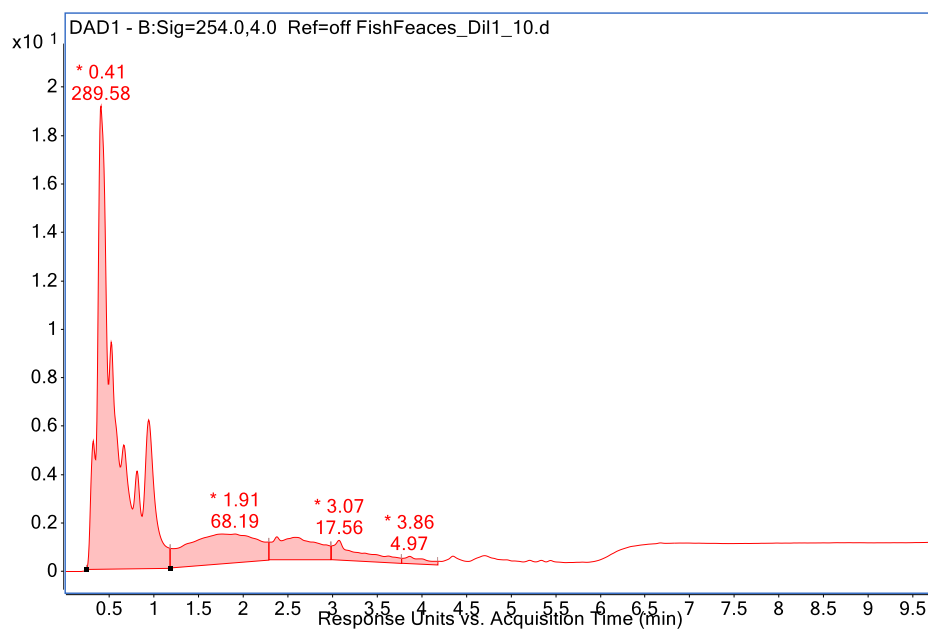
Figure A5. Integrated chromatographic peaks corresponding to the organic substances present in fish faeces filtrate.

[250]
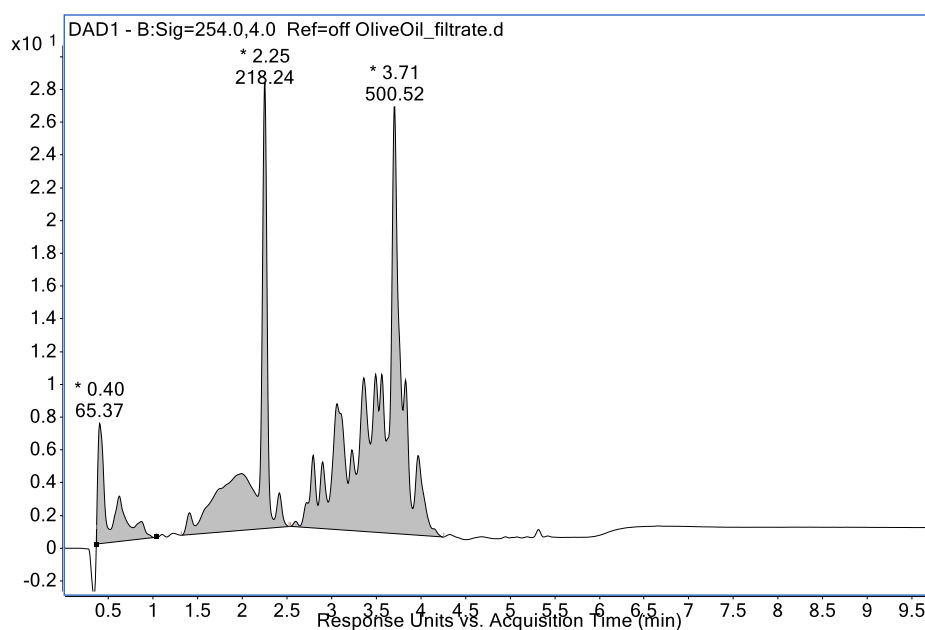
Figure A6. Integrated chromatographic peaks corresponding to the organic substances present in olive oil filtrate.
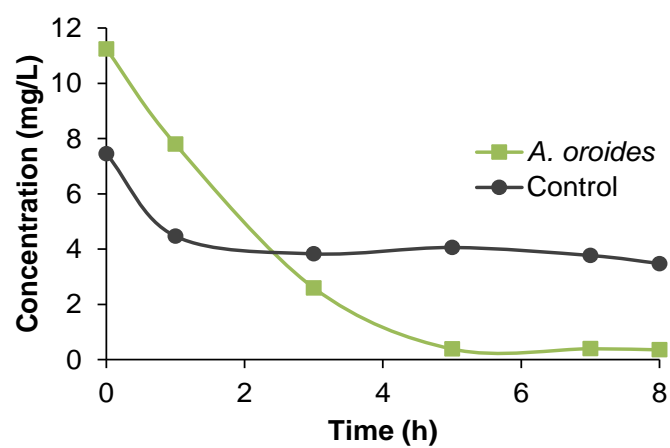
Figure A7. Concentration-time profiles of olive oil in the treatment (i.e., under the presence of the species *A. oroides*) and control experiments (i.e., without sponges) over the course of 8 h.
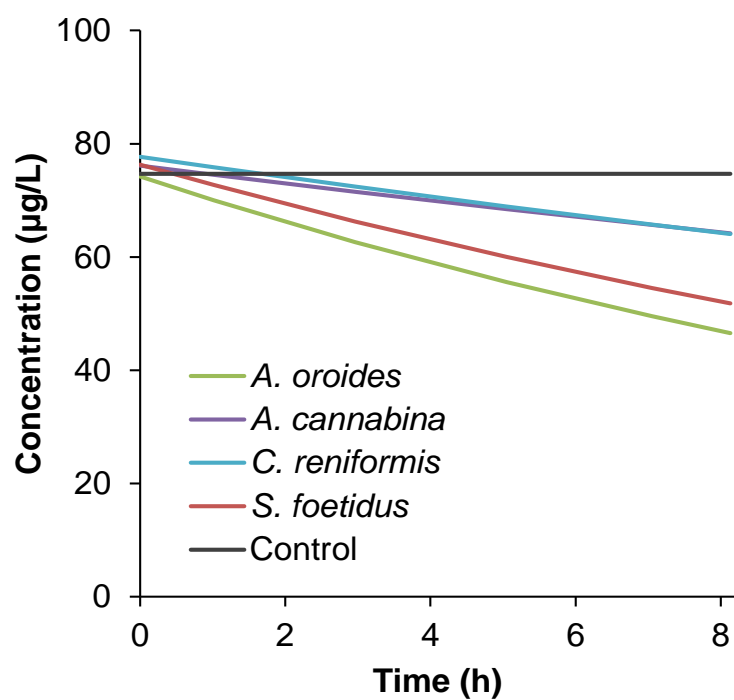
Figure A8. Concentration-time profiles simulating the decrease of phenanthrene in the treatment (i.e., under the presence of dead *A. oroides*, *A. cannabina*, *C. reniformis* and *S. foetidus* fragments) and control experiments (i.e., without sponges) solely driven by passive adsorption onto sponges biosurface over the course of 8 h.

Appendix II: Supplementary tables

Table A1. List of articles reporting sponge mariculture trials.

| Reference	Species	Country	Purpose
(Wilkinson and Vacelet, 1979)	*Aplysina aerophoba* *Aplysina cavernicola* *Chondrilla nucula* *Chondrosia reniformis* *Petrosia ficiformis*	France	Growth investigation under different *in situ conditions* (e.g., light, current)
(Verdenal and Vacelet, 1990)	*Spongia agaricina* *Spongia nitens* *Spongia officinales*	France	Bath sponges supply
(Kaandorp and de Kluijver, 1992)	*Haliclona oculata*	The Netherlands	Biomonitoring
(Battershill and Page, 1996)	*Lissodendoryx* sp.	New Zealand	Bioactive compounds supply
(Duckworth et al., 1997)	*Psammocinia hawere* *Raspailia topsenti* *Raspailia* (*Clathriodendron*) *arbuscula*	New Zealand	Growth investigation for bioactive compounds supply
(Müller et al., 1999)	*Geodia cydonium*	Croatia	Bioactive compounds supply
(Pronzato et al., 1999)	*Agelas oroides* *Axinella damicornis* *Cacospongia mollior* *Chondrosia reniformis* *Hippospongia communis* *Ircinia variabilis* *Petrosia ficiformis* *Spongia agaricina* *Spongia officinales*	Greece and Italy	Evaluation of sponge's adaptability (e.g., survival rates) near fish farm environments for bath sponges supply
(A. R. Duckworth and Battershill, 2003)	*Latrunculia* sp. nov *Polymastia croceus* *Raspailia agminata*	New Zealand	Investigation of different experimental cultivation setups for bioactive compounds supply
(A. Duckworth and Battershill, 2003)	*Latrunculia wellingtonensis* *Polymastia croceus*	New Zealand	Bioactive compounds supply
(Thoms et al., 2003)	*Aplysina cavernicola*	Italy	Monitoring of microbial diversity and natural product profiles after sponge transplantation
(van Treeck et al., 2003)	*Axinella damicornis* *Axinella verrucose* *Chondrosia reniformis* *Ircinia variabilis*	France	Growth monitoring for bioactive compounds supply
(Corriero et al., 2004)	*Spongia officinalis* var. *adriatica*	Italy	Bath sponges supply
(Duckworth et al., 2004)	*Latrunculia wellingtonensis* *Polymastia croceus*	New Zealand	Growth investigation for bioactive compounds supply
(Kelly et al., 2004)	*Spongia* (*Heterofibria*) *manipulatus*	New Zealand	Bath sponge supply
(Hadas et al., 2005)	*Negombata magnifica*	Israel	Targeted bioactive compounds supply
(Voogd, 2007)	*Aaptos suberitoides* *Amphimedon paraviridis* *Callyspongia biru* *Hyrtios reticulatus* *Ircinia ramosa* *Pseudoceratina* cf. *verrucosa*	Indonesia	Growth investigation for bioactive compounds supply

(Duckworth and Wolff, 2007)	*Coscinoderma* sp.	Australia	Bath sponges supply
(Duckworth et al., 2007)	*Coscinoderma* sp. *Rhopaloeides odorabile*	Australia	Bath sponges supply
(Johnston and Clark, 2007)	*Tedania (Tedania) anhelans*	Australia	Restoration
(Louden et al., 2007)	*Coscinoderma* sp. *Rhopaloeides odorabile*	Australia	Bath and commercial sponges supply
(Stabili et al., 2008)	*Spongia officinalis* var. *adriatica.*	Italy	Bioremediation
(Lipton and Shine, 2009)	*Callyspongia (Chladochalina) diffusa* *Callyspongia (Chladochalina) subarmigera* *Clathria (Clathria) gorgonoides*	India	Bioactive compounds supply
(Baldacconi et al., 2010)	*Spongia officinalis* Linnaeus, 1759	Italy	Bath sponges supply
(Carballo et al., 2010)	*Mycale (Carmia) cecilia*	Mexico	Targeted bioactive compounds supply
(De Caralt et al., 2010)	*Dysidea avara*	Spain	Bioactive compounds supply
(Osinga et al., 2010)	*Chondrosia reniformis* *Dysidea avara*	Turkey	Bioremediation
(Bergman et al., 2011)	*Diacarnus erythraenus*	Israel	Bioactive compounds supply
(Çelik et al., 2011)	*Spongia officinalis*	Turkey	Growth investigation for bath sponges supply
(Page et al., 2011)	*Mycale hentscheli*	New Zealand	Bioactive compounds supply
(Webster et al., 2011)	*Rhopaloeides odorabile*	Australia	Biomaterials supply
(Oronti et al., 2012)	*Hyatella pertusa* *Spongia (Spongia) tubulifera*	The Bahamas, Caribbean	Growth investigation for bioactive compounds supply
(Schiefenhövel and Kunzmann, 2012)	*Neopetrosia* sp. *Stylissa massa*	Indonesia	Investigation of different experimental setups for sponge mariculture
(Biggs, 2013)	*Aplysina cauliformis* *Aplysina* sp.	Curaçao Island, Caribbean	Restoration
(C. Ruiz et al., 2013)	*Discoderma dissolute*	Colombia	Targeted bioactive compounds supply
(Ledda et al., 2014)	*Ircinia variabilis* *Agelas oroides*	Italy	Bioremediation
(Sankar et al., 2016)	*Liosina paradoxa* *Stylissa massa*	Andaman Islands, India	Growth investigation
(Meyer et al., 2016)	*Ecionemia alata*	New Zealand	Impact of cultivation on sponge microbial diversity
(Ávila and Briceño-Vera, 2018)	*Halichondria (Halichondria) melanadocia*	Mexico	Monitoring of microbial diversity after sponge transplantation
(Padiglia et al., 2018)	*Crambe crambe*	Italy	Growth investigation for biomass supply
(Gökalp et al., 2019)	*Chondrosia reniformis*	Turkey	Investigation of different experimental cultivation setups for collagen production
(Santiago et al., 2019)	*Xestospongia* sp.	Philippines	Growth investigation for bioactive compounds supply

(Giangrande et al., 2020)	*Sarcotragus spinosulus*	Italy	IMTA assessment
(Gökalp et al., 2022)	*Chondrosia reniformis*	Turkey	Collagen production in integrated culture settings
(Charisiadou et al., 2022)	*Agelas mauritiana* var. *oxeata* *Callyspongiidae* sponges	Tanzania	Commercial production of bath sponges

Table A2. Mean wet weight of the used sponge fragments, derived from the measurements prior and after the clean-up experiments. These are presented along with standard deviation and relative standard deviation (%RSD).

	A. oroides					*A. cannabina*				
Fragment No	#1	#2	#3	#4	#5	#1	#2	#3	#4	#5
Average wet weight (g)	75.2	70.6	74.7	67.7	60.9	43.7	57.8	50.2	54.7	60.3
Standard deviation (g)	3.5	1.3	3.1	4.2	1.1	6.8	3.4	4.5	2.5	3.3
%RSD	5%	2%	4%	6%	2%	16%	6%	9%	5%	6%

	C. reniformis					*S. foetidus*				
Fragment No	#1	#2	#3	#4	#5	#1	#2	#3	#4	#5
Average wet weight (g)	82.8	90.5	74.0	103.2	79.9	134.8	100.5	77.2	94.4	139.5
Standard deviation (g)	4.7	1.9	5.0	0.4	1.7	0.7	8.1	3.6	0.3	6.6
%RSD	6%	2%	7%	0%	2%	1%	8%	5%	0%	5%

Table A3. p-values of one-way ANOVA for clearance (c) and retention rates (r) of the four sponge species against each pollutant investigated.

Chemical	p_c	p_r
OTC	0.0588	0.0649
Diuron	0.0004	0.0065
Irgarol 1051	0.0033	0.0045
2,6-DMN	0.0009	0.0495
Phenanthrene	0.0007	0.0047
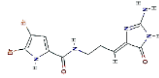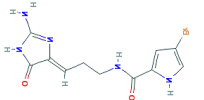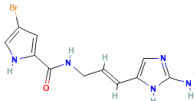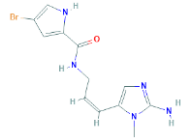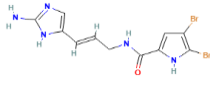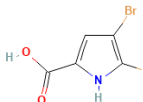
Table A4. *p*-values of one-way ANOVA for clearance (*c*) and retention rates (*r*) of each sponge species investigated against different organic pollutants.
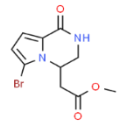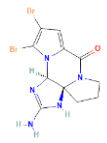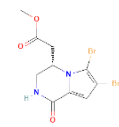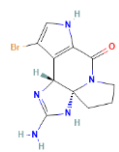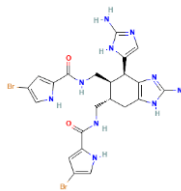
Sponge species	p_c	p_r
A. oroides	<0.0001	<0.0001
A. cannabina	<0.0001	<0.0001
C. reniformis	0.0015	0.0023
S. foetidus	0.0927	0.0629
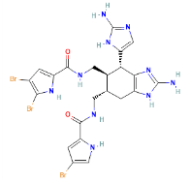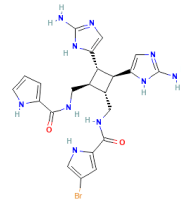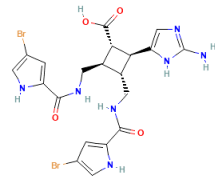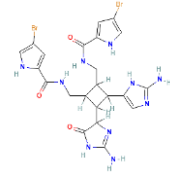
Table A5. Equations describing the relationship of pollutant hydrophobicity (x; Log(K_{OW})) and retention rates (y; Log(*r*)) of the four study sponge species.

Sponge species	Equation	Coefficient of determination, R^2
A. oroides	y=0.3129*x+1.0679	0.9459
A. cannabina	y=0.2574*x+1.1107	0.8692
C. reniformis	y=0.2888*x+0.8623	0.9505
S. foetidus	y=0.1682*x+1.3634	0.9144

Table A6. Selected compounds for *A. oroides* extracts. Experimental retention time (RT), mode polarity (mode), collision energy, precursor ion, fragment ions and bibliographic references for SRM ions.

Superclass	Class	Chemical subclass	Metabolite	RT (min)	Mode	Collision Energy (V)	Chemical formula	Chemical structure	Precursor ion (m/z)	Fragments ions (m/z)	Ref for m/z
Alkaloids	Pyrrole alkaloids	Linear pyrrole alkaloids	Dispacamide A	12.52	+	20	$C_{11}H_{11}Br_2N_5O_2$		405.934	126.067; 138.068; 155.094	(Freire et al., 2022)
			Dispacamide B	1.58	+	10	$C_{11}H_{12}BrN_5O_2$		326.025	155.09; 326.00	(Freire et al., 2022)
			Hymenidin	11.35	+	10	$C_{11}H_{12}BrN_5O$		310.029	80.050; 110.071; 122.071	GNPS
			Keramadine	11.45	+	20	$C_{12}H_{15}BrN_5O^+$		324.047	94.067; 136.088	(Freire et al., 2022)
			Oroidin	12.89	+	10	$C_{11}H_{11}Br_2N_5O$		389.938	80.05; 110.07; 122.07; 139.10; 389.94	GNPS
			4,5-Dibromopyrrole-2-carboxylic acid	16.35	–	20	$C_5H_3Br_2NO_2$		267.920	223.920	(Pontes, 2019)

	Name	RT	Ion	Value	Formula	Structure	m/z	Fragments	Reference
Fused cyclic pyrrole alkaloids	3-Debromohanishin	3.54	+	10	$C_{11}H_{14}BrN_2O_3^+$		300.990	215.968; 254.978	(Freire et al., 2022)
	Dibromophakellin	12.88	+	40	$C_{11}H_{11}Br_2N_5O$		387.940	249.975; 328.892	GNPS
	Longamide B	14.31	+	20	$C_9H_8Br_2N_2O_3$		352.897	273.978; 292.876	(Freire et al., 2022)
	Longamide B methyl ester	16.56	+	20	$C_{10}H_{10}Br_2N_2O_3$		366.913	292.877	(Freire et al., 2022)
	Monobromoisophakellin	2.06	+	10	$C_{11}H_{12}BrN_5O$		310.029	250.982	(Assmann and Köck, 2002)
Dimeric pyrrole alkaloids	Ageliferin	Not Detected	+	10	$C_{22}H_{24}Br_2N_{10}O_2^+$		623.070	148.088; 160.888; 433.094; 450.120	(Freire et al., 2022)

[258]

	Name	RT		Value	Formula	Structure	m/z 1	m/z 2	Reference
	Bromoageliferin	13.73	+	10	$C_{22}H_{23}Br_3N_{10}O_2$		701.01	700.96	(Freire et al., 2022)
	Debromosceptrin acetate	26.33	+	20	$C_{22}H_{25}BrN_{10}O_2$		541.142	177.114	GNPS
	Nakamuric acid	26.21	+	20	$C_{20}H_{21}Br_2N_7O_4$		584.008	584.000	(Hao et al., 2001)
	Oxysceptrin	Not Detected	+	40	$C_{22}H_{25}Br_2N_{10}O_3^+$		637.000	148.000; 177.000; 247.000; 466.00	(Hao et al., 2001)

[259]

	Sceptrin	18.14	+	20	$C_{22}H_{24}Br_2N_{10}O_2$		619.000	448.128; 243.131	GNPS
	Agelasidine A	26.16	+	20	$C_{18}H_{33}N_3O_2S$		356.227	183.120; 184.140	(Pontes, 2019)
Terpenoid alkaloids	Agelasine	25.71	+	20	$C_{26}H_{40}N_5^+$		422.328	177.16	(Riyanti et al., 2020)
	Agelasine A	26.62	+	20	$C_{26}H_{40}ClN_5$		458.304	457.336	PubChem
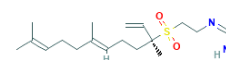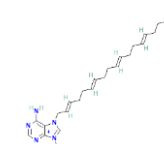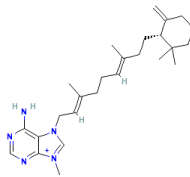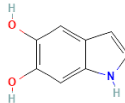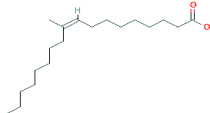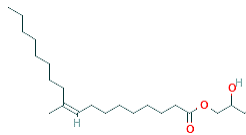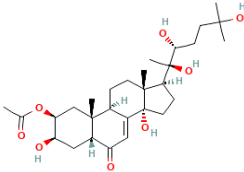
		Compound	RT	Mode	CE	Formula	Structure	m/z	Fragments	Source
		Agelasine E	Not Detected	+	40	$C_{26}H_{40}N_5^+$		422.327	95.086; 121.01; 150.077; 423.331	GNPS
Indoles		4,6-Dihydroxyindole	2.82	+	10	$C_8H_7NO_2$		150.055	132.045; 133.029	FoodB
Lipids	**Fatty acyls**	10-Methyl-9(*Z*)-octadecenoic acid	26.18	–	40	$C_{19}H_{36}O_2$		297.000	183.000	(Yu et al., 1996)
	Glycerolipids	2,3-Dihydroxypropyl(*Z*)-10-methyloctadec-9-enoate	25.37	+	10	$C_{22}H_{42}O_4$		371.000	279.000; 297.000	(Yu et al., 1996)
	Steroids	20-Hydroxyecdysone-22-acetate	26.04	+	20	$C_{29}H_{46}O_8$		523.337	299.154; 531.331	GNPS
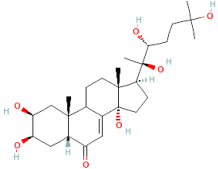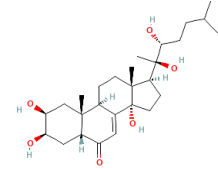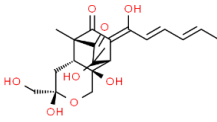
	β-ecdysterone	25.64	+	40	$C_{27}H_{44}O_7$		481.310	280.999; 299.154	GNPS
	Ponasterone-A	Not Detected	–	10	$C_{27}H_{44}O_6$		464.000	463.000; 928.000	MoNA
Polyketides	Trichodermanone C	Not Detected	–	40	$C_{20}H_{26}O_8$		393.115	205.000; 247.000	(Kang et al., 2011)

Table A7. The relative percentages of the metabolite components identified among the *A. oroides* extracts. −; not detected.

Metabolite	%Content					
	Wild #1	Wild #2	Wild #3	Farmed #1	Farmed #2	Farmed #3
Dispacamide A	2.7%	1.1%	2.4%	1.5%	1.4%	1.8%
Dispacamide B	1.2%	5.5%	2.1%	8.3%	6.3%	8.9%
Hymenidin	2.6%	0.9%	0.7%	0.6%	0.6%	1.7%
Keramadine	0.5%	5.9%	4.5%	7.2%	6.8%	10.0%
Oroidin	82.8%	75.8%	82.7%	70.8%	73.0%	66.9%
4,5-Dibromopyrrole-2-carboxylic acid	6.9%	7.2%	6.4%	7.6%	6.1%	6.0%
3-Debromohanishin	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
Dibromophakellin	0.7%	0.7%	0.8%	0.6%	0.6%	0.6%
Longamide B	0.02%	0.02%	0.05%	0.01%	0.01%	0.01%
Longamide B methyl ester	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
Monobromoisophakellin	0.01%	0.01%	<0.01%	0.01%	0.01%	0.01%

Ageliferin	<0.01%	–	–	–	–	–
Bromoageliferin	0.01%	0.02%	<0.01%	0.01%	0.01%	0.01%
Debromosceptrin acetate	0.1%	0.1%	0.01%	0.1%	0.1%	0.1%
Nakamuric acid	0.3%	0.1%	0.01%	0.2%	0.3%	0.2%
Oxysceptrin	<0.01%	–	–	–	–	–
Sceptrin	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%	–
Agelasidine A	0.1%	0.1%	0.01%	0.1%	0.1%	0.1%
Agelasine	0.1%	0.1%	0.01%	0.1%	0.1%	0.1%
Agelasine A	0.8%	0.7%	0.1%	1.1%	1.2%	0.9%
Agelasine E	<0.01%	–	<0.01%	–	<0.01%	–
4,6-Dihydroxyindole	0.01%	0.1%	<0.01%	0.01%	0.01%	0.1%
10-Methyl-9(*Z*)-octadecenoic acid	1.1%	1.9%	0.1%	1.7%	3.2%	2.4%
2,3-Dihydroxypropyl(*Z*)-10-methyloctadec-9-enoate	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%	0.01%
20-Hydroxyecdysone-22-acetate	<0.01%	<0.01%	<0.01%	<0.01%	0.01%	<0.01%

[264]
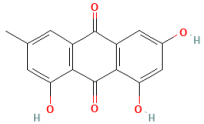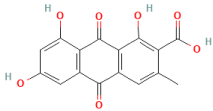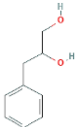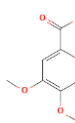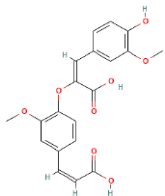
β-ecdysterone	0.01%	0.01%	<0.01%	0.01%	0.01%	<0.01%
Ponasterone-A	0.01%	−	−	−	<0.01%	−
Trichodermanone C	<0.01%	−	−	−	−	−
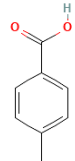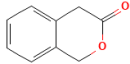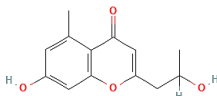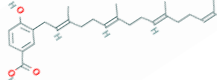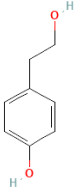
Table A8. Weight-normalized peak intensity values of the metabolite components identified among the *A. oroides* extracts, related to their production levels within sponges. −; not detected.
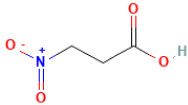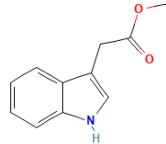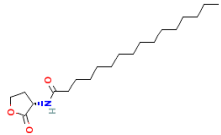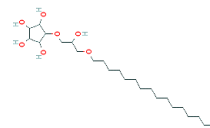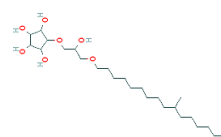
	Weight-normalized Intensity (Intensity g_{sponge}^{-1})					
Metabolite	**Wild #1**	**Wild #2**	**Wild #3**	**Farmed #1**	**Farmed #2**	**Farmed #3**
Dispacamide A	2.7E+10	10.0E+10	2.1E+11	1.8E+10	2.0E+10	2.4E+10
Dispacamide B	1.2E+10	5.2E+10	1.8E+11	9.6E+10	9.3E+10	1.2E+11
Hymenidin	2.6E+10	8.1E+09	6.4E+10	7.4E+09	9.0E+09	2.2E+10
Keramadine	4.5E+09	5.6E+10	4.0E+11	8.3E+10	1.0E+11	1.3E+11
Oroidin	8.2E+11	7.2E+11	7.3E+12	8.2E+11	1.1E+12	8.9E+11
4,5-Dibromopyrrole-2-carboxylic acid	6.8E+10	6.8E+10	5.6E+11	8.9E+10	9.0E+10	8.0E+10
3-Debromohanishin	3.1E+07	7.0E+07	2.3E+07	2.3E+07	1.2E+08	3.1E+07
Dibromophakellin	7.2E+09	6.6E+09	7.0E+10	7.4E+09	9.4E+09	7.9E+09
Longamide B	2.1E+08	2.3E+08	4.5E+09	1.3E+08	2.2E+08	9.8E+07
Longamide B methyl ester	1.1E+09	8.3E+08	1.1E+10	1.1E+09	1.1E+09	8.1E+08
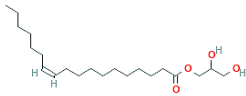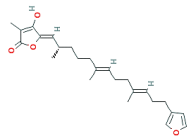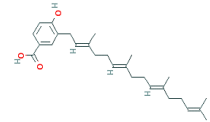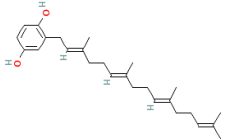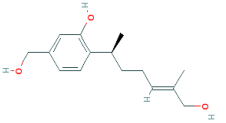
Monobromoisophakellin	1.3E+08	8.0E+07	2.8E+07	1.3E+08	8.3E+07	1.2E+08
Ageliferin	4.9E+06	–	–	–	–	–
Bromoageliferin	8.2E+07	1.5E+08	4.8E+07	1.3E+08	8.0E+07	8.8E+07
Debromosceptrin acetate	9.6E+08	7.5E+08	7.4E+08	7.4E+08	2.1E+09	1.8E+09
Nakamuric acid	2.6E+09	10.0E+08	9.5E+08	2.7E+09	4.7E+09	3.2E+09
Oxysceptrin	1.0E+07	–	–	–	–	–
Sceptrin	9.3E+06	6.7E+06	2.6E+07	3.6E+06	1.6E+07	–
Agelasidine A	9.6E+08	4.3E+08	4.8E+08	9.3E+08	1.8E+09	1.9E+09
Agelasine	1.2E+09	7.7E+08	6.5E+08	1.6E+09	1.9E+09	1.8E+09
Agelasine A	7.5E+09	7.0E+09	4.2E+09	1.2E+10	1.8E+10	1.2E+10
Agelasine E	8.2E+06	–	2.6E+07	–	9.2E+06	–
4,6-Dihydroxyindole	1.4E+08	4.8E+08	9.0E+07	9.0E+07	1.5E+08	8.7E+08
10-Methyl-9(Z)-octadecenoic acid	1.1E+10	1.8E+10	1.1E+10	1.9E+10	4.7E+10	3.2E+10
2,3-Dihydroxypropyl(Z)-10-methyloctadec-9-enoate	2.6E+07	1.8E+07	1.9E+07	3.5E+06	3.3E+07	7.5E+07
20-Hydroxyecdysone-22-acetate	4.8E+07	4.1E+07	2.2E+07	4.9E+07	8.7E+07	3.9E+07
β-ecdysterone	1.1E+08	6.7E+07	1.1E+07	5.9E+07	2.0E+08	3.0E+07
Ponasterone-A	5.8E+07	–	–	–	2.7E+07	–
Trichodermanone C	3.4E+06	–	–	–	–	–

Table A9. Selected compounds for *S. foetidus* extracts. Experimental retention time (RT), mode polarity (mode), collision energy, precursor ion, fragment ions and bibliographic references for SRM ions.

Superclass	Chemical class	Metabolite	RT (min)	Mode	Collision Energy (V)	Chemical formula	Chemical structure	Precursor ion (m/z)	Fragment ions (m/z)	Ref for m/z
Benzenoids	Anthracenes	Emodin	4.23	+	10	$C_{15}H_{10}O_5$		271.060	139.054; 225.055	GNPS
		Endocrocin	19.51	−	10	$C_{16}H_{10}O_7$		313.035	269.046; 313.037	GNPS
	Benzene & substituted derivatives	3-Phenylpropane-1,2-diol	1.54	+	10	$C_9H_{12}O_2$		153.092	91.055; 93.070; 135.081; 153.092	DrugBank
		3,4-Dimethoxybenzoic acid	1.52	+	20	$C_9H_{10}O_4$		183.180	126.046; 152.062	GNPS
		8-*O*-4'-Dehydrodiferulic acid	20.53	+	10	$C_{20}H_{18}O_8$		387.108	195.066; 351.087	HMDB

[267]

	Toluate	1.50	+	10	C₈H₈O₂		137.060	91.0555; 109.0661; 137.0597	(Bojko et al., 2019)
	3-Isochromanone	1.50	+	10	C₉H₈O₂		149.061	105.035; 121.028	(Bojko et al., 2019)
Benzopyrans	7-Hydroxy-2-(2-hydroxypropyl)-5-methylchromone (Aloesol)	1.51	+	10	C₁₃H₁₄O₄		235.097	191.071; 217.087; 235.070	HMDB
	4-Hydroxyphenylacetic acid	4.46	+	20	C₈H₈O₃		153.055	95.049; 107.049	GNPS
Phenols	Tyrosol	1.91	+	20	C₈H₁₀O₂		121.064	71.970	GNPS

[268]

	Dipeptides	3-Nitropropionic acid	1.39	+	10	$C_3H_5NO_4$		120.029	91.054; 100.001; 100.505	GNPS
	Indoles	Indole-3-methylethanoate	1.49	+	20	$C_{11}H_{11}NO_2$		190.087	103.055; 128.049; 130.065	PubChem
	Fatty acyls	*N*-hexadecanoyl-*L*-homoserine lactone	12.05	+	10	$C_{20}H_{37}NO_3$		340.283	298.275; 322.274	(Bojko et al., 2019)
Lipids	**Glycerolipids**	1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-hexadecylglycerol	27.44	+	10	$C_{24}H_{48}O_7$		471.329	471.000	(Gil et al., 2006)
		1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(10-methylhexadecyl)glycerol	28.23	+	10	$C_{25}H_{50}O_7$		485.343	245.000; 485.000	(Gil et al., 2006)

[269]

	Monovaccenin	26.24	+	10	$C_{21}H_{40}O_4$		357.000	321.279; 339.282; 357.300	HMDB
	7E,12E,20Z-Variabilin	25.61	+	20	$C_{25}H_{34}O_4$		399.254	107.083; 135.078	GNPS
	4-Hydroxy-3-tetraprenylbenzoic acid	27.75	+	10	$C_{27}H_{38}O_3$		411.287	151.038; 411.287	GNPS
Prenol lipids	1,4-Dihydroxy-2-tetraprenylbenzene	28.67	+	10	$C_{26}H_{38}O_2$		383.292	109.1007; 383.2932	GNPS
	(+)-12,15 Dihydroxycurcuphenol	23.12	+	20	$C_{15}H_{22}O_3$		251.164	59.050; 149.025	(Bojko et al., 2019)

	24-Methylcholest-7-en-3β-ol	22.68	+	40	$C_{28}H_{48}O$		400.371	85.102; 297.852	FoodB
	24-Methylcholesta-5,7-dien-3β-ol (22,23-Dihydroergosterol)	12.52	+	10	$C_{28}H_{46}O$		398.355	381.352; 399.363	HMDB
Steroids	24-Methylcholesta-7,22-dien-3β-ol (Stellasterol)	11.58	+	10	$C_{28}H_{46}O$		398.355	295.243; 399.363	FoodB
	24-Methylcholesta-5,7,22-trien-3β-ol (Ergosterol)	12.51	+	10	$C_{28}H_{44}O$		397.35	204.118; 397.116	GNPS
	Cholest-7-en-3β-yl acetate	27.15	+	10	$C_{29}H_{48}O_2$		429.372	159.115; 411.362	GNPS
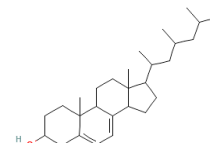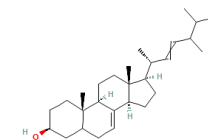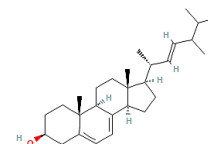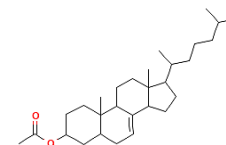
[271]
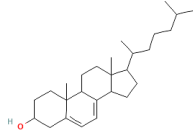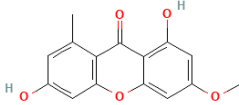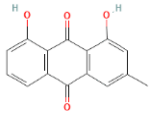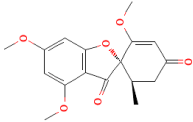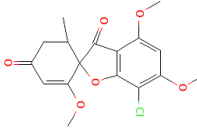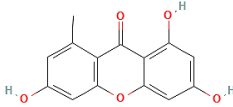
	Compound	RT		Level	Formula	Structure	m/z	Fragments	Source
	Cholesta-5,7-dien-3β-ol (Δ^7-Cholesterol)	27.85	+	10	$C_{27}H_{44}O$		385.346	161.1325; 173.1326; 367.3349	GNPS
Polyketides	3,8-Dihydroxy-6-methoxy-8-methylxanthone (Griseoxanthone C)	23.17	+	10	$C_{15}H_{12}O_5$		273.075	230.0581; 273.0766	GNPS
	Chrysophanol	1.53	+	10	$C_{15}H_{10}O_4$		255.066	209.060; 255.065	GNPS
	Dechlorogriseofulvin	14.99	+	20	$C_{17}H_{18}O_6$		319.118	165.054	GNPS
	Griseofulvin	1.53	+	10	$C_{17}H_{17}ClO_6$		353.079	385.079; 353.079	GNPS
	Norlichexanthone	21.03	–	10	$C_{14}H_{10}O_5$		257.045	257.108; 257.304; 257.0455	GNPS

[272]

Table A10. The relative percentages of the metabolite components identified among the *S. foetidus* extracts. −; not detected.

Metabolite	%Content					
	Wild #1	Wild #2	Wild #3	Farmed #1	Farmed #2	Farmed #3
Emodin	<0.01%	<0.01%	<0.01%	−	<0.01%	−
Endocrocin	<0.01%	<0.01%	0.01%	<0.01%	<0.01%	<0.01%
3-Phenylpropane-1,2-diol	0.4%	0.4%	0.5%	0.2%	0.3%	0.4%
3,4-Dimethoxybenzoic acid	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
8-*O*-4'-Dehydrodiferulic acid	1.2%	1.8%	1.8%	1.3%	1.1%	1.5%
Toluate	2.8%	2.1%	1.5%	1.8%	1.8%	1.5%
3-Isochromanone	0.1%	0.1%	0.2%	0.1%	0.1%	0.1%
7-Hydroxy-2-(2-hydroxypropyl)-5-methylchromone	0.4%	0.2%	0.5%	0.4%	0.3%	0.3%

[273]

4-Hydroxyphenylacetic acid	0.01%	<0.01%	0.01%	<0.01%	<0.01%	<0.01%
Tyrosol	0.01%	0.01%	<0.01%	0.01%	<0.01%	0.01%
3-Nitropropionic acid	0.01%	0.01%	0.02%	0.02%	0.01%	0.01%
Indole-3-methylethanoate	0.1%	0.1%	0.1%	0.1%	0.3%	0.1%
N-hexadecanoyl-*L*-homoserine lactone	56.2%	54.1%	56.3%	55.9%	56.7%	56.0%
1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(10-methylhexadecyl) glycerol	2.3%	2.4%	2.6%	2.6%	2.3%	2.5%
1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-hexadecylglycerol	0.2%	0.3%	0.2%	0.2%	0.3%	0.4%
Monovaccenin	4.2%	4.9%	4.4%	4.9%	4.7%	4.5%
7*E*,12*E*,20*Z*-Variabilin	0.03%	0.01%	0.01%	0.01%	0.01%	0.01%
4-Hydroxy-3-tetraprenylbenzoic acid	1.1%	1.8%	0.9%	2.2%	2.7%	3.0%

1,4-Dihydroxy-2-tetraprenylbenzene	0.04%	0.2%	0.4%	0.4%	0.4%	0.5%
(+)-12,15 Dihydroxycurcuphenol	<0.01%	0.01%	<0.01%	0.03%	0.06%	0.05%
24-Methylcholest-7-en-3β-ol	0.07%	0.05%	0.08%	0.05%	0.08%	0.05%
24-Methylcholesta-5,7-dien-3β-ol	0.01%	0.02%	0.02%	0.01%	0.02%	0.03%
24-Methylcholesta-7,22-dien-3β-ol	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
24-Methylcholesta-5,7,22-trien-3β-ol (Ergosterol)	29.9%	30.8%	29.3%	29.0%	28.4%	28.3%
Cholest-7-en-3β-yl acetate	–	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
Cholesta-5,7-dien-3β-ol	0.05%	0.07%	0.05%	0.06%	0.06%	0.06%
3,8-Dihydroxy-6-methoxy-8-methylxanthone	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%
Chrysophanol	0.4%	0.3%	0.3%	0.2%	0.3%	0.2%

[275]

Metabolite					
Dechlorogriseofulvin	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%	–
Griseofulvin	0.2%	0.1%	0.2%	0.1%	0.1%	0.1%
Norlichexanthone	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%

Table A11. Weight-normalized peak intensity values of the metabolite components identified among the *S. foetidus* extracts, related to their production levels within sponges. −; not detected.

	Weight-normalized Intensity (Intensity g_{sponge}^{-1})					
Metabolite	**Wild #1**	**Wild #2**	**Wild #3**	**Farmed #1**	**Farmed #2**	**Farmed #3**
Emodin	1.7E+07	7.3E+07	2.2E+08	–	2.0E+07	–
Endocrocin	3.3E+07	2.4E+08	4.2E+08	5.0E+07	4.4E+07	3.0E+07
3-Phenylpropane-1,2-diol	1.9E+10	1.9E+10	2.3E+10	1.1E+10	1.1E+10	2.4E+10

3,4-Dimethoxybenzoic acid	3.1E+09	2.9E+09	4.4E+09	3.2E+09	2.3E+09	3.1E+09
8-*O*-4'-Dehydrodiferulic acid	6.0E+10	9.7E+10	9.0E+10	7.0E+10	4.5E+10	9.7E+10
Toluate	1.4E+11	1.1E+11	7.8E+10	9.4E+10	6.0E+10	9.6E+10
3-Isochromanone	6.5E+09	6.2E+09	1.1E+10	6.2E+09	5.9E+09	8.5E+09
7-Hydroxy-2-(2-hydroxypropyl)-5-methylchromone	2.0E+10	1.3E+10	2.7E+10	1.9E+10	1.2E+10	1.7E+10
4-Hydroxyphenylacetic acid	2.7E+08	8.1E+07	2.6E+08	1.4E+08	1.1E+08	1.4E+08
Tyrosol	3.9E+08	3.5E+08	1.5E+08	3.0+08	1.0E+08	3.5E+08
3-Nitropropionic acid	7.3E+08	7.0E+08	1.2E+09	1.0E+09	6.4E+08	5.8E+08
Indole-3-methylethanoate	5.0E+09	5.4E+09	6.6E+09	3.7E+09	1.2E+10	5.8E+09
N-hexadecanoyl-*L*-homoserine lactone	2.8E+12	3.0E+12	2.8E+12	3.0E+12	2.4E+12	3.7E+12

1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-(10-methylhexadecyl)glycerol	1.2E+11	1.3E+11	1.3E+11	1.4E+11	1.0E+11	1.6E+11
1-*O*-(2,3,4,5-tetrahydroxycyclopentyl)-3-*O*-hexadecylglycerol	9.6E+09	1.6E+10	1.1E+10	1.1E+10	1.1E+10	2.4E+10
Monovaccenin	2.1E+11	2.7E+11	2.2E+11	2.6E+11	2.0E+11	2.9E+11
7*E*,12*E*,20*Z*-Variabilin	1.6E+09	6.4E+08	5.1E+08	3.0E+08	6.0E+08	9.5E+08
4-Hydroxy-3-tetraprenylbenzoic acid	5.6E+10	9.6E+10	4.5E+10	1.2E+11	1.1E+11	2.0E+11
1,4-Dihydroxy-2-tetraprenylbenzene	1.9E+09	10.0E+10	1.9E+10	2.1E+10	1.9E+10	3.3E+10
(+)-12,15 Dihydroxycurcuphenol	3.2E+07	3.3E+08	4.3E+07	1.7E+09	2.6E+09	3.1E+09
24-Methylcholest-7-en-3β-ol	3.5E+09	2.6E+09	3.9E+09	2.5E+09	3.3E+09	3.1E+09
24-Methylcholesta-5,7-dien-3β-ol	5.6E+08	1.3E+09	1.1E+09	6.1E+08	8.7E+08	2.0E+09
24-Methylcholesta-7,22-dien-3β-ol	6.4E+08	3.5E+08	6.5E+08	4.1E+08	4.1E+08	3.7E+08

24-Methylcholesta-5,7,22-trien-3β-ol (Ergosterol)	1.5E+12	1.7E+12	1.5E+12	1.6E+12	1.2E+12	1.9E+12
Cholest-7-en-3β-yl acetate	–	4.7E+07	1.4E+08	1.7E+08	9.5E+07	1.6E+08
Cholesta-5,7-dien-3β-ol	2.6E+09	3.6E+09	2.5E+09	3.4E+09	2.5E+09	4.0E+09
3,8-Dihydroxy-6-methoxy-8-methylxanthone	1.3E+10	1.6E+10	1.6E+10	1.7E+10	1.3E+10	1.8E+10
Chrysophanol	1.7E+10	1.4E+10	1.6E+10	1.1E+10	1.2E+10	1.6E+10
Dechlorogriseofulvin	1.5E+07	1.5E+07	2.2E+07	2.1E+07	6.5E+07	–
Griseofulvin	8.2E+09	5.4E+09	8.4E+09	7.1E+09	5.8E+09	9.1E+09
Norlichexanthone	4.0E+09	4.8E+09	4.1E+09	4.9E+09	4.1E+09	6.3E+09

Table A12. What to consider when selecting a bioassay to search for a selected bioactivity.

Purpose

Is it aimed at general or specific bioactivity?

How selective should it be?

Are quantitative or qualitative results needed?

How sensitive should it be (what is the requirement for the minimal amount of compound)?

Cost

Time requirement

Labour intensiveness

Cost of material

Requirement of special equipment (different modes of detection)

Effect of the extraction procedure on bioactivity

Selection of source material (amount available, possibility to reacquire)

Availability of source material (seasonal, geographic, legal)

Organic solvent or water-based

Temperature of extraction

Length of extraction

Homogenization steps

Cultivation steps

Stability of bioactive compound

Interference with materials used for extraction (e.g., plastic, solvent components)

Feasibility

Errors caused by the colour or viscosity of extracts

Reproducibility

High-throughput capacity or automation possibility

Ease of results interpretation

Other

Availability of standards

Bioactivity threshold

Capability of dereplication

Regulatory requirements (e.g., use of BSL2 or GMO organisms)

Table A13. Principles and characteristics of popular bioassays used in pre-screening and screening of bioactivities.

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Antimicrobial bioassays				
Disc diffusion = Agar disc diffusion method = Kirby-Bauer test = Disc-diffusion antibiotic susceptibility test	*In vitro* detection of effects on microbial growth or survival on solid media. A microbial inoculum suspension (e.g., 1-2 10^8CFU/mL for bacteria) is spread on agar plates and the test extract/compound is applied on impregnated paper discs. After 12-24 h incubation (bacteria) or 24 – 48 h incubation (fungi) in suitable growth conditions for the tested microbial strain inhibition zone diameters are read at the point where no growth is observed. Variations are available for yeasts and molds.	- Simple - Standardized protocols available for bacteria and yeast (CLSI, EUCAST) - Versatile (suitable for majority of bacterial pathogens) - Controls for bioassay performance available in form of antibiotics and characterized typing strains with known phenotype and antibiogram - No special equipment, only basic microbiological utilities required - Easily used in routine - Reproducible and accurate if standard protocols are followed - Inexpensive - Easy to interpret - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not appropriate for all bacterial pathogens - Diffusion of the extract/compound can be non-homogeneous and affect accuracy - Not appropriate for large molecules, amphiphilic molecules - Importance of the inoculum size and preparation - Importance of growth medium used - Not quantitative – cannot determine MIC value - Qualitative categorization into susceptible, intermediate or resistant is possible based on standardized MIC breakpoints - Cannot distinguish between bactericidal and bacteriostatic effect - Few interpretative criteria are available - Not adapted for filamentous fungi as breakpoints for standard antibiotics are not defined	(Alastruey-Izquierdo et al., 2015; Balouiri et al., 2016; Matuschek et al., 2014; Strömstedt et al., 2014)
Antimicrobial gradient method = Epsilometer testing (commercial version Etest®)	*In vitro* detection of effects on microbial growth or survival on solid media. Variant of agar diffusion method that combines the principle of dilution and diffusion methods to determine MIC. Exponential gradient of substance applied on a plastic or nitrocellulose strip (marked with concentration scale) and placed on a previously inoculated agar surface. After 12-24 h incubation (bacteria) or 24 – 48	- Simple - Used for antibiotics, also antimycobacterials	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not appropriate for all bacterial pathogens - Subjective interpretation	(Idelevich et al., 2018)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	h incubation (fungi) in suitable conditions ellipse-shaped zone of inhibition indicates the MIC that can be read off the strip.	- High sensitivity (can detect trace amount of beta-lactamase (ESBL) - Quantitative (provides MIC value) - Can be used to test interaction of two antimicrobials - Cost-effective - Useful also for yeast and filamentous fungi - No special equipment, only basic microbiological utilities required - Easy to interpret - Commercial kits available that can be used as controls	- Diffusion of the extract/compound can be non-homogeneous and affect accuracy - Not appropriate for large molecules, amphiphilic molecules - Cannot distinguish between bactericidal and bacteriostatic effect - Not used for MNPs (problematic preparation of gradient strip)	
Agar plate assay = Poisoned food method for filamentous fungi	*In vitro* evaluation of antifungal effect against filamentous fungi. The substance or extract is incorporated homogeneously into the molten agar and mycelia disc are inoculated at the center of plate. After incubation under suitable growth conditions the diameters of growth inhibition are read and compared with the unexposed control.	- Simple - Standardized protocols available (CLSI, EUCAST) - Easy to interpret - Relatively sensitive - Low cost - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - There are some commercial kits that combine identification-susceptibility testing assay for *Candida* and *Aspergillus* spp. - Resources for work with fungi - Not quantitative - Possible interference with growth medium components - Not appropriate for heat labile compounds - Requires large amounts of compounds	(Chadwick et al., 2013)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			- Time consuming	
Broth (micro)dilution for determination of MIC (Minimum Inhibitory Concentration)	*In vitro* detection of microbial growth inhibition in liquid culture containing a known concentration of drug. Two-fold dilutions of antimicrobial agent or extract are mixed with the inoculum in liquid medium and after suitable growth time period of incubation (12 – 24 h) MIC value is determined by detecting the lowest concentration that inhibited visible microbial growth. Usually performed in 96-well plates (microdilution). Detection of growth is by naked eye or colorimetric assays using tetrazolium salts, resazurin, or ATP can be used to detect metabolically active cells. Different procedures are adapted for yeasts and molds including longer incubation time (24 – 72 h).	- Standard protocols are available (CLSI, EUCAST) - Gold standard in clinical microbiology - High-capacity bioassay - Versatile - Accurate and reproducible - Applicable to both yeasts and molds - Economic if plates are produced in the laboratory - Can be used for any new discovered antimicrobials - Low sample volume required - Cost-effective - Adequate for primary screening - Appropriate for high-throughput screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Solubility of organic extract in broth medium can be challenging - Not suitable for large polycationic, amphiphilic molecules - Plastic interference of 96 well plates for peptide antimicrobial assessment - Importance of the inoculum size and preparation - Importance of growth medium used - Subjective interpretation by CLSI methodology alleviated using EUCAST protocol - Labor-intensive - Technical training requirement high - Risk of error with dilution preparation - Edge effect	(Arendrup et al., 2008; Balouiri et al., 2016; Rodriguez-Tudela et al., 2008; Strömstedt et al., 2014)
MBC (Minimum bactericidal concentration), or MFC (minimum fungicidal concentration), or MLC (minimum lethal concentration)	Common estimation of bactericidal or fungicidal activity determined after broth dilution by subculturing samples from wells with incubation time from 24 h to 72 h. It is the lowest concentration of antimicrobial agent needed to kill 99.9 % of the final inoculum after 24 h incubation in standardized conditions.	- Simple - Quantitative - Cost-effective - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Labor intensive - Importance of growth medium used - Only culturable cells are detected	(Balouiri et al., 2016)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Time-kill assay = Time-kill curve = Growth curve analysis	*In vitro* test to measure the kinetics of dynamic interaction between the compound and the microbial strain to reveal a time-dependent or a concentration dependent antimicrobial effect. The log CFU/mL of microbial/antimicrobial solution is determined on time scale depending on the bacteria strain and the media used. Alternatively, growth is followed in a microplate reader measuring optical density at 600 nm. Typically used in secondary testing.	- Existing standard guidelines CLSI and ASTM - Growth curve analysis offers many variables that may indicate mode of action: growth rate, growth dynamics - Can be used to study synergy/antagonism between substances	- BSL2 and BSL3 level microorganisms require work in suitable facility - Special software needed for growth curve analysis - Labor intensive - Specialized equipment needed - Inoculum size, growth phase, growth medium affect outcome - Possible interference with growth vessels, medium components and method of growth detection	(Balouiri et al., 2016)
Bioautography	*In vitro* direct detection of antibacterial compounds on TLC (Thin Layer Chromatography) plate based on incubation (12 – 24 h) and visualization of microbial growth using vital stains or metabolic stains or dehydrogenase-activity-detecting reagent to reveal zones of inhibition. A variation is possible using bioluminescent bacteria as reporters. Particularly adequate for monitoring.	- Simple - Rapid - Results easily visualized - Inexpensive - Applicable to both bacteria and fungi - Can be utilized for spore-producing fungi - Little amount of extract/compound required	- BSL2 and BSL3 level microorganisms require work in suitable facility - Volume of agar or broth has to be well defined otherwise resulting in poorly defined inhibition zones or irregular bacterial growth - Not quantitative - Difficult to standardize	(Balouiri et al., 2016; Choma and Grzelak, 2011; Dewanjee et al., 2015; Klöppel et al., 2008; Patil et al., 2017)
Volatile antibiotics bioassays	All versions of these bioassays use the same principle to detect volatile organic compound (VOC) activity. The source of the volatile (a living organism or chemical) is placed on one side of a chamber without direct contact with the target organism, while the target is grown or located on another side or compartment of the chamber. The effect of the volatile on the growth (inhibition) or survival of the target organism is compared to a control	- Easy to perform and interpret - Low cost - Sensitive - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not quantitative - Special equipment or material required (sealed chambers)	(Ezra et al., 2004; Liarzi et al., 2016; Tomsheck et al., 2010)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	using the same container and conditions without the volatiles.			
Antibiofilm bioassays				
Crystal violet	Gold standard for biofilm quantification in microtiter plates. Inoculum in liquid medium incubated for 24 – 72 h at selected temperature under static conditions. Washing steps and short incubation times in crystal violet, are followed by the colorimetric detection of the stained biomass.	- Adapted protocols available for different bacterial species - Different surfaces can be assayed using coupons - Versatile: both for G+ and G- - Qualitative or quantitative, but characterized control strains need to be incorporated for interpretation - Low cost - Can be used to monitor biofilm growth and biofilm eradication - High-throughput (96-well plates) - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Non-specific binding to anionic proteins and other negatively charged molecules, like capsules, lipopolysaccharides, and DNA/nucleic acids, leading to an inability to distinguish between live and dead bacterial populations and/or exopolysaccharides - Large variability between samples leading to possibly complicated interpretation - Medium composition important - Culture conditions important - Strain to strain variability is high, need to know primary biofilm phenotype - Interference of the stain with experimental setup possible	(Haney et al., 2021; O'Toole, 2011)
CFU (Colony Forming Units)	Biofilm is sonicated to dislodge adhered biomass and serial dilutions of homogenized bacterial suspension is plated onto agar plates, incubated 24 – 48 h to count the colony forming units (CFUs).	- Simple - Low cost - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment - Sonication parameters important (can reduce viability of recovered CFUs), - Sonication parameters are different for different bacterial species - aggregation can affect CFU count - Labor intensive	(Haney et al., 2021)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			- Only culturable cells are detected	
The BioFilm Ring Test	Mobility measurement of magnetic microbeads mixed with bacterial suspension in a polystyrene microplate. Without biofilm growth beads gather together in a visible central spot under magnetic action, while no spot indicates bead immobilization by biofilm formation.	- Simple - Rapid - No dyes or stains - No washing steps - Low sample volume required - High-throughput (96- well plates)	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment - Interpretation may be challenging - Qualitative	(Olivares et al., 2016)
The Calgary Biofilm device	Two-part reaction vessel containing a lid with 96 pegs that sit in channels of the reaction vessel that allows flow of medium across pegs to create consistent shear force.	- Standardized protocols available - High-throughput (96-well plates) - Quantitative	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment - Use of multiple sterile microplates for treatment and washing steps - Relies on viable cell counting for experimental validation	(Haney et al., 2021; Kırmusaoğlu, 2019)
MBEC (Minimum biofilm eradication concentration) Assay®	High-throughput screening of antibiofilm activity. Plastic lid with 96 pegs on which biofilms establish under batch conditions and the lid with pegs is transferred to a new 96 well for testing, biofilm is dislodged by sonication and CFUs are determined.	- Standardized method for *Pseudomonas aeruginosa* (ASTM E2799-17)	- BSL2 level microorganisms require work in suitable facility - Aggregation can affect CFU count - Labor intensive - Only culturable cells are detected	(ASTM, 2022; Parker et al., 2014)
SIMBA – simultaneous detection of antimicrobial and antibiofilm activity	The SIMultaneous detection of antiMicrobial and anti-Biofilm Activity (SIMBA) method combines the testing of antimicrobial and antibiofilm activity against bacteria with the evaluation of the 20-hour growth curve of the *Salmonella* Infantis ŽM9 strain determined with absorbance measurements at 600 nm in a 96-well plate.	- Simple - Rapid - No dyes or stains - Cost-effective	- Optimized for one *Salmonella* strain - Not suitable for dark colored samples - Need for specialized equipment	(Sterniša et al., 2023, 2022)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Information on both antimicrobial and antibiofilm activity in one assay - Low sample volume required - High-throughput (96-well plates) - Possibility of automation	- (spectrophotometer with temperature control and shaking capabilities)	
Cytotoxicity bioassays				
MTT (also MTS, XTT, WST)	*In vitro* colorimetric assay usually performed in 96-well plates to evaluate cellular metabolic activity - glycolytic production of NADH. Based on tetrazolium salts (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; WST, water-soluble tetrazolium salts) – difference between them is the tetrazolium salt used and the solubility and/or absorption spectrum of the formazan product. Eukaryotic cells are treated for 24 - 48 hours with different concentrations of compounds to determine the concentration of the tested compounds, which produces 50% of cytotoxicity (CC$_{50}$). Tetrazolium salt (e.g., MTT) is then added to the cells for 2 hours at 37°C. MTT is reduced by a cellular mitochondrial enzyme (succinate dehydrogenase) to violet formazan precipitates, which are subsequently solubilized by organic solvents before absorbance is read. Alternatively, water-soluble tetrazolium salts can be used, omitting the final solubilization step.	- Commercial kits with standardized protocols available - Cost-effective - Relatively simple - Assay for whole cells - Linearity between absorbance and cell count - Versatile: suitable - for both adherent and suspended cell cultures - One-step procedure variants using water soluble tetrazolium salts include XTT, MTS, WST - Possibility of automation - Appropriate for high-throughput screening	- BSL2 and BSL3 level cell lines require appropriate facility - Lengthy two-step procedure - Highly variable results depending on: the number of cells per well, and the high pH of the culture medium - Requires optimization of cell density (untreated cells have absorbance values that fall within the linear portion of the growth curve (conditions not too close to saturation) - Requires optimized incubation time - Not suitable for reducing compounds - Not for metabolically poor cells, i.e. thymocytes and splenocytes - Linearity between absorbance and cell count is lost when cells are confluent and cellular metabolism slows down - The result can be variable because metabolic activity depends not only on the number of	(Balbaied and Moore, 2020; Jo et al., 2015; McCauley et al., 2013 Riss et al., 2019)

[287]

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			cells per well but also on several other factors	
Sulforhodamine B (SRB) assay	Used for cell density determination, based on the measurement of cellular protein content. Toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader.	- Simple - Cost-effective - Results linear over a 20-fold range of cell numbers - Sensitivity comparable to those of fluorometric methods - Appropriate for high-throughput screening	- Requires microplate reader (absorbance)	(Vichai and Kirtikara, 2006)
ATP-based test	Gold standard luminescence test. See MTT for the procedure. Quantification of released intracellular ATP by enzymatic reaction between the enzyme luciferase and its substrate, luciferin, to produce luminescence. There is a linear relationship between the intensity of the light signal and the ATP concentration or cell number. It is one of the most sensitive endpoints for measuring cell viability.	- One-step procedure - Faster than MTT and MTS - Reduction of artifacts - Sensitive measure of intracellular ATP rather a specific biological effect - More sensitive than conventional biochemical methods - Sensitive compared to other cytotoxicity tests - Interferences minimal - Commercial kits available - Possibility of being automated	- BSL2 and BSL3 level cell lines require suitable facility - More expensive than MTT and MTS and fluorescent methods - The ATP assay sensitivity is usually limited by reproducibility of pipetting - Replicate samples rather than a result of the assay chemistry - Need for specialized equipment (luminescence detection)	(Aslantürk, 2017; Herzog et al., 2007; Ponti et al., 2006)
Automated fluorometric microculture cytotoxicity assay (FMCA)	Based on the measurement of fluorescence generated from cellular hydrolysis of fluorescein diacetate (FDA) to fluorescein by viable cells with intact plasma membranes	- Highly standardized and reproducible one-step procedure	- BSL2 and BSL3 level cell lines require suitable facility	(Burman et al., 2011; Lindhagen et al., 2008)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	after a 48 – 72 hour culture period in microtiter plates. See MTT for procedure.	- Possibility of being automated	- Need for specialized equipment (fluorescence detection)	
Dye exclusion method	The membrane integrity of cell is determined by its permeability to several dyes (eosin, Trypan blue, erythrosine B, Congo red assays). Trypan blue has been used the most extensively to assess the percentage of viable cells in suspension culture.	- Simple - Rapid - Small numbers of cells needed - Can be applied in non dividing cell populations	- BSL2 and BSL3 level cell lines require suitable facility - Can be challenging to process a large number of samples simultaneously, particularly when the exact timing of progressive cytotoxic effects is taken into consideration - Careful interpretation needed for living cells with metabolic activity loss (trypan blue) - Its toxic side effect of some dyes on mammalian cells (trypan blue) - Not suitable for adherent monolayer cell cultures - Labor intensive	(Aslantürk, 2017)
LDH (lactate dehydrogenase) cytotoxicity assay	LDH is a cytosolic enzyme present in many different cell types that is released upon damage to the plasma membrane. The assay quantitatively measures the activity of stable, cytosolic LDH released from damaged cells. It is a colorimetric assay.	- Suitable for both adherent and suspended cell cultures - Commercial kits available - Detects low level damage to cell membranes which cannot be detected using other methods	- BSL2 and BSL3 level cell lines require suitable facility - LDH assay is limited to serum-free or low-serum culture conditions to avoid high background readings. - Interference with serum components	(Kocherova et al., 2020)
Clonogenic cell survival assay	Determines the ability of a cell to proliferate indefinitely, retaining its reproductive ability to form a colony or a clone. These cells are considered clonogenic. Cells are seeded at low density and growth of colonies/clones is analysed after a week by staining and counting. The gold standard for measuring cellular reproductivity.	- Simple - Cost-effective - Gold standard	- BSL2 and BSL3 level cell lines require suitable facility - Suitable only for adherent cells - Not suitable for all adherent cell lines (not all cells are able to form colonies in vitro – cell-to-cell contacts and self-produced	(Munshi et al., 2005)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			growth factors are limited at low cell density)	
DNA synthesis assay 3H-labeled thymidine (3HT)	The process of DNA synthesis is relatively specific for cell division and can therefore be considered a marker of cell proliferation activity. Nucleoside analogue incorporation assays are based on the introduction of chemically or radio-labelled nucleosides that are subsequently incorporated into DNA strands synthesised during S phase. A scintillation beta counter is used to measure radioactivity in DNA recovered from cells to determine the extent of cell division that has occurred in response to a test agent. The nucleoside analogue 5-bromo-2'-deoxyuridine (BrdU) is used to avoid the use of radioisotopes and is detected with monoclonal antibodies. Alternatively, thymidine analogues are available that do not require antibody detection.	- This assay is commonly regarded as reliable and accurate. - Suitable for immunohistochemistry or immunocytochemistry, - In-cell ELISA, flow cytometry - It can be performed in experiments *in vitro* and *ex vivo*, but not *in vivo* - Not suitable for screening, used for mechanistic studies - Commercial kits available - Allows quantitative assessment of proliferation levels - Direct measures of proliferation - Appropriate for high-throughput screening	- BSL2 and BSL3 level cell lines require suitable facility - Potential use of radioisotopes - It is an endpoint assay because of the DNA extraction step, and so no further studies can be performed with the treated cells. - synthetic analogues such as 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), are usually preferred (can be used not only *in vitro* or *ex vivo* but also *in vivo*) - Both assays cannot identify cells that have undergone numerous divisions - Need for specialized equipment	(Romar et al., 2016)
Antiviral bioassays				
Flow cytometry cell count assay (FACS)	Cytotoxicity-based antiviral assay based on the detection of intact and damaged cells using a flow cytometer and dyes to stain the cells (e.g., propidium iodide, carboxyfluorescein diacetate).	- Three populations discriminated (dead, viable, injured) - Reproducible - Rapid (2-6 h to results)	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Need for specialized equipment: flow cytometry equipment - Need for trained personnel - Not easy to interpret	(Balouiri et al., 2016; Zamora and Aguilar, 2018)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			- Specific cell lines known to be susceptible to and allowing viral infection with the virus of interest.	
Cytopathic effect assay (CPE)	Suitable for primary *in vitro* antiviral screening. In this assay, cells permissive for a virus are infected with the same virus at serial dilutions. Cells are observed daily until a cytopathic effect is detected. The virus concentration is expressed as infectious tissue culture dose ($TCID_{50}$), which is the multiple of dilutions that result in CPE in 50% of wells. Direct method.	- Commercial kit available allowing standardization and automated procedures to reduce the timing - For all types of viruses that do or do not form viral plaques - Cell fixation and staining not required - Cost-effective - Operator independent - Technically simple in respect to plaque reduction assay (PRA) or virus reduction yield assay (VRA) - Labor intensive and time consuming - Reduced reading time - Appropriate for high-throughput screening - Infectious virus detection	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Method applicable only to viruses that cause morphological changes in infected cells (CPE inducing viruses) - Lengthy: the time required for the cytopathic effect to become apparent - Relatively subjective reading - Works only with specific cell lines known to be susceptible and permissible to viral infection with the virus of interest. - Equipment required to work with viruses and specialized virology trained personnel	(El Sayed, 2000; Suchman and Blair, 2007)
Plaque reduction assay (PRA)	Primary *in vitro* antiviral screening for the detection of infectious viral particles. A viral inoculum of approximately 50-70 viral plaques/well is adsorbed onto permissive cells in the presence of the test substance. After viral adsorption, the unbound virus is removed and the culture is covered with a semi-solid medium (agar, Avicel, methylcellulose). After	- Validation with a positive control, such as a commercial compound with known antiviral activity - Commonly used	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Only for viruses that form plaques - Labor intensive	(El Sayed, 2000)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	an incubation period equal to the duration of the replication cycle of the virus, the cells are fixed and stained to count the viral plaques microscopically. Titers are expressed as the number of plaque-forming units (PFU) per milliliter (PFU/ml). Direct method.	- No special equipment is required in addition to a cell culture laboratory - Results are easily visualized under a microscope or with the naked eye - Cost-effective - Sensitive - Protocols vary from laboratory to laboratory and depend on the type of cells used - Appropriate for high-throughput screening - Infectious virus detection	- Sometimes lengthy - Results not reproducible: depends on cell density, CPE and plaque size - Counting of plaques can be subjective - Specific cell lines known to be susceptible and permissible for viral infection with the virus of interest - Protocol must be adapted for each host-virus combination	
Virus reduction yield assay (VRA)	Primary *in vitro* antiviral screening to detect infectious viral particles. Permissive cell cultures are infected with a specific amount of virus, and after virus adsorption (usually 2 hours at 37°C or 33°C for temperature-sensitive viruses), the unbound virus is removed, and different concentrations of the same compound are added. After an incubation period that allows virus replication, the total viral yield is titrated and determined. Direct method.	- Less operator-dependent than the PRA - Cost-effective - Sensitive - Infectious virus detection	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Time/material-intensive - Not-automatable - Not reproducible: results depend on harvesting time - Specific cell lines known to be susceptible and permissible to viral infection of the specific virus in focus	(Collins and Bauer, 1977; Hu and Hsiung, 1989)
Focus Forming assay (FFA)	Primary *in vitro* antiviral screening for viruses that do not induce CPE. Procedure identical to PRA. FFA doses are expressed as concentration units per milliliter (FFU/mL). Direct method.	- Faster than PRA or $TCID_{50}$ - Reading time varies depending on the replication cycle of the virus	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Indirect method - Expensive	(Flint et al., 2008)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Sensitive	- Specific reagents and equipment required - Specific cell lines that are known to be susceptible and permissible to infection with the virus of interest - Reading time of foci depends on the size of the area the operator is counting. A larger area will take longer, but may provide a more accurate representation of the sample. - Based on the antibody used, no discrimination between viable viruses and non-infective ones	
Hemagglutination inhibition assay (HIA)	Primary *in vitro* antiviral screening to detect infectious and noninfectious viral particles for viruses that do not form plaques or cause CPE. For HIA, viral samples are first mixed with dilutions of compounds that take time to bind the virus. Then red blood cells (RBCs) are added to the mixture. Antiviral activity: means that there are no free virus particles and the RBCs fall to the bottom of the well by gravity, creating a distinct red spot in a conical well. No antiviral activity: the erythrocytes clump together, resulting in a lattice-like structure. Indirect method	- Simple - Does not require special equipment - Fast evaluation of virus particles - Standardized protocols available - Validation of a modified HAI: more sensitive, easy to analyse, required only a single source of erythrocytes and allowed utilisation of virus strains which are difficult to handle by the standard HAI (e.g., H3N2, H5N1 and H1N1pdm09) - Infectious virus detection	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Less sensitive than other methods - Only for hemagglutinating viruses - The red blood cells used depend on the type of influenza virus in the test - Required source of suitable red blood cells (horse, rabbit, chicken, guinea pig) - Optimization of the type and concentration of red blood cells used is necessary to obtain reliable results. - Requires skilled personnel - Manual evaluation may lead to misinterpretation of results - Non-specific inhibition of hemagglutination possible - Low sensitivity	(Joklik, 1988; Morokutti et al., 2013)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			- Semiquantitative data	
Quantitative polymerase chain reaction (qPCR)	qPCR involves amplifying short stretches of longer genomic molecules in a thermocycler, a device that exposes the reaction to a series of different temperatures for a specified time (1 amplification cycle). With each PCR cycle, the amount of target sequence (amplicon) in the reaction theoretically doubles. In quantitative polymerase chain reaction, the amplification rate is monitored in real time during PCR using nonspecific intercalating fluorescent dyes or fluorescently labeled sequence-specific DNA probes. Direct method.	- Rapid (1-4h response) - Sensitive - High specificity - Possible to validate - Quantitative or semi-quantitative - Protocol needs to be adapted for each virus, but the general guidelines are the same	- Cell lines and/or viruses of BSL2 and BSL3 levels require a suitable facility - More complex compared to PRA - Need for specialized equipment: flow cytometry equipment - Need for trained personnel - Positive detection does not equate to viable (or infectious) virus, therefore not recommended for initial screening - Expensive	(Engstrom-Melnyk et al., 2015; Kralik and Ricchi, 2017)
Antioxidant assays				
DPPH (2,2'-diphenyl-1-picrylhydrazyl radical) assay	Based on the reaction of the tested antioxidant with the stable synthetic radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), accompanied by a color shift of the latter. Aliquots of the extracts are mixed with a methanolic solution containing DPPH radicals, and the mixture is incubated in the dark for 30 min. Absorbance is measured with a spectrophotometer at 517 nm. Usually, quercetin is used as a reference standard, and DPPH results are expressed as quercetin equivalents (QE) in µmol per 100 mL.	- Commercial kits available - Simple - Cost-effective - Good repeatability - Quantitative - Adequate for primary screening - Appropriate for high-throughput screening	- Applicable only for compounds soluble in organic solvents - Radical strongly affected by light, oxygen, pH and type of solvent - Steric hindrance effects for bulky antioxidants - Narrow linear range - Limited relevance to biological systems - Need for specialized equipment (spectrophotometer, multiplate reader)	(Apak et al., 2006; Awika et al., 2003; Molyneux, 2003)
ABTS/TEAC (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox equivalent antioxidant capacity	With the help of an oxidizing agent, the colorless ABTS salt is converted into its radical cation with characteristic blue-green color, which is then reduced back to its original colorless ABTS form by reaction with the tested antioxidant. Antioxidant activity is defined as the amount of ABTS·+ quenched after a given time (usually 5 minutes) and is expressed in Trolox (6-hydroxy-2,5,7,8-	- Rapid - Simple - Sensitive - Reproducible	- Limited relevance to biological systems - Difficulties in the formation of the colored radical and limited stability	(Apak et al., 2007; Awika et al., 2003; Erel, 2004; Lee et al., 2015; Re et al., 1999)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	tetramethylochroman-2-carboxylic acid) equivalents as TEAC (Trolox Equivalent Antioxidant Capacity).	- More sensitive than DPPH assay, high response to antioxidants - Can be performed in a 96-well microplate. - Diverse, flexible usage in multiple media (pH, solvents) - Applicable to both lipophilic and hydrophilic anti-oxidants - Commercial kits available - Quantitative - Adequate for primary screening	- Steric hindrance effects for bulky antioxidants - Specialized equipment required (spectrophotometer, multiplate reader)	
Cupric ion (Cu $^{2+}$) reducing assay (CUPRAC)	*In vitro* assay for measurement of the absorbance of the colored Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction between the chromogenic oxidizing CUPRAC reagent (i.e., Cu(II)-Nc) and the chain-breaking antioxidant under study. Trolox is used as the standard.	- Applicable to both lipophilic and hydrophilic antioxidants - Selective detection of antioxidants - Simulates antioxidant action under nearly physiological conditions - Ffavorable redox potential - High stability of reagents - No steric hindrance effects - Commercial kits available - Quantitative - Adequate for primary screening	- Unable to react with compounds having isolated hydrocarbon double bonds or alternating double and single bonds (e.g., ferulic acid, β-carotene) - An incubation at elevated temperature may be required for slow-reacting compounds (e.g., naringin and naringenin) - Need for specialized equipment (spectrophotometer, multiplate reader)	(Apak et al., 2007, 2006; Gulcin, 2020; Özyürek et al., 2011)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Appropriate for high-throughput screening		
Folin-Ciocalteu	The Folin-Ciocalteu phenolic reagent is used to obtain a rough estimate of the total amount of phenolic compounds present in an extract. Specifically, the phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in the reaction mixture, yielding a blue color proportional to the amount of phenols. The assay can be performed in a 96-well microplate. The absorbance is read at 760 nm and quantification is based on a calibration curve generated using gallic acid standards (GA).	- Adequate for primary screening - Simple - Reproducible - Excellent correlation between measured "antioxidant capacity" and "total phenolic content" - Quantitative - Commercial kits available - Adequate for primary screening	- Non-specific to phenolics (it reacts with many non-phenolic compounds) - not applicable to lipophilic components - Need for specialized equipment (spectrophotometer, multiplate reader)	(Apak et al., 2007; Bravo et al., 2016; Singleton et al., 1999)
Oxygen radical absorbance capacity (ORAC)	This method is based on the ability of antioxidants to protect fluorescein, a highly fluorescent protein, from oxidative damage caused by peroxyl radicals. The experimental procedure of ORAC involves the addition of the extract under study and a free radical, usually AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), which forms a moiety together with fluorescein, followed by heating in a phosphate buffer. Thermal decomposition produces free radicals that react with antioxidant compounds, resulting in loss of fluorescence due to decrease in radical concentration. The test can be performed in a 96-well microplate.	- Easily automated and largely standardized - adaptable for numerous sample matrices - High biological relevance - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- It is based on fluorescence detection and it requires more expensive instrumentation - Need for specialized equipment (fluorescence detection, multiplate reader)	(Awika et al., 2003; Bravo et al., 2016; Ou et al., 2001)
Anti-aging enzyme-based assays				
Anti-elastase	This *in vitro* assay is performed in Tris-HCl buffer and at room temperature using porcine pancreatic elastase (PPE; E.C.3.4.21.36) and N-succinyl-Ala-Ala-Ala-*p*-nitroanilide	- Rapid	- High cost and limited lifetime of enzymes used	(Pastorino et al., 2017; Thring et al., 2009)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	(Suc-Ala3-*p*NA) as substrate. Inhibition of PPE by natural extracts is determined spectrophotometrically by monitoring the release of p-nitroaniline from Suc-Ala3-pNA at 410 nm. Can be performed in a 96-well microplate. Epigallocathechin-3-gallate (EGCG) is commonly used as a positive control.	- Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	
Anti-collagenase	The ability of the extracts to inhibit collagenase activity is evaluated by a spectrophotometric method based on hydrolysis of the synthetic substrate N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) using collagenase from *Clostridium histolyticum* (ChC – EC.3.4.23.3). Can be performed in a 96-well microplate. EGCG is usually used as positive control.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	(Thring et al., 2009; Van Wart and Steinbrink, 1981)
Anti-hyaluronidase	*In vitro* assay that determines activity indirectly by measuring the amount of undegraded hyaluronic acid (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37°C.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Standardized protocol	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (turbidimeter)	(Bailey and Levine, 1993; Kim et al., 1995)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Commercial kits available - Quantitative		
Anti-tyrosinase	The ability of the extracts to inhibit the catalytic action of tyrosinase in the oxidation of L- DOPA, a precursor of melanin biosynthesis, is usually determined by an enzymatic procedure using the substrate L- DOPA and fungal tyrosinase followed by incubation in a phosphate buffer. The absorbance of the final solutions is measured at 492 nm using a microplate reader. Kojic acid (500 mM) is usually used as a reference inhibitor.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and in vivo conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	(Momtaz et al., 2008)
Anti-aging Fibroblast-based assays				
Cytotoxicity/cytoprotection	Cultured human fibroblast cell lines are pretreated with the samples and subjected to UV irradiation. Cell viability is measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The amount of formazan is measured by recording the absorbance changes at 570 nm with a spectrophotometer.	- Rapid - Precise - Avoids manipulation of radioactive isotopes - Constitutes a vital cellular setting and a real-life model for simulating oxidative damages and assessing the protective role of natural extracts/compounds	- Handling and preservation of human fibroblast cell lines can be cumbersome - Results should be interpreted with caution as the biological effect is evaluated against a specific type of cells (the interaction of the tested substance with other cell types are not taken into account) - Need for specialized equipment (cell culture, absorbance detection)	(Mosmann, 1983; Ramata-Stunda et al., 2013; Ratz-Lyko et al., 2012; Riss et al., 2019, 2004)
Regenerative potential	This assay involves exposure of seeded human fibroblast cells to extracts followed by washing with chemical reagents and measurement of procollagen type I or hyaluronic acid content in cell-free supernatants by enzyme-linked immunosorbent assay (ELISA).	- Constitutes a vital cellular setting and a real-life model for simulating oxidative damages and assessing the protective role of natural extracts/compounds	- Expensive - Results should be interpreted with caution as the biological effect is evaluated against a specific type of cells (the interaction of the	(Koudan et al., 2022)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			tested substance with other cell types are not taken into account) - Need for specialized equipment	
Pesticidal bioassays				
Feeding bioassay = poisoned food assay	Compound is incorporated into food (mixing in an artificial diet or producing a genetically modified plant) or spread/sprayed over food. Different parameters can be followed after exposure depending on the pest – e.g., survival, weight gain, size gain, offspring count, food consumption or a specific trait	- Simple - Easy interpretation - Qualitative or quantitative – depending on the set up	- Live animals (e.g., arthropods, gastropods) are used so a rearing facility is required - Dependent on test insect availability – laboratory cultures or seasonal collection - Time-consuming - Development of artificial diet or GM food can be challenging	(Burgess et al., 2020; Phan et al., 2020; Portilla, 2020; Razinger et al., 2014; Sanané et al., 2021; Šmid et al., 2015)
Volatile organic compounds (VOCs) Anti-insect activity test	The bioactivity of metabolites can be based on different mechanisms, two of which that are most often studied are to repel or to kill the insect.	- Simple - Easy interpretation - Qualitative or quantitative – depending on the set up	- Live animals (e.g., arthropods, gastropods) are used so a rearing facility is required - Dependent on test insect availability – laboratory cultures or seasonal collection - Time-consuming - Need for specialized equipment	(Daisy et al., 2002; Sternberg et al., 2014)
Other				
Enzymatic activity or inhibition of enzymatic activity	To determine enzymatic activity, the sample is incubated with the substrate in an appropriate buffer and at an appropriate temperature, and the reaction is followed by measuring absorbance or fluorescence change (depending on the substrate used). For inhibition of enzymatic activity, the sample is added to an enzyme in a suitable buffer, and after pre-incubation period of 10 to 60 min the substrate is added and the reaction is followed with a spectrophotometer or fluorimeter kinetically or at a selected endpoint (incubation time).	- For some enzymes SOPs (Standard Operating Procedures) available - Simple - Versatile - Quantitative or qualitative - Mechanism of action can be determined	- High cost and limited lifetime of enzymes used - Can be time-consuming - Optimization of conditions (buffer, pH, temperature, cofactors, incubation time) needed for each enzyme - Prone to false positive and false negative results	(Brooks et al., 2012; Mohan et al., 2018; Pohanka, 2019; Sabotič et al., 2009; Sepčić et al., 2019)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Commercial kits available for selected enzymes - High-throughput	- Enzyme inhibitors in the extracts may affect activity - Specific for each enzyme-substrate pair	
In-gel detection of enzymatic activity	Sample is resolved in polycrylamide gel under nondenaturing conditions and gel is then incubated in a series of solutions until colored or fluorescent bands appear.	- Additional info on size of enzyme - Can be simple one-step but also multiple step staining - Qualitative, can be semiquantitative	- Not all enzymes withstand the conditions of in-gel separation - Optimization of each enzymatic reaction required with many variables - Can take variable time for signal development (e.g., from minutes to days)	(Covian et al., 2012; Rivoal et al., 2002; Sabotič et al., 2007; Sepčić et al., 2019; Sims, 1965; Žun et al., 2017)

Table A14. The summary of selected validation guidelines and corresponding organizations.

Organization	Abbreviation	Sample Guideline(s)	Area of Interest	Remarks and References
European Medicines Agency	EMA	Guideline on bioanalytical method validation (EMEA/CHMP/EWP/192217/2009)	Bioanalytical assays for drug development studies (with all clinical trials)	Biological matrices such as blood, urine, tissues etc. (European Medicines Agency, 2011)
European Network of Forensic Science Institutes	ENFSI	Guidelines for the single laboratory Validation of Instrumental and Human Based Methods in Forensic Science	Forensic	Biological matrices such as blood, urine, tissues etc. (De Baere et al., 2014)
International Council for Harmonisation	ICH	Validation Of Analytical Procedures: Text And Methodology Q2(R1)	Pharmaceutical QC analyses	Pharmaceutical samples such as; Active Pharmaceutical Ingredient (API), finished drug samples (ICH Expert Working Group, 2005)
		Bioanalytical method validation and study sample analysis (M10)	Bioanalytical assays for drug development studies	Biological matrices such as blood, urine, tissues etc., Draft document (EMA, 2019)
United States Food and Drug Administration	USFDA	Bioanalytical Method Validation-Guidance for Industry	Bioanalytical assays for drug development studies (with all clinical trials) and for veterinary drug development as well	Biological matrices such as blood, urine, tissues etc. (USFDA, 2018)
Association of Analytical Communities	AOAC	Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals	Food & Feed Quality	Food and feed stuffs (Harnly et al., 2012)
International Union of Pure &Applied Chemistry	IUPAC	Harmonized Guidelines for Single laboratory Validation of Methods of Analysis	General terminology on analytical method characteristics	Sample matrices are not specified (Thompson et al., 2002)
European Directorate for the Quality of Medicines & HealthCare-The Directorate-General for Health and Food safety	EDQM/DG-SANTE	Analytical Quality Control and Method Validation; Procedures for Pesticide Residues Analysis in Food and Feed (SANTE/12682/2019)	Food & Feed Quality	Specified on the pesticide analysis in food and feed samples (Pihlström et al., 2019)

Organization	Abbreviation	Sample Guideline(s)	Area of Interest	Remarks and References
EURACHEM	n/a	The Fitness for Purpose of Analytical Methods- A Laboratory Guide to Method Validation and Related Topics	General terminology on analytical method performance characteristics	Sample matrices is not specified (Magnusson and Örnemark, 2014)
European Commission Joint Research Centre Institute for Health and Consumer Protection	ECJRC-IHCP	Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials (EUR 24105 EN - 1st edition/2009)	Food Quality	Migration analysis (from the food contacting part of the packing materials) (Bratinova et al., 2009)
United States Pharmacopeia	USP	General Chapter <1225> Validation of Compendial Procedures	Pharmaceutical QC analyses	Pharmaceutical samples such as Active Pharmaceutical Ingredient (API) and finished drug samples (USP 40, 2017)
United States Environmental Protection Agency	USEPA	Guidance for Methods Development and Methods Validation for the RCRA Program	Environmental analysis	Test Methods for Evaluating Solid Waste (SW-846) Methods (US EPA Office of Solid Waste, 1992)