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Unlocking marine treasures: isolation and mining strategies of natural products from sponge-associated bacteria†

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Marine sponges form unique ecosystems through symbiosis with diverse microbial communities, producing natural products including bioactive compounds. This review comprehensively addresses the key steps in the discovery of natural products from sponge-associated microorganisms, encompassing microbial isolation and cultivation, compound identification, and characterisation. Various cultivation methods, such as floating filter cultivation, microcapsule-based cultivation, and *in situ* systems, are examined to highlight their applications and strategies for overcoming limitations of conventional approaches. Additionally, the integration of genome-based methodologies and compound screening is explored to enhance the discovery of novel bioactive substances and establish a sustainable platform for natural product research. This review provides insights into the latest trends in sponge-associated microbial research and offers practical perspectives for expanding the utilization of marine biological resources.

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1. Introduction

1.1. The importance of marine sponges and symbionts

Sponges (Phylum: Porifera) are one of the oldest existing multicellular organisms, with fossil records indicating their appearance at least 600 million years ago.[1,2] Approximately 15 000 species of sponges have been described, primarily categorized into Calcarea, Hexactinellida, Homoscleromorpha, and Demospongiae.[3,4] Among these, Demospongiae represents the largest group, comprising the majority of currently known species.[5,6] The histological structure of sponges is unique, lacking homologous organs found in other animal species. The outer epithelial layer (exopinacoderm) consists of flattened exopinacocytes, while the aquiferous system, a network of canals responsible for water circulation and filtration, is lined with endopinacocytes and choanocytes. Choanocytes, equipped with flagella and microvilli, facilitate water pumping, nutrient uptake, and capturing microorganisms. The mesohyl, a connective tissue located between the outer epithelial layer and the aquiferous system, contains an extracellular matrix and various mobile cells. Among these, archaeocytes serve as macrophage-like stem cells, while lophocytes secrete the extracellular matrix, and spongocytes and sclerocytes generate skeletal elements.[7] Sponges are sessile filter feeders that efficiently capture suspended food particles

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Dr Jae Kyu Lim obtained his PhD in Marine Biotechnology from the University of Science and Technology, South Korea, in 2013. He is currently a scientist at the Marine Biotechnology Center, Korea Institute of Ocean Science & Technology (KIOST). His research focuses on metagenomic exploration of sponge-associated symbiotic microorganisms that produce bioactive natural products. He investigates microbial genes involved in biosynthetic pathways, including PKS, NRPS, and terpene cyclases. His current work emphasizes developing cultivation techniques for previously unculturable bacteria and applying heterologous gene expression to enable the biotechnological production of novel marine-derived compounds.

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Dr Si-sun Choi is a research Professor at the Inha University, South Korea. She received her bioengineering PhD at Inha University, focusing on isolation, genetic manipulation and characterization of the Tautomycetin biosynthetic gene cluster in Streptomyces *sp. CK4412. She also carried out postdoctoral research at Inha University, focusing on bioactive compound production from metabolic engineered* Streptomyces *and Corynebacteria. The main research interests of her are (i) genetic engineering for production of nature compound in* Streptomyces *(ii) discovery of microbial fungicide using actinomycetes genomics-driven approaches.*

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from the water.[8] This filtration introduces diverse microorganisms into their canals, allowing them to form mutualistic symbiotic relationships.[9] Sponge-associated microbial communities include archaea, cyanobacteria, heterotrophic bacteria, fungi, and microalgae, establishing complex symbioses.[10,11] These relationships involve extracellular, intracellular, and even intranuclear symbiosis, constituting 50–60% of the host's biomass.[12] Depending on their persistence, symbionts are classified as either transient or resident. Upon establishing stability and functionality, transient microbes become resident symbionts, forming integrated relationships with the sponge host.[13–15]

Sponges have evolved unique chemical defence systems in response to ecological pressures such as competition, overgrowth, infection, and predation. These defences involve secondary metabolites, including amino acids, nucleosides, polyethers, alkaloids, macrolides, porphyrins, terpenes, and steroids, many of which exhibit potent anticancer, anti-inflammatory, antifungal, and antiviral activities.[16] Between 2010 and 2019, a total of 2659 compounds were identified from sponges, making them a "goldmine" of bioactive compounds.[17] Interestingly, these bioactive compounds are primarily produced by sponge-associated microorganisms rather than the sponges. Among these, sponge-associated bacteria are the most extensively studied symbionts due to their ease of cultivation and the superior properties of their metabolites.[11] Structural similarities between natural products derived from sponges and bacterial metabolites suggest that many bioactive compounds likely originate from sponge-associated bacteria.[18–21]

In this context, numerous sponge-derived compounds have served as lead structures for pharmaceutical development, including clinically approved drugs such as cytarabine and eribulin.[22] However, translating these discoveries into therapeutics often faces the critical barrier of sustainable supply, as compound yields from natural sponge biomass are typically low, and large-scale harvesting raises ecological concerns.[23,24] Identifying the true producer, whether sponge or symbiont, is essential for enabling sustainable production strategies, such as microbial fermentation or synthetic biology. Moreover, the metabolite structure similarity between host sponges and their symbionts makes biosynthetic origin assignments increasingly complex, highlighting the need for integrated chemical and genomic approaches.[25–30]

Given these challenges, there is a growing need for integrated knowledge that bridges natural product chemistry, microbial symbiosis, and modern biotechnological tools. This review aims to provide an integrated overview of progress in the discovery of natural products from sponges and examines strategies for isolating and cultivating sponge-associated bacteria. Additionally, it discusses genome-based approaches for identifying biosynthetic gene clusters (BGCs) and their heterologous expression, as well as chemical-based approaches for the discovery of natural products.

1.2. Major natural products and their biological activities found in sponge symbionts in the last 5 years

Research on natural products derived from marine sponges has been extensively documented in numerous studies. Therefore, this section focuses on natural products reported in the past five years, categorizing them based on their biological activities to examine recent research trends (Table 1 and Fig. S1–S5†). Through this, we aim to gain a clearer understanding of the biological activity patterns and structural diversity of these compounds while highlighting the latest advancements in the field. Additionally, this review seeks to contribute to future natural product discovery by providing insights into research directions targeting specific biological activities.

1.2.1. Antimicrobial and antiparasitic activities. Marine sponge-derived natural products have yielded a wide array of antibacterial compounds, many of which show activity against clinically relevant pathogens, including multidrug-resistant strains. Several terpenoid compounds have demonstrated notable broad-spectrum or Gram-positive antibacterial activity. Secodinorspongin A **1**, a 3,4-*seco*-3,19-dinorspongian diterpenoid lactone from *Spongia* sp., exhibited inhibitory activity against *Staphylococcus aureus*, suppressing bacterial growth by 43%, 54%, and 75% at concentrations of 50, 100, and 200 μM, respectively.[31] Scalimides J **2** and K **3**, scalarane sesterterpenes from the same genus, exhibited antimicrobial activity against *Micrococcus luteus* and *Bacillus subtilis*, respectively, with MIC values ranging from 4 to 16 μg mL^{-1}.[32] In contrast, Phyllospongianes A **4**, B **5**, and D **6**, isolated from *Phyllospongia foliascens*, exhibit broad-spectrum antibacterial activity with MIC values ranging from 1 to 8 μg mL^{-1} against *S. aureus*, *E. coli*, *B. subtilis*, *Enterococcus faecalis*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Pseudomonas aeruginosa*.[33] Other compounds with unique structural features have also been reported. Sceptrin **7**, a bromopyrrole alkaloid from *Stylissa massa*, and stonikacidin A **8**, a 4-bromopyrrole alkaloid containing an L-idonic acid core from *Lissodendoryx papillosa*, both exhibit antibacterial activity against *S. aureus* and *E. coli*. The latter acts *via* sortase A inhibition, a novel antibacterial mechanism.[34,35] Additionally, 3,5-dibromo-6-(3′,5′-dibromo-2′-hydroxyphenoxy) phenol **9** from *Lamellodysidea herbacea* showed strong antibacterial activity with a minimum inhibitory dose (MID) of 0.078 μg per disk against *S. aureus* and *Klebsiella pneumoniae*.[36] A number of compounds target antibiotic-resistant bacteria, particularly MRSA and VRE. Aspergetherins A **10** and C **11**, isolated from *Aspergillus terreus*, inhibited methicillin-resistant *S. aureus* (MRSA) with MICs of 64–128 μg mL^{-1}.[37] Bacillimidazole F **12**, an imidazolium-containing compound from sponge-associated *Bacillus* sp. WMMC1349, also inhibited MRSA with a MIC of 38.3 μM.[38] Notably, xanthoelegansin **13**, a viomellein derivative from *Aspergillus elegans*, showed potent bactericidal activity against MRSA, vancomycin-resistant *Enterococcus* (VRE), and *E. faecalis*, with MICs ranging from 0.5 to 2 μg mL^{-1}.[39] Xishaeleganin B **14**, a sesquiterpene hydroquinone from *Dactylospongia elegans*, exhibited comparable potency to vancomycin, with MIC values of 1.5 μg mL^{-1} against *S. aureus* and *Streptococcus pyogenes*, and 3.0 μg mL^{-1} against *E. faecium*.[40] Several sponge-associated sources have yielded alkaloids and sterols with moderate to weak antibacterial activities. (+)- and (−)-Tedanine **15**, enantiomeric indolone alkaloids from *Tedania* sp., inhibited *E. faecalis* with a minimum inhibitory concentration (MIC) of 64 μg mL^{-1}.[41] 2-Ethylhexyl 1*H*-imidazole-4-

Table 1 List of natural products reported in the past five years

Activity classification	Compound	Origin	Activity	Ref.
Antimicrobial and antiparasitic activities	Secodinorspongin A **1**	*Spongia* sp.	Antibacterial activity against *S. aureus*	Tai *et al.*[31]
	Scalimides J **2** and K **3**	*Spongia* sp.	Antibacterial activity against *Bacillus subtilis* and *Micrococcus luteus*	Shin *et al.*[32]
	Phyllospongianes A **4**, B **5**, and D **6**	*Phyllospongia foliascens*	Antibacterial activity against *S. aureus*, *E. coli*, *B. subtilis*, *E. faecalis*, *V. vulnificus*, *V. parahaemolyticus*, and *P. aeruginosa*	Yu *et al.*[33]
	Sceptrin **7**	*Stylissa massa*	Antibacterial activity against *S. aureus* and *E. coli*	Kosgahakumbura *et al.*[34]
	Stonikacidin A **8**	*Lissodendoryx papillosa*	Antibacterial activity against *S. aureus* and *E. coli*	Tabakmakher *et al.*[35]
	3,5-Dibromo-6-(3′,5′-dibromo-2′-hydroxyphenoxy)phenol **9**	*Lamellodysidea herbacea*	Antibacterial activity against *S. aureus* and *K. pneumoniae*	Hanif *et al.*[36]
	Aspergetherins A **10** and C **11**	*Aspergillus terreus*	Antibacterial activity against MRSA	Li *et al.*[37]
	Bacillimidazole F **12**	*Bacillus* sp. WMMC1349	Antibacterial activity against MRSA	Yan *et al.*[38]
	Xanthoelegansin **13**	*Aspergillus elegans*	Antibacterial activity against MRSA, VRE, and *E. faecalis*	Kumla *et al.*[39]
	Xishaeleganin B **14**	*Dactylospongia elegans*	Antibacterial activity against *S. aureus*, *S. pyogenes*, and *E. faecium*	Chen *et al.*[40]
	(+)- and (−)-Tedanine **15**	*Tedania* sp.	Antibacterial activity against *E. faecalis*	Guo *et al.*[41]
	2-Ethylhexyl 1*H*-imidazole-4-carboxylate **16**	*Verrucosispora* sp.	Antibacterial activity against *H. pylori*, *S. aureus*, and *K. pneumoniae*	Chen *et al.*[42]
	3β-hydroxy-5α,6β-methoxyergosta-7,22-dien-15-one **17**	*Aspergillus asclerogenus*	Antibacterial activity against *S. aureus*	Wen *et al.*[43]
	Aspergilluone A **18**	*Aspergillus* sp. LS57	Antibacterial activity against *Mycobacterium tuberculosis H37Rv* and *S. aureus*	Liu *et al.*[44]
	Duryne **19**	*Petrosia* sp.	Antimycobacterial activity against *M. tuberculosis H37Rv*	Chhetri *et al.*[45]
	Odoripenoids A **20** and B **21**	*Streptomyces* spp.	Antifungal activity against *Candida albicans*	Wen *et al.*[46]
	Oceanalin B **22**	*Oceanapia* sp.	Antifungal activity against *Candida glabrata*	Makarieva *et al.*[47]
	7-Dechloronidulin **23**	*Aspergillus nidulans*	Antibacterial and antifungal activities against Gram-positive bacteria and *C. albicans*	Thi Hoang Anh *et al.*[48]
	Chlocarbazomycin C **24**	*Streptomyces diacarni* LHW51701	Antibacterial and antifungal activity against MRSA, *Mycobacterium smegmatis*, *Bacillus mycoides*, and *C. albicans*	Cheng *et al.*[49]
	Marmaricines A–C **25–27**	*Agelas* sp. aff. *marmarica*	Antibacterial and antifungal activity against MRSA and *C. albicans*	Youssef *et al.*[50]
	Bacillisporin A **28** and B **29**	*Talaromyces pinophilus* KUFA 1767	Antibacterial activity against MRSA and *S. aureus*, antibiofilm activity	Machado *et al.*[51]
	Spinolactone **30** Tenellic acid C **31** Neospinosic acid **32**	*Neosartorya spinosa* KUFA 1047	Inhibition of biofilm formation by *E. coli*, *S. aureus*, and *E. faecalis*	de Sá *et al.*[52]

Table 1 (*Contd.*)

Activity classification	Compound	Origin	Activity	Ref.
Ant and cytotoxic activities	Citronamine A **33**	*Citronia astra*	Actiparasitic activity against *Plasmodium falciparum*	Prebble *et al.*[53]
	Saframycin Y3 **34** Juglomycin E **35**	*Streptomyces* sp.	Inhibited *P. falciparum* lysyl-tRNA synthetase, showing high binding affinity	Gamaleldin *et al.*[54]
	Plakortinic acids C **36** Plakortinic acids D **37**	*Plakortis symbiotica* *Xestospongia deweerdtae*	Antiparasitic activity against *Plasmodium berghei*, with favourable ADMET profiles	Amador *et al.*[55]
	Furospinulosin-1 **38**	*Hippospongia fistulosa*	Induces apoptosis in triple-negative breast cancer spheroids	Guzmán *et al.*[56]
	Neviotin A **39**	*Haliclona fascigera*	Induces oxidative stress and mitochondrial dysfunction, leading to cytotoxicity in MCF-7 breast cancer cells	Saquib *et al.*[57]
	Phakefustatin A **40**	*Phakellia fusca*	Affects the PI3K/Akt pathway; modulates RXRα receptor	Wu *et al.*[58]
	(+)-Spondomine **41**	*Tedania anhelans*	Inhibits Wnt and HIF1 signalling; cytotoxicity against K562 leukemia cells	Jin *et al.*[59]
	Swinhopeptolides A **42** and B **43**	*Theonella swinhoei*	Disrupt Ras–Raf interactions	Kim *et al.*[60]
	2*Z*- and 6*Z*-onnamides A **44–45**	*Theonella conica*	Potent activity against HeLa and P388 cells	Nakamura *et al.*[61]
	Neopetrothiazide **46**	*Neopetrosia* spp.	Selectively inhibits PAX3-FOXO1-mediated transcription	Wang *et al.*[62]
	Scalarane-type sesterterpenoid (featuring C-24 pentenone E-ring) **47**	*Hyrtios erectus* and *Dysidea* spp.	Cytotoxicity against MDA-MB-231 breast cancer cells	Shin *et al.*[63]
	Neopetrosidines A–D **48–51**	*Neopetrosia chaliniformis*	Inhibit proliferation in HeLa/Fucci2 cells	Hitora *et al.*[64]
	Sarcotragusolides A1 **52**, A2 **53**, and B1 **54**	*Sarcotragus* sp.	Cytotoxicity against K562 leukemia and ASPC-1 pancreatic cancer cells	Xu *et al.*[65]
	24*R*,25*S*-luffariellin A **55** 24*R*-*O*-methyl-25*S*-luffariellin A **56** 24*R*-*O*-methyl-25*S*-manoalide **57**	*Luffariella* spp.	Cytotoxicity against leukemia cell lines	Lai *et al.*[66]
	24*R*,25*S*-manoalide **58**		Cytotoxicity against leukemia cell lines and induced mitochondria-dependent apoptosis and inhibited human topoisomerase I and II	
	Phyllospongiane C **59**	*Phyllospongia foliascens*	Cytotoxicity across multiple cancer cell lines	Yu *et al.*[33]
	Phyllofolactone S **60**	*Phyllospongia foliascens*	Selective activity against HCT116 and HN6 cells	Lu *et al.*[67]
	Ascandinine D **61**	*Aspergillus candidus* HDN15-152	Selectively inhibited HL-60 leukemia cells	Zhou *et al.*[68]
	(3*R*)-(3′,5′-Dihydroxyphenyl) butan-2-one **62**	*Ascomycota* sp. VK12	Cytotoxicity across various cancer cell lines; anti-inflammatory activity *via* NO inhibition in microglial cells	Quang *et al.*[69]
	Unguisol B **63**	*Aspergillus unguis*	Cytotoxicity in MDA-MB-231 cells; induced apoptosis with S-phase arrest, associated with down-regulation of BCL2L1 and AKT1	Bashari *et al.*[70]

Table 1 (*Contd.*)

Activity classification	Compound	Origin	Activity	Ref.
	19-Methoxy-dictyoceratin-A **64**	*Dactylospongia elegans*	Cytotoxicity activity across several cancer cell lines	Yu *et al.*[71]
	Halaminol E **65**	*Haliclona chrysa*	Cytotoxicity in the NCI-60 cancer cell panel; notable selectivity toward melanoma and colon cancer cell lines	Grkovic *et al.*[72]
	Batzelladines O **66** and P **67**	*Monanchora pulchra*	Cytotoxicity against drug-resistant prostate cancer lines; induced both apoptosis and autophagy	Dyshlovoy *et al.*[73]
	Pelorol **68**	*Dactylospongia elegans*	Cytotoxic to melanoma cells; caused G1 arrest and apoptosis through modulation of apoptosis-related genes and miRNAs	Carpi *et al.*[74]
	5-*epi*-ilimaquinone **69**	*Dactylospongia elegans*		
Immunomodulation and host defence-related activities	Secodinorspongin D **70**	*Spongia* spp.	Inhibited neutrophil-derived superoxide and elastase release	Tai *et al.*[31]
	Sponginolide **71**			
	Fistularin-1 **72**	*Ecionemia acervus*	Significant anti-inflammatory effects; reduced NO and prostaglandin E_2 production	Ji *et al.*[75]
	11,19-Dideoxyfistularin-3 **73**			
	17-Dehydroxysponalactone **74**	*Spongia* sp.	Inhibited superoxide anion generation and elastase release without cytotoxicity	Tai *et al.*[76]
	(−)-Sponalisolide B **75**	*Spongia* sp.	Inhibited superoxide anion generation	Tai *et al.*[76]
	Spongianol **76**	*Spongia* sp.	Suppressed superoxide and elastase release	Tai *et al.*[77]
	3β,5α,9α-Trihydroxy-24*S*-ethylcholest-7-en-6-one **77**	*Spongia* sp.	Exhibited superoxide inhibition	Tai *et al.*[77]
	(22*E*,24*S*)-Ergosta-7,22-dien-3β,5α-diol-6,5-olide **78**			
	Thalysiaketide A **79** and B **80**	*Clathria* (*Thalysias*) *vulpina*	Dual inhibition of COX-2 and 5-LOX	Chakraborty & Francis[78]
	Stylimassalins A **81** and B **82**	*Stylissa massa*	Reduced macrophage migration in a $CuSO_4$-induced zebrafish inflammation model	Liu *et al.*[79]
	(−)-Subertieslide B **83**	*Suberties* sp.	Suppressed NO production in LPS-stimulated RAW 264.7 macrophages	Li *et al.*[80]
	Ircinialactam J **84**	*Ircinia felix*	Inhibited human adenovirus type 5 by interfering with the viral life cycle prior to nuclear genome entry	Ruiz-Molina *et al.*[81]
	2-Bromoaldisine **85**	*Stylissa massa*	Inhibitory activity against HIV-1 Vpr-mediated suppression of cell proliferation in the TREx-HeLa-Vpr model	Win *et al.*[82]
	Ascandinine C **86**	*Aspergillus candidus* HDN15-152	Displayed potent activity against influenza A virus	Zhou *et al.*[68]
	Spiroetherone A **87**	*Dysidea etheria*	Inhibited angiogenesis in a zebrafish model	Jiao *et al.*[83]
	Cyclobutastellettolides A **88** and B **89**	*Stelletta* sp.	Significantly increased ROS production in murine peritoneal macrophages	Kolesnikova *et al.*[84]

Table 1 (*Contd.*)

Activity classification	Compound	Origin	Activity	Ref.
	Assimiloside A **90**	*Hymeniacidon assimilis*	Stimulated lysosomal activity and elevated intracellular ROS levels in RAW 264.7 macrophages	Kudryashova *et al.*[85]
Neuroactive and neuroprotective activities	Veranamine **91**	*Verongula rigida*	Selective binder to 5HT2B and sigma-1 receptors, exhibiting antidepressant activity in the forced-swim test without interacting with serotonin, norepinephrine, or dopamine transporters	Kochanowska-Karamyan *et al.*[86]
	Aaptamine **92**	*Aaptos aaptos*	Demonstrated analgesic effects as a delta-opioid receptor agonist in a neuropathic pain model	Sung *et al.*[87]
	Lamellosterols A–C **93–95**	*Lamellodysidea cf. chlorea*	Identified as anti-prion compounds, exhibiting inhibition of α-synuclein aggregation	Jennings *et al.*[88]
	Frondoplysins A **96** and B **97**	*Dysidea frondosa*	Inhibit protein tyrosine phosphatase 1B	Jiao *et al.*[89]
	Erectcyanthins A–C **98–100**	*Hyrtios erectus*	Inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase and 5-lipoxygenase, with erectcyanthin B exhibiting strong inhibition of HMGCR	Chakraborty & Francis[78]
	Eutypoid F **101**	*Penicillium* sp. SCSIO 41413	Inhibits PI3K, with potential as an anticancer lead compound	Ye *et al.*[90]
Other functional activities	Aaptodines A–D **102–105**	*Aaptos suberitoides*	Inhibit RANKL-induced osteoclastogenesis	Wang *et al.*[91]
	Tedaniophorbasins A **106** and B **107**	*Tedaniophorbas ceratosis*	Exhibited strong yellow colouration, UV absorbance, cyan fluorescence, and potential ecological functions such as UV shielding or bioluminescence	Hiranrat *et al.*[92]
	9,11-Dihydrogracillinone A **108**	*Dendrilla antarctica*	Antifouling activity	Prieto *et al.*[93]

carboxylate **16**, isolated from *Verrucosispora* sp., exhibited antibacterial activity against *Helicobacter pylori* (MIC = 8 μg mL^{-1}), *S. aureus* (16 μg mL^{-1}), and *K. pneumoniae* (64 μg mL^{-1}).[42] A novel ergostane-type steroid, 3β-hydroxy-5α,6β-methoxyergosta-7,22-dien-15-one **17**, derived from *Aspergillus asclerogenus*, inhibited *S. aureus* with an MIC of 64 μg mL^{-1}.[43] Aspergilluone A **18**, a chromone derivative from *Aspergillus* sp. LS57 isolated from the sponge *Haliclona* sp., exhibited antibacterial activity against *Mycobacterium tuberculosis* H37Rv (MIC = 32 μg mL^{-1}) and moderate activity against *S. aureus* (MIC = 64 μg mL^{-1}), while showing weak activity against *B. subtilis* and *E. coli* (both MICs = 128 μg mL^{-1}).[44]

Several sponge-derived compounds have demonstrated activity against fungal pathogens, mycobacteria, and in some cases, also exhibited antibiofilm effects. Among compounds with antimycobacterial or antifungal activity, Duryne **19**, a linear acetylene compound from *Petrosia* sp., was active against *M. tuberculosis* H37Rv, with a MIC of 1.4 μM.[45] Odoripenoids A **20** and B **21**, geosmin-related metabolites from *Streptomyces* spp. isolated from mesophotic sponges, showed antifungal activity against *Candida albicans* with MIC values of 16 and 32 μg mL^{-1}, respectively.[46] Likewise, oceanalin B **22**, a bipolar sphingoid tetrahydroisoquinoline β-glycoside from *Oceanapia* sp., inhibited *Candida glabrata* with a MIC of 25 μg mL^{-1}, and its activity was proposed to be linked to interference with the sphingolipid pathway.[47] In addition, 7-dechloronidulin **23**, a chlorinated depsidone isolated from *Aspergillus nidulans*, exhibited strong activity against Gram-positive bacteria and *C. albicans*, with MIC values ranging from 2–8 μg mL^{-1}.[48] Chlocarbazomycin C **24**, a chlorinated carbazole alkaloid from *Streptomyces diacarni* LHW51701, exhibited broad-spectrum antimicrobial activity against MRSA, *Mycobacterium smegmatis*, *Bacillus mycoides*, and *C. albicans*, with MICs of 32 μg mL^{-1} for all strains, and showed no cytotoxicity against human lung cancer cells.[49] Similarly, marmaricines A–C **25–27**, brominated pyrrole alkaloids from the Red Sea sponge *Agelas* sp. aff. *marmarica*, were active against MRSA and *C. albicans*. Specifically, marmaricines A and B inhibited MRSA with MIC values of 8 μg mL^{-1}, while B and C showed equivalent inhibition of *C. albicans* at the same concentration.[50]

In addition to direct antimicrobial effects, several sponge-derived metabolites demonstrated antibiofilm activity. Bacillisporins A **28** and B **29**, isolated from *Talaromyces pinophilus* KUFA 1767, exhibited antibacterial and antibiofilm activity, inhibiting MRSA and *S. aureus* with MIC values of 4–16 μg mL^{-1}, and effectively suppressing biofilm formation.[51] Furthermore, Fungal metabolites such as spinolactone **30**, tenellic acid C **31**, and neospinosic acid **32**, produced by *Neosartorya spinosa* KUFA 1047 isolated from a Thai sponge, significantly inhibited biofilm formation by *E. coli*, *S. aureus*, and *E. faecalis*, despite showing no direct growth inhibition (MIC > 64 μg mL^{-1}). At 64 μg mL^{-1}, tenellic acid C and neospinosic acid reduced biofilm biomass by up to 82.78 ± 0.51% for *E. coli*, 85.46 ± 0.25% for *S. aureus*, and 85.06 ± 0.42% for *E. faecalis*.[52]

Marine sponges have also yielded compounds with notable antiparasitic activities, particularly against *Plasmodium* species. Citronamine A **33**, an isoquinoline alkaloid isolated from *Citronia astra*, exhibited moderate antiplasmodial activity against both drug-sensitive (*Plasmodium falciparum* 3D7, IC_{50} = 4.3 μM) and drug-resistant (*P. falciparum* Dd2, IC_{50} = 5.8 μM) strains. It showed low cytotoxicity toward HEK293 cells (66% inhibition at 40 μM), indicating a selectivity index greater than nine. The compound features a novel pentacyclic spiro[indene-2,2′-pyrrolo[2,1-*a*]isoquinoline] scaffold, highlighting its potential as a lead for the development of antiplasmodial drugs.[53] From *Callyspongia siphonella*-associated *Streptomyces*, saframycin Y3 **34** and juglomycin E **35** were identified as potential inhibitors of *P. falciparum* lysyl-tRNA synthetase, showing high binding affinity in docking studies.[54] Plakortinic acids C **36** and D **37**, isolated from a two-sponge association of *Plakortis symbiotica* and *Xestospongia deweerdtae*, exhibited antiplasmodial activity against *Plasmodium berghei* (EC_{50} = 5.3 μM), along with favourable ADMET properties including high intestinal absorption and hepatic metabolism.[55]

1.2.2. Anticancer and cytotoxic activities. Marine sponge-derived natural products have yielded a wide array of cytotoxic compounds with significant anticancer potential. Several of these compounds demonstrate well-defined mechanisms of action. For example, furospinulosin-1 **38**, isolated from *Hippospongia fistulosa*, selectively induces apoptosis in triple-negative breast cancer spheroids, despite limited activity in 2D cultures.[56] Neviotin A **39**, isolated from *Haliclona fascigera*, induces oxidative stress and mitochondrial dysfunction, leading to cytotoxicity in MCF-7 breast cancer cells. At 25 μg mL^{-1}, it caused a 23.5% increase in late apoptosis and triggered the activation of *p53*, *BAX*, and *caspase-3*.[57] Phakefustatin A **40**, a kynurenine-bearing cycloheptapeptide from *Phakellia fusca*, acts as an RXRα modulator and down-regulates PI3K/Akt signalling, inducing G2/M arrest and apoptosis in cancer cells. It showed potent cytotoxicity against MCF-7 (IC_{50} = 3.4 μM), HeLa (6.2 μM), and NCI-H460 (7.1 μM), with no effect on normal cells at 100 μM.[58] Similarly, (+)-Spondomine **41**, isolated from *Tedania anhelans*, exhibits strong cytotoxicity against K562 leukemia cells (IC_{50} = 2.2 μM) and dual inhibition of Wnt (72.4%) and HIF1 (70.6%) signalling pathways.[59] Swinhopeptolides A **42** and B **43**, cyclic depsipeptides from the marine *sponge Theonella swinhoei*, inhibit Ras–Raf interactions with IC_{50} values of 5.8 and 8.5 μM, respectively.[60] Similarly, 2Z- and 6Z-onnamides A (**44** and **45**), isolated from *Theonella conica*, exhibited strong cytotoxicity against HeLa cells with IC_{50} values of 0.17 and 0.15 μM, and against P388 cells with IC_{50} values of 1.8 and 4.8 μM, respectively.[61] Neopetrothiazide **46**, a structurally unique isoquinoline–quinone alkaloid containing a thiazide moiety, was isolated from *Neopetrosia* spp. It selectively inhibits PAX3–FOXO1-mediated transcription, a key oncogenic driver in rhabdomyosarcoma, with an IC_{50} value of 1.4 μM, although nonspecific cytotoxicity was observed at concentrations ≥4.6 μM.[62]

Many sponge-derived cytotoxic compounds fall into structurally distinct classes. Among scalarane sesterterpenes isolated from *Hyrtios erectus* and *Dysidea* spp., a compound featuring a C-24 pentenone 'E-ring' (compound **7**) **47** exhibited potent cytotoxicity against MDA-MB-231 breast cancer cells (GI_{50} = 4.21 μM), while other analogues with differing 'E-ring'

modifications showed weaker activity.[63] Neopetrosidines A–D **48–51**, pyridine alkaloids from *Neopetrosia chaliniformis*, inhibited the proliferation of HeLa/Fucci2 cells with IC_{50} values of 1.2–2.6 μM. Among them, neopetrosidine A delayed the cell cycle from 16.9 to 36.2 hours by decreasing mitochondrial membrane potential and suppressing ATP production, suggesting a cytostatic mechanism *via* perturbation of cellular bioenergetics.[64] Sarcotragusolides A1 **52**, A2 **53**, and B **54**, butenolide sesterterpenes from *Sarcotragus* sp., showed moderate cytotoxic activity. Notably, A1 and A2 inhibited K562 leukemia cells (IC_{50} = 4.38 and 2.91 μM, respectively), while B showed activity against ASPC-1 pancreatic cancer cells (IC_{50} = 4.71 μM).[65] Among the isolated stereoisomers from *Luffariella* spp., 24*R*,25*S*-luffariellin A **55**, 24*R*-*O*-methyl-25*S*-luffariellin A **56**, 24*R*,25*S*-manoalide **57**, and 24*R*-*O*-methyl-25*S*-manoalide **58** showed the most potent anti-leukemic activity, with IC_{50} values ranging from 0.50 to 7.67 μM across Molt 4, K562, Sup-T1, and U937 cell lines. Notably, 24*R*,25*S*-manoalide induced mitochondria-dependent apoptosis and inhibited human topoisomerase I and II (IC_{50} = 1.80 and 1.18 μM, respectively), and significantly reduced Molt 4 tumor volume by 66.11% in a xenograft mouse model.[66] Of the phyllospongianes A–E isolated from *P. foliascens*, only phyllospongiane C **59** exhibited notable cytotoxicity across multiple cancer cell lines (IC_{50} = 0.7–2.0 μM), while others, such as A, B, and D, were primarily reported for their antibacterial activity.[33] In addition, phyllofolactone S **60**, a bishomoscalarane sesterterpenoid from the same sponge, demonstrated selective activity against HCT116 and HN6 cells.[67]

Fungal symbionts associated with marine sponges have also been a source of notable cytotoxic compounds. Ascandinine D **61**, an indole diterpenoid from *Aspergillus candidus* HDN15-152, selectively inhibited HL-60 leukemia cells (IC_{50} = 7.8 μM).[68]. (3*R*)-(3′,5′-Dihydroxyphenyl)butan-2-one **62**, a phenolic compound isolated from *Ascomycota* sp. VK12, exhibited moderate cytotoxicity against HepG2, MCF-7, and SK-Mel2 carcinoma cells with IC_{50} values ranging from 48.6 to 96.5 μM, and showed anti-inflammatory activity by inhibiting NO production in LPS-stimulated BV2 microglial cells (IC_{50} = 24.2 μM).[69] From *Aspergillus unguis*, a sponge-associated fungus, Unguisol B **63** showed cytotoxicity in MDA-MB-231 cells and induced apoptosis with S-phase arrest, associated with down-regulation of BCL2L1 and AKT1.[70]

Recent studies have also identified new compounds with selective or context-dependent cytotoxicity. A 19-methoxy-dictyoceratin-A **64** from *Dactylospongia elegans* showed moderate activity across several cancer cell lines (IC_{50} = 17.4–37.8 μM).[71] Halaminol E **65**, isolated from *Haliclona chrysa*, exhibited moderate cytotoxicity in the NCI-60 cancer cell panel with a mean GI_{50} value of 6.76 μM. Notably, it demonstrated selective activity against melanoma and colon cancer cell lines.[72] Batzelladines O **66** and P **67**, guanidine alkaloids from *Monanchora pulchra*, exhibited potent cytotoxicity against prostate cancer cell lines (PC3, PC3-DR, and 22Rv1) at low micromolar concentrations (IC_{50} = 1.4–2.1 μM), and induced apoptosis *via* caspase-3 and PARP cleavage, as well as cytoprotective autophagy characterised by LC3B-II upregulation and mTOR suppression.[73] Additionally, Pelorol **68** and 5-*epi*-ilimaquinone **69**, two meroterpenoids isolated from *Dactylospongia elegans*, exhibited cytotoxicity against 501Mel melanoma cells with IC_{50} values of 3.02 μM and 1.72 μM, respectively (72 h). Both compounds induced G1 cell cycle arrest and triggered apoptosis *via* modulation of apoptosis-related genes (*e.g.*, *BAX*, *BCL2*, *BIRC5*, *MCL-1*) and microRNAs (*e.g.*, upregulation of *miR-16-5p*, *miR-193a-3p*, and down-regulation of *miR-214-3p*).[74]

1.2.3. Immunomodulation and host defence-related activities. Recent studies have increasingly highlighted the potential of sponge-derived natural products as immune modulators and therapeutic agents for inflammatory conditions. Among these, secodinorspongin D **70** and sponginolide **71**, isolated from *Spongia* spp., belong to the 3,4-*seco*-3,19-dinorspongian and classical spongian diterpenoid lactone classes, respectively. Both compounds were shown to inhibit neutrophil-derived superoxide (20.4% and 22.0% inhibition, respectively) and elastase release (30.8% and 22.5%, respectively).[31] Similarly, a series of bromotyrosine alkaloids from *Ecionemia acervus* demonstrated significant anti-inflammatory effects in a Caco-2/THP-1 co-culture model. Notably, fistularin-1 **72** and 11,19-dideoxyfistularin-3 **73** markedly reduced nitric oxide (NO) and prostaglandin E_2 production, down-regulated iNOS and COX-2 expression, suppressed phosphorylation of MAPKs (p38, JNK, ERK1/2), and blocked nuclear translocation of NF-κB p65, leading to the overall reduction of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α.[75] Other sponge-derived compounds with neutrophil-targeted anti-inflammatory activity include 17-dehydroxysponalactone **74**, which inhibited superoxide anion generation (IC_{50} = 3.37 μM) and elastase release (IC_{50} = 4.07 μM) in fMLF/CB-induced human neutrophils without cytotoxicity. Similarly, (−)-sponalisolide B **75** exhibited superoxide inhibition (IC_{50} = 5.31 μM).[76] Spongianol **76**, a chlorinated, polyoxygenated labdane-type diterpenoid from *Spongia* sp., significantly inhibited neutrophil-derived inflammatory responses, suppressing superoxide anion generation by 76.7% and elastase release by 85.6% at 10 μM without cytotoxicity. In addition, two newly identified steroidal compounds, 3β,5α,9α-trihydroxy-24*S*-ethylcholest-7-en-6-one **77** and (22*E*,24*S*)-ergosta-7,22-dien-3β,5α-diol-6,5-olide **78**, exhibited moderate superoxide inhibition, showing 47.4% and 57.2% inhibition, respectively, at the same concentration.[77] Polyketides thalysiaketide A **79** and B **80**, isolated from *Clathria* (*Thalysias*) *vulpina*, showed dual inhibition of COX-2 (IC_{50} = 0.93 and 1.03 mM, respectively) and 5-lipoxygenase (5-LOX) (IC_{50} = 0.87 and 0.98 mM), with thalysiaketide A exhibiting stronger COX-2 selectivity than ibuprofen.[94] Likewise, stylimassalin A **81** and B **82**, two pyrrole-derived alkaloids from *Stylissa massa*, significantly reduced macrophage migration in a $CuSO_4$-induced zebrafish inflammation model at concentrations of 5 μM and 10 μM, respectively.[79] Another compound, (−)-subertieslide B **83**, a butenolide alkaloid isolated from *Suberties* sp., suppressed NO production in LPS-stimulated RAW 264.7 macrophages with an IC_{50} value of 40.8 μM.[80]

In addition to anti-inflammatory effects, several sponge-derived compounds have exhibited notable antiviral activity. For example, ircinialactam J **84**, isolated from *Ircinia felix*,

potently inhibited human adenovirus type 5 by interfering with the viral life cycle prior to nuclear genome entry.[81] 2-Bromoaldisine **85**, a brominated pyrrolactam alkaloid isolated from *Stylissa massa*, showed inhibitory activity against HIV-1 Vpr-mediated suppression of cell proliferation in the TREx-HeLa-Vpr model at 10 μM, with a proliferation recovery comparable to that of the positive control damnacanthal.[82] Furthermore, ascandinine C **86**, isolated from *Aspergillus candidus* HDN15-152, displayed potent activity against influenza A virus, with an IC_{50} value of 26 μM.[68]

Sponge-derived compounds have also shown potential in vascular activity regulation. Spiroetherone A **87** (IC_{50} = 2.4 μM) and B (IC_{50} = 8.7 μM), two sesquiterpene naphthoquinones from *Dysidea etheria*, inhibited angiogenesis in a zebrafish model. Notably, spiroetherone A also showed selective cytotoxicity toward NCI–H929, HepG2, A549, and SK-OV-3 cells (IC_{50} = 7.4–12.2 μM), but was non-cytotoxic to HEK293 cells at 32 μM.[83]

Lastly, a number of compounds have demonstrated immunostimulatory or ROS-modulating activities. Cyclobutastellettolides A **88** and B **89**, two C_{19} norterpenoids from the sponge *Stelletta* sp., significantly increased ROS production in murine peritoneal macrophages at non-cytotoxic concentrations. At 10 μM, cyclobutastellettolide A and B enhanced ROS levels by 93% and 90%, respectively, suggesting their potential as immunostimulatory agents.[84] Similarly, assimiloside A **90**, a glycolipid isolated from *Hymeniacidon assimilis*, stimulated lysosomal activity (25–57%) and elevated intracellular ROS levels (15–35%) in RAW 264.7 macrophages at non-cytotoxic concentrations ranging from 0.01 to 10 μM.[85]

1.2.4. Neuroactive and neuroprotective activities. Certain marine sponge-derived natural products target the central nervous system and show promising potential for the treatment of neurological and psychiatric disorders. Veranamine **91**, a compound with a unique heterocyclic structure derived from *Verongula rigida*, has been identified as a selective binder to 5HT2B and sigma-1 receptors. *In vivo* experiments exhibited antidepressant activity in the forced-swim test and displayed a distinct mechanism of action by not interacting with serotonin, norepinephrine, or dopamine transporters.[86] Aaptamine **92**, an alkaloid isolated from *Aaptos aaptos*, demonstrated analgesic effects in a neuropathic pain model. Studies have shown that it functions as a delta-opioid receptor agonist within the spinal cord and was reported to reduce pain sensitivity in a chronic constriction injury model. Additionally, it was suggested that aaptamine modulates neuropathic pain by down-regulating vascular endothelial growth factor and lactate dehydrogenase A expression.[87] Meanwhile, Lamellosterols A–C **93–95**, polyoxygenated sterol sulfates isolated from *Lamellodysidea cf. chlorea*, exhibited potent anti-prion activity against the [PSI^+] yeast prion (EC_{50} = 12.7, 13.8, and 9.8 μM, respectively). Among them, lamellosterol A additionally inhibited α-synuclein aggregation by 68.0% and directly bound to the protein in mass spectrometry assays. This suggests potential therapeutic relevance to prion and Parkinson's disease-related pathologies.[88]

1.2.5. Enzyme inhibitory activities. Marine sponge-derived natural products have yielded structurally diverse enzyme inhibitors with therapeutic potential. Frondoplysins A **96** and B **97**, terpene–alkaloid bioconjugates isolated from *Dysidea frondosa*, exhibit potent inhibitory activity against protein tyrosine phosphatase 1B, with IC_{50} values of 0.39 μM and 0.65 μM, respectively. Notably, frondoplysin A exhibited *in vivo* antioxidative activity over five times stronger than vitamin C.[89] Three cyanthiwigin-type diterpenoids, erectcyanthins A–C **98–100**, were obtained from *Hyrtios erectus* and evaluated for their activity against 3-hydroxy-3-methylglutaryl coenzyme A reductase. Erectcyanthin B showed the strongest inhibitory effect (IC_{50} = 0.07 mM), comparable to atorvastatin (IC_{50} = 0.08 mM), and also displayed moderate antioxidant (IC_{50} = 0.4 mM) and 5-lipoxygenase inhibitory activity (IC_{50} = 0.88 mM), indicating its potential as a multifunctional therapeutic candidate. Erectcyanthins A and C exhibited slightly weaker activity, but all three shared a common structural scaffold useful for structure–activity relationship analysis.[78] In addition, eutypoid F **101**, a new butenolide derivative isolated from the sponge-derived fungus *Penicillium* sp. SCSIO 41413 collected from the Beibu Gulf, exhibited potent phosphatidylinositol 3-kinase (PI3K) inhibitory activity (IC_{50} = 1.7 μM). Molecular docking studies supported its binding to the PI3K catalytic site, suggesting its mechanism of action and potential as an anticancer lead compound.[90]

1.2.6. Other functional activities. Several marine sponge-derived natural products have shown unique biological activities beyond conventional categories such as antimicrobial, cytotoxic, or neuroactive effects. These include anti-osteoclastic, fluorescence, and antifouling properties, which expand the functional diversity of sponge-derived metabolites. Aaptodines A–D **102–105**, hybrid alkaloids with a haptacyclic spiro naphthyridine-furooxazoloquinoline scaffold isolated from *Aaptos suberitoides*, were reported to inhibit RANKL-induced osteoclastogenesis. Among them, aaptodine D exhibited the strongest activity by significantly reducing TRAP-positive multinucleated osteoclasts and resorption pits in a dose-dependent manner (ED_{50} = 2.3 μM) without cytotoxicity, suggesting its potential application in bone disease therapeutic.[91]

Tedaniophorbasins A **106** and B **107**, isolated from the marine sponge *Tedaniophorbas ceratosis*, represent a structurally distinct class of pteridine alkaloids incorporating a rare thiomorpholine ring, a feature not previously observed in marine natural products.[92] Tedaniophorbasin A possesses a 2-imino functional group, while B is its corresponding 2-oxo derivative, and both structures display substitution at the C-7 position, a modification unprecedented among known pteridines of natural origin. Although these compounds exhibited no detectable activity in antimalarial, cytotoxic, or antitrypanosomal assays at concentrations up to 40 μM, their strong yellow colouration and pronounced UV absorbance prompted further analysis of their photophysical behaviour. Both tedaniophorbasins showed striking cyan fluorescence with dual excitation wavelengths (λ_{ex} = 280 and 419 nm) and a shared emission maximum at 490 nm, accompanied by large Stokes shifts (~14 000 cm^{-1}). These properties point to notable electronic transitions and potential excited-state rearrangements. The combination of broad UV absorption and intense visible

fluorescence suggests that these metabolites may function in roles beyond direct antimicrobial defence. Specifically, their photophysical traits support potential ecological functions such as UV shielding, serving as biological sunscreens, or as donor luminophores involved in marine bioluminescence systems. These findings underscore the importance of investigating not only bioactivity but also physicochemical traits when evaluating the functional diversity of sponge-derived natural products.

9,11-Dihydrogracillinone A **108**, a nor-diterpenoid from the Antarctic sponge *Dendrilla antarctica*, showed potent antifouling activity in a 90-day marine field assay. When incorporated into rosin-based paint at 25 mg%, it reduced macrofouling organism coverage on submerged panels to <10%, compared to ~80% in the control group. This result demonstrates its potential as an eco-friendly antifouling agent for marine applications.[93]

1.3. Trends in sponge-associated NP research

Over an extended period, natural product research from marine sponges has focused mainly on identifying novel compounds with biological activity, particularly through compound-first, bioactivity-guided isolation.[95,96] While this approach has yielded a remarkable array of structurally diverse metabolites, it presents several inherent limitations: the true producers of many compounds remain unknown, yields are often low and irreproducible, and many sponge-associated microorganisms remain uncultured.[95] These factors hinder sustainable supply, biosynthetic understanding, and the translation of promising metabolites into drug candidates.

In response to these challenges, the research focus is gradually shifting from compound-centred discovery toward microbe-centred and gene-guided strategies. Key developments include improved methods for separating and cultivating sponge symbionts, genome-based tools for predicting BGCs, and chemical-based approaches such as *in situ* detection and metabolomics.[20,97,98] These methods are increasingly used together to improve the reliability and scope of natural product discovery.[99–102]

Recent reviews have provided valuable insights into the structural diversity and biological activities of sponge-associated microbial metabolites, with particular attention to antimicrobial compounds and the taxonomic classification of their microbial sources.[103,104] Some have also discussed cultivation strategies or genome-based discovery in specific contexts.[105,106] However, these aspects have typically been treated separately, and a comprehensive perspective that integrates the full range of methodologies, from microbial separation to chemical-based detection, remains lacking.

This review builds on previous efforts by outlining how evolving methodologies such as microbial separation, cultivation, genome-based discovery, and chemical approaches are being applied in a complementary way. Instead of focusing solely on compound inventories, it brings together these strategies to provide a broader understanding of how sponge-associated microbial natural products are explored today.

2. Separation of bacteria from sponges

2.1. Importance of separating sponge-associated bacteria

Marine sponges form complex ecological networks through symbiosis with diverse microbial communities. Symbiotic microorganisms play essential roles in the survival of sponges, contributing to key biological functions such as pathogen suppression, nutrient cycling, and the production of bioactive compounds.[8,107] To accurately evaluate the physiological roles and ecological contributions of symbiotic microorganisms, it is essential to separate sponge-associated microorganisms from sponge hosts. The separation of symbiotic microorganisms from sponges is crucial for several reasons. First, separating microbial communities from sponges is essential for accurately identifying the producers of natural products. While most sponge-derived natural products are known to be produced by symbiotic microorganisms, there is an ongoing debate about whether certain compounds originate from the sponge itself or its symbiotic microbes.[108] Clarifying the origin of these compounds is crucial for the sustainable development of sponge-derived drugs and the establishment of stable supply chains for future drug discovery. For instance, swinholide A, a compound with potent cytotoxic activity, was discovered in *Theonella swinhoei*. A study using density gradient centrifugation revealed that swinholide A was detected exclusively in fractions containing unicellular heterotrophic bacteria rather than in sponge tissue.[109] This suggests that swinholide A originates from symbiotic bacteria rather than the sponge itself. This demonstrates that separating microbial communities is crucial for accurately determining the true producers of bioactive compounds. Second, separating microbial communities from sponges is essential for accurately evaluating the ecological roles of sponge-associated microorganisms. Sponges derive 56–99% of their daily carbon intake from dissolved organic matter (DOM),[110–116] making them significant contributors to marine carbon cycling and important ecological components of aquatic ecosystems worldwide. Sponge-associated microorganisms also play essential roles in nutrient cycling and nitrogen retention, making them crucial subjects of study for understanding the ecological functions of sponges.[117–121] Various studies have attempted to evaluate the individual contributions of sponge host cells and microbial symbionts by measuring DOM assimilation using stable isotope probing of phospholipid fatty acids (PLFAs) without separating the sponge and its microbial symbionts.[122,123] However, this approach only measures the fraction of DOM incorporated into PLFAs and fails to account for the total DOM assimilation. Recently, a study employed physical separation of sponges and their microbial symbionts to quantify DOM uptake, revealing biomass. In contrast, microbial symbionts recycle nitrogenous waste produced by the host, playing a complementary role in nutrient cycling.[124] In conclusion, separating microbial communities enables researchers to accurately identify microbial taxa and their associated metabolic pathways, as well as to better understand the complexity of nutrient exchange that sponge host cells primarily assimilate

DOM into cellular processes. This, in turn, facilitates a deeper understanding of the impact of sponge-microbe symbioses on marine biogeochemical cycles.

Finally, ensuring the reliability of metagenomic analysis of sponge-associated microbial communities requires minimizing contamination from host DNA and unrelated microbial DNA. Schmieder and Edwards (2011) pointed out that host-derived DNA acts as a major noise source in metagenomic data analysis, compromising the accuracy of similarity assessments and functional pathway interpretations.[125] Although they demonstrated that host DNA can be removed using the bioinformatics filtering tool DeconSeq, they indirectly suggested that the physical separation of host tissues and symbiotic microbes during sampling is essential for fundamentally improving data quality. Knights *et al.* (2011) further supported this view by showing that data refinement processes, such as host DNA removal, significantly enhance the accuracy of metabolic pathway analysis and similarity assessments of microbial communities.[126] They reported that datasets excluding host DNA more clearly revealed the structure and contributions of metabolic pathways. Pereira-Marques *et al.* (2019) reported that a high proportion of host DNA reduces the sensitivity of metagenomic sequencing, making it difficult to detect low-abundance microbial species.[127] Marotz *et al.* (2018) demonstrated that the chemical removal of host DNA increases the microbial DNA ratio, improving analytical accuracy.[128] These studies emphasize that combining physical separation with chemical host DNA removal during the sample preparation stage critically enhanced data quality. In conclusion, bioinformatics filtering and physical host DNA removal play complementary roles in metagenomic data analysis. By thoroughly excluding host DNA or combining physical and chemical separation methods during the initial sampling stage, researchers can reduce data noise and more clearly evaluate microbial communities' genetic contributions and metabolic pathways.

Separating microbial communities from host tissues in sponges is a critical step for the success of natural product discovery, ecological role research, and advanced genomic analysis. This separation improves data reliability, facilitates the interpretation of complex ecological networks, and maximizes the potential value of sponge-associated microorganisms.

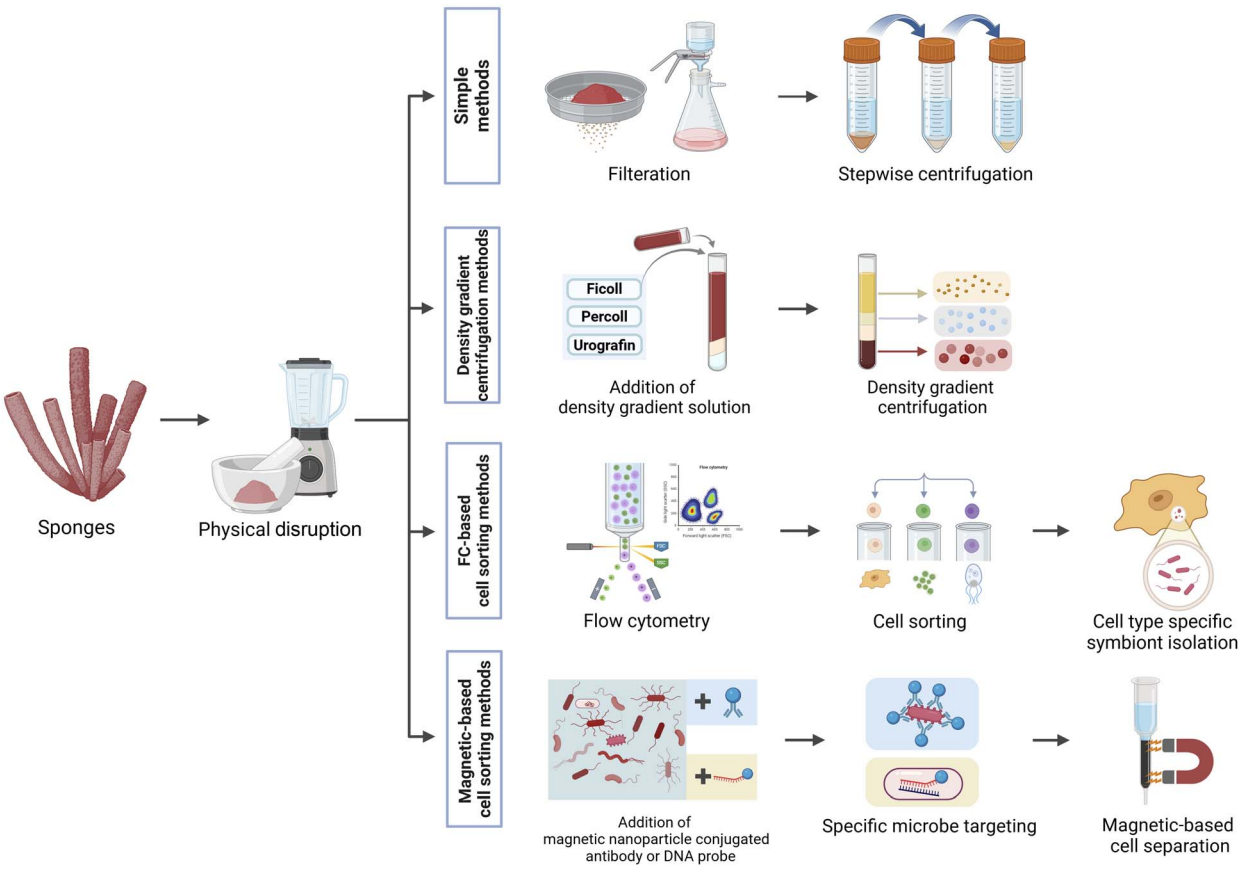

Fig. 1 Methods for isolating sponge-associated bacteria. This figure illustrates various approaches used to isolate sponge-associated bacteria following the physical disruption of sponge tissue. Simple methods allow for the preliminary separation of microbial cells from sponge debris. Density gradient centrifugation methods utilize solutions like Ficoll, Percoll, and Urografin to achieve finer separation. Flow cytometry-based cell sorting enables the separation of microbial population on size, complexity, and fluorescence characteristics, making it particularly useful for isolating intracellular symbionts residing within sponge cells. Magnetic-based cell sorting further enhances specificity by using magnetic nanoparticle-conjugated antibodies or DNA probes to capture target bacteria selectively. Created with BioRender.com.

Fig. 1 illustrates an overview of the major microbial separation techniques used in recent studies, highlighting their key steps and methodologies.

2.2. Simple methods for isolation and its limitations

Physical disruption, filtration, and centrifugation are widely used methods for isolating symbiotic microorganisms from sponge tissues. These methods are simple, cost-effective, and require no specialized skills, making them highly accessible and among the most commonly used techniques in sponge symbiont research. This approach typically involves cutting sponge tissues into small pieces and homogenizing them using a blender or a mortar and pestle. For example, in studies on *Aplysina aerophoba* and *Dysidea avara*, homogenized samples were filtered through a 100 μm nylon mesh to remove large tissue fragments. Low-speed centrifugation (770*g* or 1100*g*) was then used to pellet sponge cells, resulting in microbial cell fractions with over 99% purity.[129] Similarly, in a study on *Cymbastela concentrica*, sponge tissues were cut into ~1 cm^3 pieces, homogenized, and filtered through a 125 μm metal sieve to remove large debris. Subsequently, centrifugation at 100×*g* for 15 minutes eliminated the remaining sponge cells. Additional filtration through 11 μm and 3 μm filters captured microbial-sized particles, and high-speed centrifugation concentrated the bacterial cells into high-purity fractions.[130]

Following physical disruption and filtration, stepwise centrifugation further enhances the purity and recovery of microbial communities. In a study on *Halichondria okadai*, stepwise centrifugation (500*g*, 1000*g*, 3000*g*, 8000*g*) progressively removed sponge cells and enriched microbial fractions, reducing host-derived DNA to 0.02%. Metagenomic analysis confirmed that the enriched pellet was predominantly composed of bacteria.[131] Similarly, in research on *Discodermia dissoluta*, collagenase treatment (500 μg mL^{-1}) was employed to digest sponge tissues enzymatically, followed by blending and a combination of stepwise centrifugation and filtration, successfully increasing the purity of microbial communities.[132]

However, these simple methods have several limitations. The heat generated during physical disruption may negatively affect certain sensitive microorganisms, potentially hindering their cultivation. Excessive pressure during filtration can also cause microbial cell damage or loss. Furthermore, while stepwise centrifugation provides high-purity fractions, it cannot completely remove sponge cells, leaving trace amounts of host contaminants (*e.g.*, host DNA) that may introduce inaccuracies into analysis results. Despite these limitations, these methods remain a primary approach in sponge symbiont research due to their simplicity and ability to handle large sample volumes efficiently.

2.3. Density gradient centrifugation method

Sponges host diverse microorganisms, which can be categorized into intracellular symbionts residing within sponge cells and extracellular symbionts located outside the cells. Sponges consist of major cell types, such as archaeocytes and choanocytes, that harbor intracellular symbionts. Density gradient centrifugation is commonly employed to distinguish these microbial communities. This method isolates cells based on their physical properties, such as density and size, enabling effective separation of each cell type and facilitating independent studies of microbial communities associated with specific sponge cell types.[7,133]

Density gradient media such as Ficoll, Percoll, and Urografin are widely used for sponge cell fractionation, as they create gradients with varying densities and viscosities, enabling the effective separation of sponge cells and symbiotic microorganisms. Among these, Ficoll is particularly favoured in sponge research due to its ability to form stable density gradients while minimizing cell damage.[133–138] A study on *Eurypongia arenaria* demonstrates the application of Ficoll-based density gradients.[133] In the experiment, differential centrifugation at 100×*g* was first performed to remove most extracellular microorganisms, followed by a gradual increase in centrifugal force to fractionate the microbial community. Subsequently, Ficoll density gradient centrifugation was used to isolate archaeocytes from the 20–30% Ficoll layer and choanocytes from the 10–20% Ficoll layer. Each cell type was fractionated with over 70% purity, and DNA sequencing analysis of these fractions revealed that archaeocytes and choanocytes harbored distinct microbial communities. Specifically, Chlamydiae, Spirochaetes, and Candidatus Tectomicrobia were detected in the archaeocyte fraction, suggesting that these bacteria are likely endosymbionts. In contrast, a distinct microbial community was also observed in the choanocyte fraction, but it did not include the specific bacterial taxa found in archaeocytes. These findings suggest that each sponge cell type maintains a unique symbiotic microbial community and may have functionally distinct roles.

Percoll consists of colloidal silica particles coated with polyvinylpyrrolidone and can maintain its density gradient even under high-speed centrifugation, making it useful for specific cell fractionation studies. Due to these properties, Percoll may be a better choice than Ficoll for fractionation based on cell size and density differences.[139,140] This approach was applied in a study on *Haliclona* sp., where Percoll density gradient centrifugation was used to effectively fractionate sponge cells and symbiotic microorganisms.[139] The research team applied Percoll density gradients to separate nine major cell layers (A1–A9) based on size and density differences. Transmission electron microscopy (TEM) analysis revealed that layers A1–A4 primarily contained sponge cells, including choanocytes, archaeocytes, and mesohyl cells, whereas layers A5–A9 were composed mainly of symbiotic dinoflagellates and nematocysts. Chemical analysis of each cell layer showed that the cytotoxic alkaloids haliclonacyclamines A and B were detected exclusively in layers A1–A4, while no traces were found in the symbiotic dinoflagellate-containing layers A5–A9. Based on these findings, the researchers concluded that the cytotoxic alkaloids of *Haliclona* sp. are more likely to originate from sponge cells rather than symbiotic dinoflagellates. These results suggest that density gradient centrifugation can serve as a crucial tool for accurately identifying the producer cells of specific secondary metabolites.

A study has applied an approach utilizing the sequential use of Ficoll and Percoll to perform both initial fractionation and fine-scale fractionation. In the study by Flowers *et al.* (1998), a two-step density gradient centrifugation method incorporating Ficoll and Percoll was employed to achieve a higher-purity separation of sponge cells and the symbiotic cyanobacterium *Oscillatoria spongeliae* in *Dysidea herbacea*.[138] They first applied a discontinuous Ficoll density gradient ranging from 15% to 46%, fractionating the sample into seven distinct cell layers (A1–A7). Subsequently, layers A4–A7, which were identified to contain cyanobacteria, were further purified using a Percoll density gradient (20–80%), yielding four additional major cell layers (B1–B4). Upon further fractionation with the Percoll gradient, the B4 layer still contained some sponge cells, whereas layers B5–B7 exhibited a higher purity of cyanobacteria. Notably, in layers B6 and B7, *O. spongeliae* was fractionated with over 99% purity. These findings demonstrated that an initial fractionation using Ficoll followed by additional purification with Percoll enables the separation of sponge cells and symbiotic microorganisms with higher precision.

Urografin is an iodinated contrast agent with high density and viscosity, which can enhance the stability of density gradients due to its high viscosity. It can also be used as a density gradient medium for the separation of cells and bioactive compounds. Song *et al.* (2011) utilized Urografin density gradient centrifugation to study the intracellular localization of the bioactive compounds debromohymenialdisine (DBH) and hymenialdisine (HD) in *Axinella* sp. The research team first performed an initial cell fractionation using differential centrifugation, followed by the application of a 10–30% Urografin density gradient to separate four major cell layers (C1–C4).[141] TEM analysis revealed that the C1 layer was primarily composed of dark brown spherulous cells. High-performance liquid chromatography analysis showed that DBH (10.9%) and HD (6.1%) were detected at the highest concentrations in the C1 layer. Based on these findings, the researchers suggested that spherulous cells serve as the primary storage sites for DBH and HD. This demonstrates that Urografin can be used as an important tool for understanding the cellular localization and origin of metabolites.

Studies utilizing density gradient centrifugation have contributed to a more detailed understanding of sponge cells and their symbiotic microorganisms. As various density gradient media and methods continue to be applied, research on sponge-microbe interactions is expected to become more refined in the future.

2.4. Targeted cell sorting by using flow cytometry

Flow cytometry can be an effective technique for separating sponge cells and targeting symbiotic microorganisms. This method analyses and isolates individual cells based on differences in forward scatter (FSC) and side scatter (SSC) signals. These vary according to cell size, internal complexity, and fluorescence expression as cells pass through a laser beam in a fluid stream. This process is refined through fluorescence-activated cell sorting (FACS), which directly separates cells exhibiting specific fluorescence signals, enabling the high-purity isolation of target cell populations.[142]

Mukherjee *et al.* (2015) utilized flow cytometry to classify cells from the sponge *Eunapius carteri* into three major cell populations (P1, P2, P3) based on cell size and internal complexity.[143] The analysis revealed that the P1 group consisted of small cells such as choanocytes, small amoebocytes, and blast-like cells, while the P2 group comprised large amoebocytes and archaeocytes characterised by their complex internal structures. The P3 group included granular cells and sclerocytes, distinguished by their highly complex internal structures and numerous vesicles. Notably, the researchers observed the production of superoxide anions (O_2^-) and NO in the P2 group, indicating strong immune responses. This finding suggests that these cells play a critical role in maintaining symbiotic microbial populations while eliminating external pathogens.

In another study, sponge cells and the symbiotic bacterium *O. spongeliae* from *D. herbacea* were separated using flow cytometry.[144] The researchers obtained initial cell pellets from sponge tissues through centrifugation and successfully isolated *O. spongeliae* with over 95% purity using flow cytometry based on the natural fluorescence signal of phycoerythrin exhibited by the symbiotic cyanobacterium. Organic solvent extraction of the isolated symbiont fraction revealed that the major brominated metabolite, 2-(2′,4′-dibromophenyl)-4,6-dibromophenol, was detected exclusively in this fraction. These findings indicate that this metabolite is derived from the symbiotic cyanobacterium *O. spongeliae*.

In conclusion, these studies demonstrated that flow cytometry is a powerful tool for leveraging natural fluorescence signals to achieve high-purity separation of symbiotic microorganisms from marine sponges and to facilitate efficient research on their metabolites.

2.5. Targeted cell sorting by using magnetic-activated cell sorting

Magnetic-based cell separation techniques can separate and enrich a wide range of substances, from ions to cells. These techniques are relatively simple and cost-effective, making them an alternative to flow cytometry, which requires expensive equipment and highly skilled operators.[145–148] While direct applications of magnetic-based separation in sponge symbiotic microorganism research have not yet been reported, magnetic-based separation holds significant potential for high-efficiency selective cell sorting and is therefore introduced in this section as a promising approach.

Magnetic-activated cell sorting (MACS) is a technique that uses magnetic nanoparticles to bind to cell surface antigens and employs an external magnetic field to retain the labeled cells in a magnetic column (*e.g.*, MACS® Column), allowing for the separation of target cells.[149,150] A study applied MACS with pipette tips to demonstrate its potential for bacterial separation.[151] Researchers designed a highly magnetized 3D-printed pipette tip (MACS Tip) using commercial nickel mesh and neodymium magnets. They coated magnetic nanoparticles with

Table 2 Comparison of microbial separation techniques from marine sponges

Method	Principle	Advantages	Limitations
Simple physical methods	Physical breakdown of tissue and stepwise separation by size/density	- Easy and cost-effective - No specialized equipment required - Handles large sample volumes	- Possible microbial cell damage - Residual host contamination - Limited separation precision
Density gradient centrifugation	Separation based on cell density in a stable gradient medium	- Enables high-resolution fractionation - Reduces host DNA contamination - Allows cell-type-specific analysis	- Requires gradient optimization - Lower throughput - Specialized reagents needed
Flow cytometry	Cell sorting using FSC/SSC and fluorescence signals	- High precision and purity - Enables live cell separation - Useful for functionally distinct populations	- Expensive instrumentation - Requires trained personnel - Often needs fluorescent labeling
Magnetic-activated cell sorting	Magnetic labeling of target cells using antibodies or RNA probes	- Selective and high-purity enrichment - Relatively low-cost and scalable - Applicable to genome-level studies	- Requires target antigen or sequence - Cell fixation may be needed (for RNA probes)

mannose-binding lectin, which specifically binds to mannose residues on the lipopolysaccharides (LPS) of the *E. coli* outer membrane. By passing the bacterial mixture through the MACS Tip, selective separation of *E. coli* was achieved with a capture efficiency of approximately 90.5% ± 7.3%. These results demonstrate that magnetic-based cell separation techniques can exhibit excellent performance in removing non-target bacteria and enabling selective cell sorting. However, if target microorganisms lack specific antigens on their cell surface or possess extracellular capsules, antibody-based targeting can be challenging. Furthermore, non-specific binding and the presence of matrix contaminants may reduce the efficiency of MACS.

To overcome these limitations of MACS, magnetic probe-based cell fishing was developed.[152] Unlike traditional MACS, this technique targets intracellular genetic sequences rather than cell surface antigens for selective bacterial separation. Researchers targeted the 23S rRNA sequences of *E. coli* DH10B and *Acinetobacter* sp. ADP1 to generate biotin-labeled RNA probes, which were hybridized *in situ* with intracellular ribosomal RNA. Streptavidin-coated paramagnetic beads were then added to bind with biotin, imparting magnetism to the cells. These magnetized cells were introduced into a magnetic cell sorting device containing NdFeB films. Through this process, *E. coli* DH10B, initially at a concentration of only 0.04%, was enriched to a high purity of 98.46%, with a cell recovery rate of approximately 50%. Similarly, *Acinetobacter* sp. ADP1 was enriched to a purity of 85%, with a recovery rate of around 40%. These results indicate that magnetic probe-based cell fishing significantly improves both cell recovery and selective separation efficiency compared to traditional MACS technologies. This study highlights that magnetic cell sorting, when applied using nucleic acid-targeted approaches, can achieve higher sensitivity and accuracy than antibody-based MACS. However, the requirement for cell fixation during *in situ* hybridization of RNA probes limits its applicability to live cell separation. Therefore, this technique is a useful tool for genome-based sponge symbiotic microorganism research and can be complementarily used with antibody-based MACS, depending on research objectives. The various microbial separation techniques discussed in Section 2 are summarized in Table 2.

3. Cell culture attempts for sponge-associated bacteria

As previously mentioned, sponge-associated microorganisms are considered key producers of various bioactive compounds, making the cultivation of sponge-associated bacteria one of the most direct and essential approaches for obtaining natural products.[8,153–155] These symbionts depend on sponge-derived nutrients and chemical signals, allowing them to thrive in complex microenvironments. However, replicating their natural living conditions in the laboratory remains challenging. Due to these difficulties, current cultivation success rates are typically reported to range between 0.1% and 14%, significantly limiting the practical utilization of these valuable microbial strains.[156–158] To overcome these obstacles, researchers are actively developing innovative cultivation methods and tailored media formulations to improve the recovery and cultivation of new microbial strains. This section will introduce these cultivation methods and novel strategies to improve cultivation (Fig. 2).

3.1. Fine control of medium composition (*in vitro* cultivation methods)

3.1.1. Effects of low-nutrient media. Several studies have demonstrated the effectiveness of low-nutrient media and sponge extract-based media in enhancing microbial diversity.

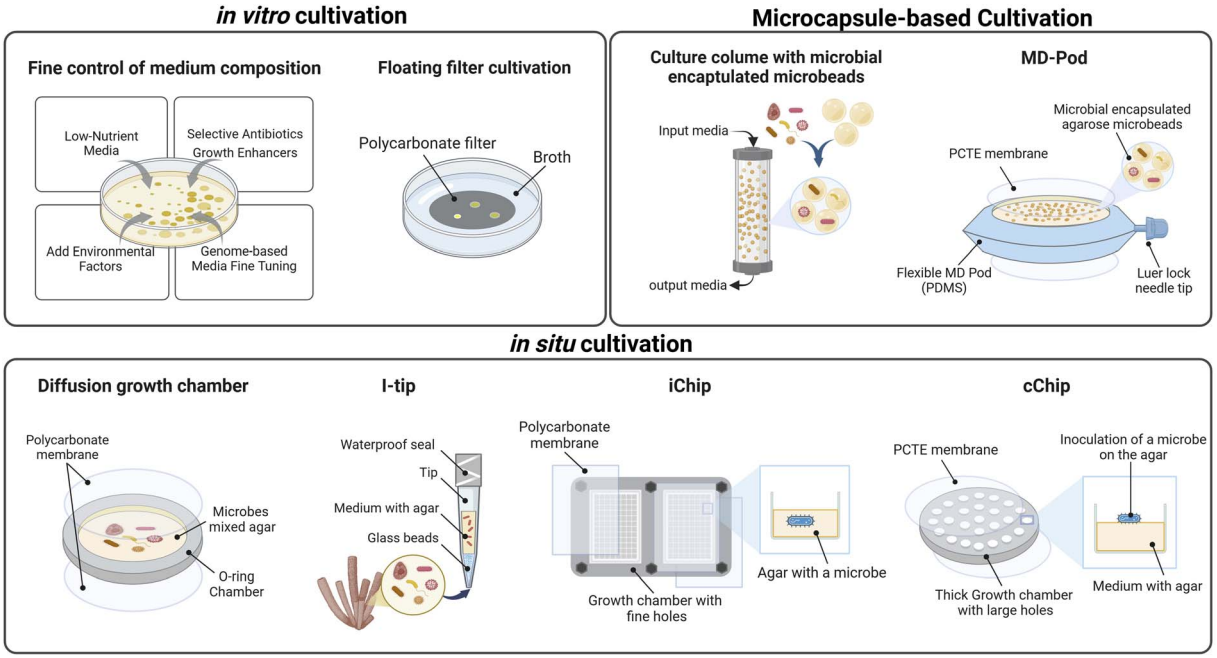

Fig. 2 Various cultivation methods for sponge-associated microorganisms. *In vitro* cultivation included fine control of medium composition and floating filter cultivation using polycarbonate membranes to enhance microbial growth. *In situ* cultivation using diffusion growth chambers, I-tip for microbial enrichment from sponges, and chip-based systems (iChip, cChip) that enable selective microbial growth through controlled environmental exposure. Microcapsule-based cultivation, including culture columns and MD-Pod systems, encapsulates single microbial cells in microbeads, allowing stable interaction with external growth factors while minimizing competition with other microbes. Created with BioRender.com.

The marine environments where sponges thrive are generally nutrient-poor, and this favours the dominance of slow-growing bacteria. For example, Sipkema *et al.* (2011) utilized low-nutrient media supplemented with sponge extracts and siderophores to isolate rare bacteria, such as Planctomycetes and Verrucomicrobia.[157]

Similarly, Esteves *et al.* (2016) replicated the natural sponge environment using diluted marine broth and sponge extracts to create nutrient conditions that mirror the sponge habitat.[159] Building on earlier findings that anaerobic metabolism, such as denitrification, and ammonium oxidation are prevalent in sponges,[160] this study employed nitrate (NO_3^-)-enriched media for anaerobic cultivation and ammonium (NH_4^+)-added media for aerobic cultivation. As a result, microorganisms involved in nitrogen cycling, including bacteria known for nitrate reduction such as Photobacterium and Desulfovibrio, were successfully isolated.

Lavy *et al.* (2014) applied high-nutrient and low-nutrient media under various oxygen conditions to observe cultivation efficiency.[161] Their results showed that 34% of OTUs grew in high-nutrient media, 49% in low-nutrient media, and 17% in both, indicating higher cultivation efficiency in low-nutrient media.

These findings indicate that low-nutrient media, those tailored to specific metabolic pathways, are crucial for enhancing the cultivation success of diverse sponge-associated bacteria.

3.1.2. Use of selective antibiotics and growth enhancers. The use of selective antibiotics and growth promoters during the cultivation process can suppress the growth of fast-growing dominant bacterial strains and effectively isolate rare, slow-growing microorganisms. In particular, inhibiting the rapid proliferation of Proteobacteria allows for the more effective isolation of Actinobacteria, a major microbial group involved in natural product production.

Selvin *et al.* (2009) incorporated sponge extracts, antibiotics, and signal molecules such as *N*-acyl-homoserine lactones (GMDs) into actinomycete-selective media to isolate Actinobacteria.[162] Among these, AHLs function as quorum-sensing molecules, activating microbial metabolic processes, regulating growth, and providing a competitive advantage to slow-growing bacteria. The application of these cultivation conditions significantly increased the diversity of isolated strains, with Actinobacteria accounting for 46% of all isolates.

Versluis *et al.* (2017) applied 13 individual antibiotics or antibiotic combinations to inhibit the growth of certain Proteobacteria strains and successfully isolated 27 previously uncultured OTUs.[163] Among them, 14 belonged to the Actinobacteria group, suggesting that selective antibiotic pressure can facilitate the isolation of specific bacterial taxa.

Montalvo *et al.* (2014) utilized various culture media and antibiotics to isolate 434 pure bacterial strains from *Xestospongia muta* and *Xestospongia testudinaria*, identifying over 90 bacterial species.[158] To maximize the diversity of culturable microorganisms from sponge tissues, the researchers applied 18 different culture media, with some media supplemented with selective antibiotics (*e.g.*, cycloheximide, nalidixic acid, nystatin) to regulate the growth of specific bacterial groups.

Through these methods, a diverse microbial community, including Actinobacteria, Firmicutes, and Proteobacteria, was successfully cultivated, with Actinobacteria exhibiting the highest diversity.

3.1.3. Application of environmental factors and specialized media. Integrating environmental factors is a crucial strategy for selectively cultivating microorganisms with specific metabolic capabilities. For example, Keren *et al.* (2015) designed an arsenic-enriched medium (AsO_4^{3-} and AsO_3^{3-}) to mimic the high arsenic accumulation characteristic of *T. swinhoei*.[164] In this study, the researchers first fractionated sponge cells and bacterial cells and found that arsenic was present in both fractions, though it accumulated at higher concentrations in the bacterial fraction. These results suggest that sponge-associated microorganisms may play a significant role in arsenic accumulation processes. To better replicate the natural sponge environment in the laboratory, the researchers formulated various culture media incorporating sponge skeletons and arsenic. By applying seven different culture media, they successfully isolated 54 bacterial strains, which were classified into 15 OTUs. Among these, 20 OTUs exclusively grew in media containing sponge skeletons, suggesting that certain microorganisms may possess physiological traits associated with sponge structures. This study highlights the importance of integrating environmental factors, such as sponge skeletons and chemical gradients, in the cultivation of sponge-associated microorganisms, demonstrating their effectiveness in isolating bacteria with unique physiological adaptations.

3.1.4. Insights from genome-based studies. Genome-based studies help improve the cultivation of sponge-associated microorganisms by revealing their metabolic needs and guiding the creation of suitable culture conditions. These approaches go beyond conventional methods, uncovering key metabolic pathways and environmental adaptation mechanisms of unculturable sponge symbionts, thereby supporting the development of more specialized and effective cultivation strategies.

For example, the major sponge symbiont Poribacteria possesses genes required for CO_2 fixation *via* the Wood–Ljungdahl pathway, suggesting its adaptation to carbon-limited environments.[165] Based on this, culture media with high CO_2 concentrations or low organic carbon content can be designed to promote its growth. Similarly, the sponge-associated archaeon *Cenarchaeum symbiosum* contains genes related to ammonia oxidation and CO_2 fixation.[166] This indicates that ammonia-supplemented media under low-oxygen conditions may facilitate its growth.

Furthermore, genomic analysis results of the sponge symbiotic cyanobacterium "*Candidatus* Synechococcus spongiarum" across three lineages revealed an abundance of genes related to DNA modification and recombination compared to free-living cyanobacteria, while genes associated with inorganic ion transport, metabolism, cell wall biosynthesis, and signal transduction were reduced.[167] Decreasing antioxidant enzymes and low-weight peptides in Photosystem II suggest unique physiological adaptations, such as siderophore-mediated iron transport and external methionine dependency. Consequently, culture conditions enriched with iron and supplemented with methionine may enhance their growth. Genomic analysis of sponge-associated *Entotheonella* spp. indicates that these microorganisms are likely mixotrophs, capable of utilizing various carbon sources but synthesizing only a limited set of proteinogenic amino acids (Cys, Met, Lys, Thr, Ser). Therefore, growth conditions may need to include diverse carbon sources and free amino acids to accommodate their metabolic characteristics. Additionally, their abilities for anaerobic respiration and sulfate reduction suggest that providing sulfate and maintaining controlled oxygen levels or anaerobic conditions could enhance their cultivation success.

Metagenomic studies of *A. aerophoba* revealed microbial groups enriched in genes associated with carnitine and sulfated polysaccharide metabolism, components abundant in the extracellular matrix of sponges.[168] Incorporating carnitine and sulfated polysaccharides into culture media could support the growth of sponge symbionts reliant on these resources.

To date, there have been few cases where genome-based research has directly led to the successful cultivation of sponge-associated microorganisms. However, genomic analysis provides crucial insights into their metabolic pathways, environmental adaptation mechanisms, and nutritional requirements. This information can help overcome the limitations of traditional cultivation methods and suggests the potential for designing tailored cultivation strategies based on specific metabolic traits. Future research should integrate experimental validation with genomic data to effectively enhance the cultivation success of sponge-associated microorganisms.

3.1.5. Floating-filter cultivation. The floating-filter cultivation method involves inoculating microbial cells onto a filter with a fine pore size and suspending the filter on the surface of a culture medium. The filter provides a growth surface for the cells while maintaining contact with the medium to ensure nutrient availability. Unlike conventional *in vitro* cultivation techniques conducted entirely under controlled laboratory conditions, this method partially simulates environmental exposure by allowing microbial cells to interact with diffusible factors across the medium–air interface.

Sipkema *et al.* (2011) applied the floating-filter cultivation method to culture sponge-associated microorganisms derived from *Haliclona* sp.[157] They used black polycarbonate filters (0.1 μm pore size) and observed that two types of colonies formed on the filter: macrocolonies and microcolonies. While microcolonies were barely visible to the naked eye, they accounted for approximately 80% of the total colonies, indicating a high frequency of occurrence. A total of 60 OTUs were isolated using the floating-filter method, among which 23 OTUs had not been cultured previously using conventional solid or liquid media.

Wiegand *et al.* (2018) successfully applied the floating-filter cultivation method to isolate microorganisms belonging to the Planctomycetes phylum from marine and freshwater environments.[169] The researchers placed culture media in six-well culture plates and used black polycarbonate filters with a 0.1 μm pore size to culture microorganisms in a floating state. The culture media contained various antibiotics, including cycloheximide, carbenicillin, and streptomycin, which were used to

inhibit the growth of fast-growing bacteria and selectively isolate specific microorganisms. Using this approach, the researchers isolated four novel species of Planctomycetes, which were previously unculturable using conventional methods. 16S rRNA gene sequencing confirmed that these isolates represented previously unreported microbial species.

These studies suggest that the floating-filter cultivation method can serve as an alternative approach for isolating microorganisms that cannot be cultured using traditional methods. In particular, this method may be a valuable tool for studying the physiological characteristics of specific microbial groups, such as Planctomycetes.

3.2. *In situ* cultivation methods

Researchers have developed *in situ* cultivation techniques that utilize natural habitats to overcome the limitations of laboratory-based cultivation methods. This approach directly places cultivation devices in marine ecosystems, allowing essential factors such as microbial interactions, nutrients, and signalling molecules to be maintained, providing optimal growth conditions for previously uncultivable bacteria.

3.2.1. Diffusion growth chambers. Kaeberlein *et al.* (2002) designed a diffusion growth chamber using two polycarbonate membranes with a pore size of 0.03 μm to more effectively cultivate environmentally challenging microorganisms.[170] This chamber employed a structure where the membranes were secured using circular stainless steel, and the interior was filled with agar medium containing microorganisms. This setup allowed for free chemical exchange with the external environment (diffusion of nutrients and signalling molecules) while preventing the direct movement of microorganisms. In this study, they used the diffusion growth chamber to culture microorganisms derived from intertidal marine sediment. The chamber was placed on the sediment layer inside an aquarium filled with seawater and incubated for one week. As a result, 40 ± 13% of the inoculated cells formed colonies, achieving a maximum recovery rate up to 300 times higher than conventional Petri dish cultivation methods. Additionally, some of the microorganisms cultured within the chamber acquired the ability to sustain growth on petri dishes afterward. This suggests that the diffusion growth chamber provides a favourable environment for the proliferation and isolation of microorganisms that were previously uncultivable using conventional methods.

Bollmann *et al.* (2007) applied the diffusion growth chamber to culture microorganisms in a freshwater pond sediment rather than a marine sediment environment.[171] The chamber used in this study consisted of a 0.03 μm pore-size polycarbonate membrane attached to a stainless-steel O-ring, with an interior filled with agar medium mixed with environmental samples. The chamber was then sealed and placed back into the natural environment for incubation. Over 16 weeks, four generations of diffusion growth chambers were used consecutively. Analysis of the distribution of cultured strains revealed that 59% of the newly isolated strains were detected exclusively through the diffusion growth chamber method. Specifically, 62 species of Alphaproteobacteria, 69 species of Betaproteobacteria, 7 species of Gammaproteobacteria, 1 species of Deltaproteobacteria, 12 species of Bacteroidetes, 5 species of Verrucomicrobia, 2 species of Acidobacteria, 4 species of Spirochaetes, 6 species of Firmicutes, and 6 species of Actinobacteria were successfully cultured. Among these, Verrucomicrobia and Acidobacteria required at least two generations of diffusion growth chambers before they could be successfully cultivated.

Steinert *et al.* (2014) were the first study to apply the diffusion growth chamber for the cultivation of symbiotic microorganisms within sponge tissue, utilizing a chamber with a structurally distinct design from previous studies.[172] Instead of the stainless-steel ring-based design, this study employed a modified centrifuge microfilter section with an integrated 0.2 μm pore-size filter, allowing direct insertion into sponge tissue. The research team homogenized *Rhabdastrella globostellata* sponge tissue and introduced a suspension of microbial cells mixed with various concentrations of culture media into the diffusion growth chamber. The chamber was then inserted into the sponge tissue and incubated for four weeks. As a result, a total of 255 sequences of 16S rRNA genes were obtained. Analysis of these sequences revealed the presence of 83 sequences from Alphaproteobacteria, 96 from Gammaproteobacteria, 19 from Bacteroidetes, 14 from Actinobacteria, and 43 from Firmicutes. Additionally, 15 of the sequences represented previously uncultivated strains. This study marked the first case of inserting a diffusion growth chamber into sponge tissue for cultivating sponge-associated microorganisms, demonstrating a novel approach distinct from previous studies based on marine or freshwater sediment environments.

3.2.2. Isolation tip (I-tip). The I-tip method is an innovative *in situ* cultivation technique developed by Jung *et al.* (2014) and first applied to freshwater sponges in Lake Baikal.[173] I-tip uses standard micropipette tips as primary components, creating a simple yet efficient cultivation device that allows microbes to grow by absorbing nutrients and signalling molecules naturally diffused from their habitats. The narrow end of the pipette tip is filled with fine glass beads and agar medium to prevent large particles from entering, while the broader end is sealed with waterproof adhesive. The design enables the device to function as a solid support structure that absorbs environmental nutrients, promoting microbial growth when inserted into sponge tissue. The research team successfully isolated 103 bacterial strains from *Baicalospongia* sp. and *Lubomirskia baicalensis* sponges using the I-tip method. Major bacterial phyla identified included Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Firmicutes, and Gammaproteobacteria. Actinobacteria and Alphaproteobacteria were notably absent in Petri dish cultures but were successfully isolated using the I-tip method. Additionally, another study showed that using I-tip cultivation on the sponge *T. swinhoei* resulted in higher microbial diversity and the isolation of new species from the Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria phyla, compared to standard direct cultivation methods. However, some microbial groups such as Cyanobacteria, Planctomycetes, and Verrucomicrobia were rarely isolated, indicating that the I-

tip method may require complementary cultivation techniques.[174]

3.2.3. Isolation chip (iChip). The iChip system, introduced by Nichols *et al.* (2010), leverages natural environments as cultivation chambers while physically isolating microbial cells to promote the formation of single-strain colonies.[175] The iChip consists of a central plate containing hundreds of small wells, each serving as a miniature microbial cultivation chamber. Environmental samples are diluted so that approximately one bacterial cell is introduced into each well. The plate is then covered with semipermeable membranes that allow nutrients and growth factors to diffuse from the environment while blocking microbial movement. The device is returned to the natural habitat for incubation.

Nichols *et al.* (2010) demonstrated that the iChip system enabled the cultivation of over 40% of bacterial cells in seawater samples and over 50% in soil samples.[175] This increase in cultivation efficiency represents a fivefold improvement in microbial recovery rates. Additionally, Berdy *et al.* (2017) reported that microbial recovery using iChip varied from 5- to 300-fold higher than traditional methods, depending on the specific environmental sample, further emphasizing its effectiveness in cultivating previously uncultivable species.[84,176]

The iChip system also supports high-throughput screening by simultaneously cultivating thousands of micro-wells. This feature has proven useful for large-scale antimicrobial activity screening, including the first discovery of the antibiotic teixobactin by Ling *et al.* (2015).[177] They tested approximately 10 000 microbial isolates obtained from soil samples using the iChip system for activity against *S. aureus*. This led to the discovery of a new beta-proteobacterium species, *Eleftheria terrae*, which exhibited strong antimicrobial activity. MacIntyre *et al.* (2019) demonstrated the successful application of iChip technology to marine sponges, specifically *Xestospongia muta*.[178] Researchers implanted iChips into sponge tissues and isolated a novel bacterial strain, *Alteromonas* sp. RKMC-009, which produced a unique *N*-acyltyrosine derivative with potent antimicrobial properties against Gram-positive bacteria.

Recent advancements have explored combining iChip technology with co-culture techniques to further enhance microbial cultivation. Lodhi *et al.* (2018) hypothesized that while iChip is effective in isolating single microbial species, some bacteria may depend on metabolic interactions with neighboring species for growth. Accordingly, they proposed that integrating co-culture techniques with iChip could enhance the cultivation of previously unculturable microorganisms by facilitating metabolic interactions between different bacterial species. However, further experimental validation is required to confirm the effectiveness of this approach.[179]

3.2.4. Culturing chip (cChip). Based on iChip, Lodhi *et al.* (2023) developed a cChip for the isolation of microorganisms from the aquatic environment.[180] The cChip was designed to enhance interaction with the environment by adjusting features such as the well diameter, chip thickness, number of membrane barriers, and operating protocol. These modifications create conditions that provide more stable growth in the aquatic environment for bacteria. This system demonstrated approximately five times higher microbial diversity than conventional Petri dish cultures, and a total of 45 new strains were isolated from the cChip. Notably, species such as Verrucomicrobia, rarely detected in standard cultures, were successfully isolated using cChip.

3.3. Microcapsule-based cultivation methods

3.3.1. Culture column with microbial encapsulated microbeads. Microcapsule-based cultivation is a single-cell cultivation system designed to encapsulate microbial cells within semipermeable gel materials such as agarose. This structure protects the cells while allowing the diffusion of nutrients and signal molecules from the external environment. This method addresses limitations of traditional cultivation, such as difficulties in isolating slow-growing strains in mixed cultures dominated by fast-growing microbes, and challenges in cultivating strains requiring essential environmental growth factors. In simple terms, microcapsules are produced using microfluidic devices and emulsion techniques, with each capsule serving as an independent cultivation chamber. Environmental cell samples are mixed with agarose, emulsified in a matrix through a microfluidic device, and formed gel microdroplets (GMDs). These GMDs are immobilized in columns that prevent external contamination while allowing the influx of nutrients and signal molecules for microbial growth.

Zengler *et al.* (2002) isolated microbial cells from marine samples and encapsulated them individually using agarose gel.[181] These cells were cultivated under low-organic medium in a growth column designed to mimic a flow-through system. After cultivation, microcolony included GMDs were discriminated by using flow cytometry. As a result, previously uncultivable microbial lineages such as Planctomycetes and the Cytophaga-Flavobacterium-Bacteroides group were successfully cultivated.

Toledo *et al.* (2006) separated symbiotic microbial cells from the marine sponge *Mycale armata* and encapsulated them in agarose-based gel microcapsules.[182] These capsules were cultured for five weeks in media containing sterile seawater diluted with sponge homogenates. Afterward, microcolonies formed within the capsules were isolated using flow cytometry, and Fourier-transform infrared (FT/IR) spectroscopy was employed for dereplication of redundant strains. Ultimately, the microcapsule-based cultivation method demonstrated approximately six times higher novel species isolation efficiency than traditional Petri dish methods, resulting in the discovery of five potential candidate strains which produce novel secondary metabolites.

3.3.2. Microbe domestication pod. Alkayyali *et al.* (2021) introduced the Microbe Domestication Pod (MD Pod), a system combining microbial encapsulation with *in situ* cultivation to address the challenges of cultivating hard-to-grow microorganisms.[183] The MD Pod encapsulates microbial cells in agarose microbeads through a microfluidic process, forming microbeads approximately 80–100 μm in size. These microbeads provide a protective barrier while promoting cell-to-cell interactions and maintaining microbial metabolic activity. To

facilitate nutrient exchange and signal molecule diffusion while preventing microbial migration, the MD Pod is equipped with a polycarbonate track etch (PCTE) membrane with 30 nm pores. The semipermeable membrane connects the internal cultivation environment to the external habitat, enabling continuous nutrient supply and natural metabolic signals. Various MD Pod designs were tested, and researchers ultimately selected a flexible MD Pod design due to its quick assembly and durability, making it well-suited for long-term deployments in marine environments. The MD Pod demonstrated up to five times higher microbial diversity compared to traditional cultivation methods, leading to the successful isolation of 45 novel bacterial strains. Notably, previously undetectable microbial species such as Verrucomicrobia were successfully cultivated using the MD Pod, emphasizing its effectiveness in improving microbial recovery from marine environments.

The micron-scale Microbial Domestication Pod (μMD Pod) was developed as an enhanced version of the original MD Pod, enhancing its adaptability for *in situ* cultivation within small marine invertebrates while maintaining its core design principles.[184] The μMD Pod features porous wall structures with 10 μm pores, fabricated using two-photon polymerization technology. These structures facilitate efficient nutrient and signal molecule exchange, eliminating the need for fragile filter membranes used in the MD Pod. This improvement allows the μMD Pod to perform effectively in challenging environments, such as marine sediments and small invertebrates, where conventional cultivation devices face limitations. Another distinguishing feature of the μMD Pod is its replacement of semipermeable membranes with porous structures, simplifying the microbial encapsulation and nutrient delivery process while maintaining natural interactions with the surrounding environment. In experiments, microbial strains such as *Sphingomonas phyllosphaerae* and *Streptomyces* sp. were encapsulated in agarose beads and incubated. Fluorescence microscopy confirmed stable cell growth within the μMD Pod, confirming its viability as an *in situ* cultivation device. In conclusion, the μMD Pod builds on the MD Pod's foundational concept, enhancing structural durability and cultivation efficiency, thereby broadening the potential for microbial isolation and bioprospecting in previously inaccessible environments.

4. Genome-based approach

Marine sponges harbor diverse microbial communities that play essential roles in the biosynthesis of bioactive secondary metabolites. Advances in genomic technologies have significantly enhanced our ability to investigate the biosynthetic potential of these sponge-associated microorganisms. Due to the challenges associated with cultivating marine microorganisms, genome-based strategies such as metagenomics, single-cell genomics, and heterologous expression have been developed. These approaches enable the identification and functional characterisation of BGCs leading to the discovery of novel natural products with pharmaceutical and biotechnological significance. This chapter discusses these genome-based methodologies and their applications in natural product discovery from sponge-derived bacteria (Fig. 3).

4.1. Metagenome-based NPs biosynthesis gene discovery

Despite continuous advancements in microbial cultivation techniques, more than 99% of all microbial species, including marine microorganisms, remain uncultivable. To overcome this limitation, metagenomics has emerged as a powerful approach for exploring BGCs from marine microbes that are difficult or impossible to culture.[185] By analysing total environmental DNA extracted directly from natural habitats, metagenomics enables the reconstruction of individual microbial genomes and facilitates the identification of gene clusters associated with specific metabolic functions.

Marine environmental DNA can be sequenced using advanced technologies such as next-generation sequencing, PacBio, and nanopore sequencing, generating sequence data that can be assembled into contigs. These contigs are classified based on genomic characteristics such as GC content and codon usage patterns, allowing the reconstruction of microbial

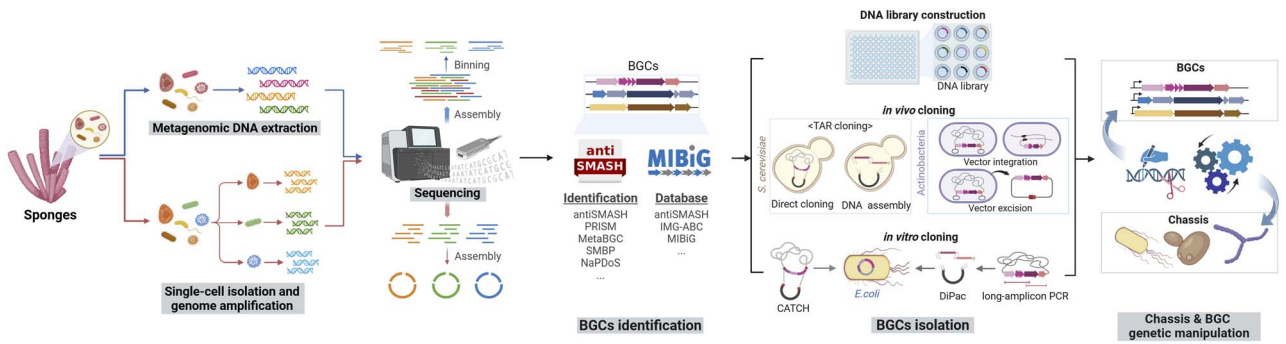

Fig. 3 Genome-based approach for biosynthetic gene cluster (BGC) identification and isolation from sponge-associated bacteria. The process begins with metagenomic DNA extraction or single-cell genome amplification, followed by sequencing, assembly and binning to reconstruct microbial genomes. Identified BGCs are analysed using *in silico* tools. BGC isolation involves constructing a DNA library and employing *in vivo* (TAR cloning, DNA assembly, vector integration/excision in Chassis) of *in vitro* (CATCH, *E. coli*-based cloning, DiPac, long-amplicon PCR) cloning strategies. BGCs can be introduced into heterologous chassis for expression, facilitating the discovery of novel natural products from sponge-derived microbial communities. Created with BioRender.com.

genomes. This process, known as binning, plays a crucial role in distinguishing genome fragments belonging to different microbial taxa. The identification of biosynthetic gene clusters through metagenomics is performed using bioinformatics tools such as antiSMASH, PRISM, MetaBGC, SMBP, and NaPDoS. These identified gene clusters can then be compared against large-scale databases such as antiSMASH, IMG-ABC, and MIBiG to analyse homology with known gene clusters and elucidate novel biosynthetic pathways.[186–194]

Although the identification of biosynthetic gene clusters from marine microbes remains a challenging task, metagenomics has proven instrumental in the discovery of bioactive natural products derived from marine invertebrates such as sponges, tunicates, and bryozoans.[195] Comparative metagenomics, which involves comparing metagenomic datasets from different environments, is a useful approach for identifying unique biosynthetic gene clusters within specific microbial communities. Recent comparative metagenomic studies have identified 5082 BGCs in high-microbial-abundance sponges, with most of them classified as novel clusters showing no homology to previously known gene clusters.[97] This study demonstrated that BGCs found in sponges are markedly distinct from those in surrounding marine environments and that certain BGCs are consistently present in specific sponge species. Notably, non-ribosomal peptide synthetase (NRPS) and ribosomally synthesized and post-translationally modified peptides (RiPPs) were found to be predominant, suggesting that these biosynthetic pathways are evolutionarily conserved among sponge-associated microbes.[97]

Targeted metagenomics is another approach that focuses on specific biosynthetic pathways or gene clusters associated with particular natural products, providing an advantage over conventional random metagenomic analysis in identifying functionally relevant biosynthetic gene clusters. This method selectively analyses target genes or pathways of interest, enhancing the identification of biosynthetic origins. It has played a critical role in determining the true microbial producers of marine-derived natural products with previously unclear origins. For example, the anticancer compound bryostatin, originally thought to be derived from the marine bryozoan *Bugula neritina*, was later confirmed to be produced by the symbiotic bacterium Candidatus *Endobugula sertula* through targeted metagenomic analysis.[196] Similarly, ET-743 (Yondelis®), a well-known anticancer drug initially attributed to the sea squirt *Ecteinascidia turbinata*, was later found to be biosynthesized by the symbiotic bacterium Candidatus *Endoecteinascidia frumentensis*.[197] By using metagenome-based biosynthetic pathway analysis, the complete biosynthetic route of ET-743 was identified, allowing its large-scale semi-synthetic production using an industrial microbial strain. This approach, adopted by the Spanish pharmaceutical company PharmaMar, enabled the commercialization of ET-743 as a novel anticancer drug, demonstrating the potential of metagenomics in natural product discovery. In addition to these examples, patellazole (antifungal), polytheonamide (anticancer), and calyculin A (phosphatase inhibitor) were also identified through targeted metagenomic analysis, significantly expanding our understanding of the complex biosynthetic pathways found in marine microorganisms.[198–200]

Currently, metagenomic research on marine-derived natural products is primarily focused on elucidating biosynthetic pathways and analysing functional genes encoding biosynthetic enzymes, yet several major challenges remain. In particular, while metagenomic sequencing has identified numerous biosynthetic gene clusters, a significant proportion of these clusters remain uncharacterised and are classified as silent biosynthetic gene clusters, which are not naturally expressed under standard conditions. To overcome this issue, efforts have been made to optimize heterologous expression systems and apply synthetic biology techniques to activate gene expression.

4.2. Single cell-based NPs biosynthesis gene discovery

In natural environments, uncultivated microorganisms constitute the majority of microbial diversity, and traditional culture-based studies have limitations in fully elucidating their physiological characteristics and genomic information. While microbial communities harbor numerous BGCs, analysing these genes in uncultivable species remains challenging. Conventional metagenomic approaches allow for the broad exploration of genetic diversity within microbial communities but are limited in resolving the genomic context of individual microorganisms. To address this limitation, single-cell genomics has emerged as a powerful approach, enabling the isolation of individual cells *via* FACS, followed by whole-genome amplification (WGA) and sequencing for BGC analysis. This method provides a means to directly analyse the genomes of individual microbes, overcoming the constraints of traditional techniques.[201]

Kvist *et al.* (2007) introduced a technique for targeting and isolating single cells from uncultivated microbial populations, followed by whole-genome amplification *via* multiple displacement amplification.[100] In this study, researchers employed fluorescence *in situ* hybridization (FISH) to label specific microbial cells, which were subsequently isolated using a micromanipulator, demonstrating the feasibility of genome analysis at the single-cell level even in non-cultivable microbes. While FISH-based cell sorting is effective for targeting specific microbes, it suffers from low throughput. To overcome this limitation, recent single-cell genomic studies have increasingly adopted FACS-based approaches to achieve rapid and high-throughput isolation of individual cells.[202]

In a study by Pachiadaki *et al.* (2019), this method was applied to isolate 12 715 single-amplified genomes (SAGs) from tropical and subtropical marine environments, enabling a systematic analysis of the biosynthetic potential of marine prokaryotes.[203] The study revealed that many SAGs contained diverse BGCs encoding terpenes, bacteriocins, polyketides (PKS), and NRPS, suggesting that marine microorganisms possess significant genetic potential for bioactive compound production. Moreover, single-cell genome analysis allowed for the phylogenetic characterisation of NRPS and PKS clusters, uncovering these biosynthetic pathways in microbial lineages not previously represented in public databases. This finding

expands the potential for discovering secondary metabolites from uncultivated marine microbes, reinforcing the value of single-cell genomics as a powerful tool for natural product discovery and biosynthetic pathway analysis.

A more recent study introduced MERMAID (Microfluidic Encapsulation, Raman Microscopy, and Integrated Digital Genomics), a novel method that integrates Raman microscopy with single-cell genomics for the targeted identification of secondary metabolite producers. MERMAID involves encapsulating individual cells within a microfluidic system, analysing their metabolic signatures using Raman microscopy, and selecting specific cells for single-cell genome sequencing. Kogawa *et al.* (2022) applied this technique to the symbiotic microbiome of the marine sponge *T. swinhoei*, successfully identifying an aurantoside producer that had previously remained undetected through metagenomic binning.[204] This led to the discovery of a novel Chloroflexi symbiont, 'Candidatus *Poriflexus aureus*,' which harbors a 14 Mbp genome enriched with multiple BGCs. Among these, the researchers identified a PKS-NRPS hybrid BGC responsible for aurantoside biosynthesis, along with 19 additional BGCs, underscoring the greater-than-expected chemical diversity within sponge-associated microbiomes.

Compared to metagenomic approaches, single-cell sequencing offers cleaner and less contaminated genomic sequences, facilitating the identification of previously undetected BGCs and enhancing the potential for novel natural product discovery. However, the WGA process introduces risks of DNA loss and amplification errors, and successful cultivation of strains identified through single-cell genomics remains rare. Despite these challenges, the integration of FACS-based single-cell approaches with metagenomics is expected to play a crucial role in advancing NP BGC discovery.[202]

4.3. Heterologous expression of NP BGCs

The first step in heterologous expression of NP BGCs is capturing the target BGC. Traditionally, this has been done through cosmid-, fosmid-, or BAC-based genomic libraries, which allow for the isolation of multiple BGCs. More recently, Transformation-Associated Recombination cloning in *Saccharomyces cerevisiae* has become an attractive option due to yeast's robust recombination capabilities, enabling the cloning of large BGCs exceeding 200 kb. Another method involves integrating *E. coli*-host shuttle vectors and restriction enzyme sites adjacent to native BGC loci, allowing for precise excision and manipulation of target BGCs. Advances in genetic engineering techniques, including Large PCR, CRISPR/Cas9, Gibson Assembly, and In-Fusion cloning, have further improved the efficiency of direct *in vitro* cloning, although challenges remain in handling large BGCs.[205,206]

Once isolated, BGCs can be expressed in their native form or undergo modifications to improve production yields and generate novel analogues. Engineering strategies involve modifying promoters, regulatory genes, post-modification genes, or even PKS domain structures to optimize BGC performance.[207–209]

Actinobacteria, particularly *Streptomyces* species, are the primary chassis used for NP heterologous expression due to their established genetic tools and secondary metabolite production capabilities. *S. coelicolor* and *S. lividans* are widely used model strains, while *S. albus* and *S. venezuelae* have gained attention due to their rapid growth and abundant precursor supply.[209,210]

To enhance chassis productivity, several modifications are commonly implemented, including the removal of native BGCs to reduce competition for precursors, mutation of ribosome binding sites to increase translation efficiency, elimination of negative global regulators, and introduction of additional attachment sites to amplify BGC copy numbers.

Marine-derived natural products pose unique challenges due to the specialized metabolic systems of their microbial producers. Recognizing this limitation, researchers have developed MGCEP 1.0 (Marine Gene Cluster Expression Platform 1.0), an optimized chassis strain derived from *Streptomyces atratus* SCSIO ZH16. Expression trials using MGCEP 1.0 successfully produced nineteen compounds, including the novel angucycline compound, demonstrating the effectiveness of marine-specific chassis systems for NP production.[211]

Despite these advancements, heterologous expression still faces limitations, especially for the expression of silent or cryptic BGCs from marine microorganisms. As a complementary strategy, BGC awakening approaches have emerged as an alternative to directly induce expression of these clusters. Traditional OSMAC (One Strain, Many Compounds) methods, such as altering salinity, temperature, pH, or co-culturing with other microbes, have shown success in activating silent gene clusters.[212] Moreover, recent synthetic biology techniques, including promoter refactoring, overexpression of pathway-specific regulators, and chassis optimization, enable targeted activation of these silent BGCs.[212] Integrating awakening strategies with marine-specific heterologous expression platforms is expected to greatly expand the accessible chemical space of marine-derived natural products, thereby accelerating novel compound discovery.

5. Chemical-based bacterial NPs mining method

5.1. *In situ* chemical capture using artificial marine sponge devices

An *in situ* capture method using artificial sponge devices was initially introduced as an alternative to traditional sponge sampling techniques.[213] This method allows for the direct collection of bioactive compounds from the environment without microbial cultivation.

La Clair *et al.* (2014) designed an artificial sponge device that mimics the physical structure and functional properties of natural marine sponges, and deployed it in marine environments for extended periods to successfully capture bioactive compounds.[213] The device is centred around a hollow-fiber bioreactor, which provides a surface for microbial colonization and includes a resin (Amberlite XAD-18) capable of

adsorbing metabolites. It operates in three main stages: (1) inoculation through the introduction of seawater from sponge-rich environments, (2) incubation for several days *in situ*, and (3) recovery of the device followed by extraction of adsorbed metabolites from the internal resin. This approach enables the collection of microbial metabolites while preserving the native microbial community, minimizing ecological disturbance, and offering a sustainable strategy for natural product exploration.

A notable compound group collected using this device includes jasplakinolide-class depsipeptides, which were found to have identical structures to those previously isolated from sponges in the same region. This result suggests that device-based strategies can complement or even replace traditional sponge sampling techniques in certain contexts. Such device-based *in situ* compound capture strategies were later adapted into simpler resin-based systems, as exemplified by technologies like SMIRC.

5.2. Small molecule *in situ* resin capture

While artificial sponge devices offer a means to collect metabolites from marine environments, their application typically requires complex equipment and external deployment. In contrast, the Small Molecule *in situ* Resin Capture (SMIRC) method provides a simpler and more direct approach by inserting resin adsorbents into sponge tissue itself, allowing compound capture to occur within the native microenvironment.

SMIRC enables the direct capture of small molecules from marine environments using resin adsorbents, bypassing the need for microbial cultivation (Fig. 4).[98] This innovative method integrates high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) to identify novel compounds while leveraging genomic tools to explore their biosynthetic origins. In this study, the researchers deployed the resin adsorbent HP-20, known for its efficacy in capturing marine toxins across various polarities, in diverse marine environments. Among the identified compounds were cabrillostatin, which demonstrated selective bioactivity against cancer cell lines, and the newly discovered compounds cabrillospirals A and B, which exhibited no notable bioactivity. To explore potential links between these compounds and corresponding BGCs, the researchers extracted DNA from environmental samples and conducted the untargeted shotgun metagenomic analysis. This approach identified 1843 BGCs, grouped into 631 Gene Cluster Families based on sequence similarity. Many of these BGCs were classified as NRPS, terpenes, and RiPPs, with most representing novel pathways not listed in the MIBiG database. In their search for a PKS-NRPS hybrid BGC potentially responsible for producing cabrillostatin, the researchers examined 43 BGCs with similar characteristics but found no matches capable of synthesizing the same structure. Based on the chemical structure of cabrillospirals A and B, they predicted that its biosynthesis might involve a modular T1PKS, halogenases, a *p*-hydroxybenzoic acid starter unit, and methyltransferases responsible for the C-2 hydroxymethyl and C-8 methyl groups. Among the 68 T1PKS BGCs identified, two promising candidate genes were found in high-quality MAGs from the phylum Planctomycetes, though cultivation attempts for these Planctomycetes were unsuccessful. These results show the persistent challenges in linking

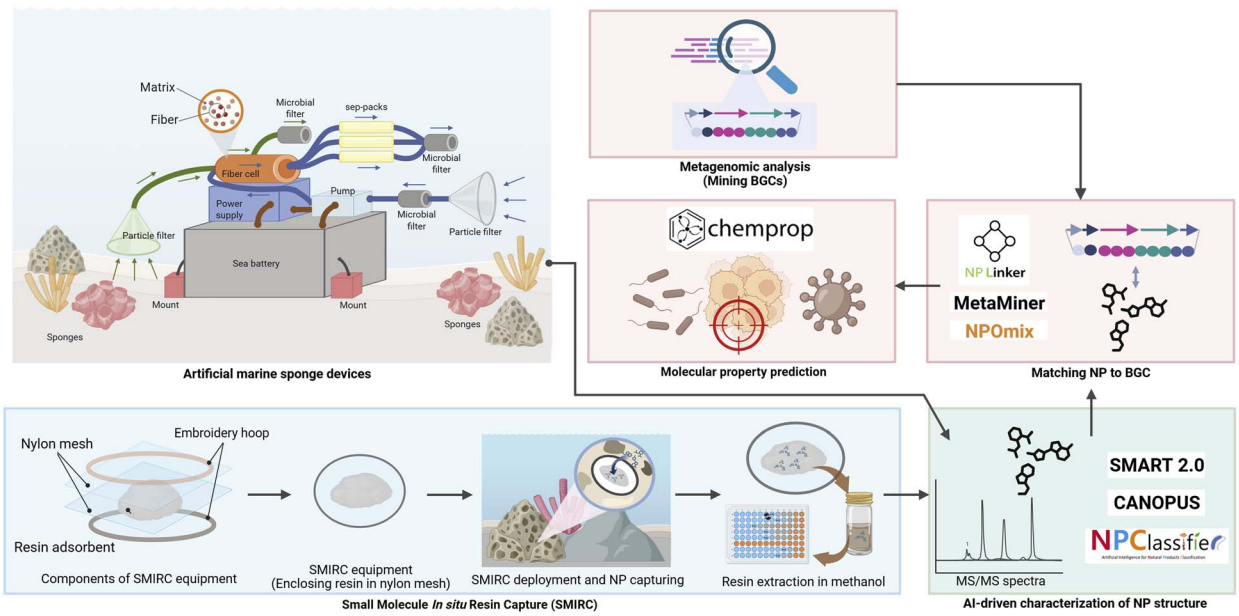

Fig. 4 A chemical-based strategy for bacterial NP discovery. This figure illustrates an integrated workflow combining *in situ* compound capture (artificial sponge devices and SMIRC), MS/MS- and NMR-based metabolomics, and genome mining to discover natural products from sponge-associated bacteria. AI-assisted tools such as SMART 2.0, CANOPUS, NPClassifier, and Chemprop are used to predict compound structures, biosynthetic origins, and bioactivities. Metabolite-BGC linkage is established through platforms including NPLinker, MetaMiner, and NPOmix, enabling the discovery of novel compounds without microbial cultivation. Created with BioRender.com.

chemical structures to BGCs within complex microbial communities. Although challenges in linking compounds to BGCs remain, SMIRC offers a cultivation-independent framework for discovering chemically diverse metabolites.

5.3. Integration of metabolomics and genome mining

Recent studies have begun integrating metabolomics and genome mining to rapidly elucidate the structures of unknown natural products and trace their biosynthetic origins.[214,215] At the core of this integrated strategy lies tandem mass spectrometry (MS/MS)-based metabolomic analysis. Data generated through MS/MS are utilized by platforms such as GNPS (Global Natural Products Social Molecular Networking), MolNetEnhancer, and CANOPUS to cluster compounds, classify metabolic features, and predict structures.[216–218] However, structure prediction alone is insufficient to identify the corresponding BGCs, and therefore, integration with genomic information is essential.

Recently, Louwen *et al.* (2023) developed NPLinker, a platform designed to facilitate this integration, and NPClassScore, a complementary scoring system that prioritizes meaningful links between MS/MS spectra and BGCs.[102] NPClassScore assigns likelihood scores to BGC-spectrum pairs based on chemical class ontology, thereby reducing false positives and enhancing link prioritization. Notably, NPClassScore quantitatively connects structure-based classifications (*e.g.*, ClassyFire, NPClassifier) with genome-based predictions (*e.g.*, antiSMASH), maintaining 96% of experimentally validated BGC-MS/MS links while reducing the number of candidate links by an average of over 63%, significantly alleviating the burden of manual inspection.

In cases such as RiPPs, where BGC architectures are often atypical and metabolic diversity is high, metabolomics-based information offers critical guidance to complement genome mining. For instance, MetaMiner enables structural-genomic linkage of RiPP compounds by comparing substructures derived from MS/MS spectra with predicted peptide sequences from BGCs.[219] This tool demonstrates high accuracy in predicting ribosomal core peptides and modification patterns, outperforming traditional spectrum-based comparison approaches. MetaMiner analysed approximately 10 million MS/MS spectra collected from eight diverse environmental sources, identifying 31 known RiPPs and 7 novel RiPPs. Among these, wewakazole was successfully isolated and structurally confirmed *via* NMR. Notably, in a sponge symbiont dataset derived from *Theonella swinhoei*, the genome of *Candidatus Entotheonella* sp. TSY1 was linked with ~223 000 MS/MS spectra, allowing the identification of several BGCs (including four RiPP-type clusters) and suggesting structural relationships with the observed metabolites.

NPOmix is a *k*-nearest neighbor-based model that integrates chemical fingerprints and biosynthetic features to predict links between MS/MS spectra and BGCs.[220] Utilizing 1040 paired omics datasets for training, NPOmix accurately predicted 13 MS/MS spectra corresponding to 11 experimentally validated BGC-spectrum links, including structural analogues. The model achieved a 92.9% top-3 accuracy, highlighting its high predictive power. Compounds such as albicidin, its analogues, and brasilicardin A were among those accurately linked, demonstrating the method's robustness.

These integrated approaches are especially effective in exploring unknown metabolites produced by complex microbial consortia such as sponge-associated microbiomes. By leveraging MS/MS data alongside genomic information, researchers can simultaneously infer structural characteristics and biosynthetic origins. Together, these tools enable metabolite-BGC linkage even in microbiomes where cultivation is not feasible.

5.4. AI-driven strategies for natural product discovery

Recent advances in artificial intelligence (AI) are reshaping multiple aspects of natural product-based drug discovery, particularly in chemically focused areas such as metabolomic analysis, structure elucidation, bioactivity prediction, and target identification. Among these domains, AI has demonstrated notable success in addressing the complexities of chemical data interpretation and in accelerating the identification of promising compound leads.

In metabolomics, AI has been instrumental in automating the analysis of large-scale MS/MS datasets and prioritizing compounds from complex mixtures. Tools such as CANOPUS and NPClassifier classify natural products into chemical classes and predict their biosynthetic origins directly from MS/MS spectra, even in the absence of complete structural information.[217,221] These classifiers have been successfully applied to the annotation of novel compounds such as rivulariapeptolide, offering insights into functional roles within structurally related families.[222]

AI has also advanced the field of structure elucidation. For example, SMART 2.0, a platform based on convolutional neural networks, enables rapid and accurate structural determination from NMR and MS data. This tool was used to resolve the structure of the complex macrolide symplocolide A with high efficiency.[223]

In addition, AI models are increasingly applied to predict the biological activity and potential molecular targets of natural products. A prominent example is Chemprop, a graph neural network-based framework that infers properties such as antimicrobial activity, toxicity, and protein target specificity from molecular structures.[224] This approach facilitated the discovery of halicin and abaucin as antibiotic candidates, both of which were subsequently validated and shown to act through mechanisms distinct from traditional antibiotics.[225,226]

These examples collectively illustrate AI's ability to enhance both the precision and scalability of chemically driven natural product discovery. When integrated with conventional methodologies, AI can produce synergistic gains across the pipeline, from dereplication and annotation to lead prioritization. Looking ahead, the convergence of AI with metabolomics, structure elucidation, and activity prediction is expected to yield unified platforms capable of accelerating early-stage discovery from natural sources while expanding the chemical and biological space accessible to researchers.

6. Conclusion

Sponge-associated microorganisms are key producers of bioactive compounds and have garnered attention as valuable resources for natural product discovery and understanding marine ecosystems. This review highlights the latest trends and technological advancements in sponge symbiont-based natural product research, showcasing the potential for continued progress in this field.

The precise isolation and cultivation of symbiotic microorganisms are necessary to discover natural products from the sponge. Recent advancements in physical and chemical separation techniques, along with fine-controlled cultivation strategies, have significantly improved cultivation rates and compound productivity by addressing the unique metabolic pathways and physiological requirements of these microorganisms. Cell separation techniques such as density gradient centrifugation and flow cytometry enhance the accuracy of microbial separation and clarify the origins of natural products.

The genome- and chemistry-based approaches are powerful tools for understanding the metabolic pathways for cultivation, exploring the BGCs and chemical properties of natural products from sponge-associated microorganisms. Genomic analysis enables the prediction of metabolic pathways and BGCs, providing critical insights into novel natural product structures and functions. Techniques like single-cell genomics and heterologous expression systems allow researchers to elucidate individual microbial gene functions and produce natural products under laboratory conditions. At the same time, chemical-based approaches bypass the need for cultivation by directly identifying natural products from environmental samples, facilitating the discovery of compounds that are otherwise challenging to access. Together, these approaches accelerate the research pipeline from sponge symbionts to drug development and expand the potential for discovering novel bioactive compounds.

Recent studies on sponge-associated microorganisms have transcended traditional limitations by incorporating techniques, offering a more systematic understanding of complex host–microbe interactions and natural product biosynthesis mechanisms. These multidisciplinary approaches provide a strong foundation for discovering new compounds and understanding the ecological roles of microorganisms.

Future research must integrate genomic analysis, chemical exploration, and advanced biological cultivation techniques to achieve a deeper understanding of the complex ecological interactions and metabolic pathways of sponge symbionts. This effort should prioritize developing novel technologies targeting unculturable microorganisms and creating sustainable strategies for natural product production and utilization. Such integrated approaches are expected to significantly contribute to the development of new therapeutics based on bioactive compounds from sponge-associated microorganisms while enhancing our understanding of microbial roles in marine ecosystems.

7. Data availability

No primary research results, software, or code have been included, and no new data were generated or analysed as part of this review.

8. Conflicts of interest

The authors declare no conflict of interest.

9. Acknowledgements

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