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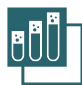

Taking Synthetic Biology to the Seas: From Blue Chassis Organisms to Marine Aquaforming

Lennart Schada von Borzyskowski*[a]

chassis organisms

genetic tools

marine synthetic biology

bioproduction

bioremediation

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Review
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Oceans cover 71% of Earth's surface and are home to hundreds of thousands of species, many of which are microbial. Knowledge about marine microbes has strongly increased in the past decades due to global sampling expeditions, and hundreds of detailed studies on marine microbial ecology, physiology, and biogeochemistry. However, the translation of this knowledge into biotechnological applications or synthetic biology approaches using marine microbes has been limited so far. This review highlights key examples of marine bacteria in synthetic biology and metabolic engineering, and outlines possible future work based on the emerging marine chassis organisms *Vibrio natriegens* and *Halomonas bluephagenesis*. Furthermore, the valorization of algal polysaccharides by genetically enhanced microbes is presented as an example of the opportunities and challenges associated with blue biotechnology. Finally, new roles for marine synthetic biology in tackling pressing global challenges, including climate change and marine pollution, are discussed.

1. Introduction

The oceans constitute over 90% of the habitable space on the planet and are home to at least 250000 known species.[1] The majority of those species are microbial organisms; it is estimated that 35000 different taxa of bacteria and archaea and 150000 taxa of micro-eukaryotes are living in marine habitats.[2] In the past decade, knowledge about these microorganisms has grown immensely, both due to the results of large-scale ocean sampling expeditions such as Tara Oceans[3] and a multitude of studies on specific marine microbial isolates by the global community of marine microbiologists. More recently, the priorities for ocean microbiome research were outlined, including the sustainable application of resources from oceanic microorganisms.[4] Indeed, new resources derived from the marine microbiota are increasingly applied in blue biotechnology. This field includes all biotechnological applications derived from aquatic environments.[5] In principle, blue biotechnology is built on the same scientific and technological principles as other areas of biotechnology, but the source, process, and/or final product is aquatic.[6] In extension, blue synthetic biology can be defined as synthetic biology that deals with aquatic chassis organisms, feedstocks, pathways, or other targets of biological engineering, as opposed to the terrestrial hosts and substrates that dominate conventional synthetic biology.

As an example of blue synthetic biology, it has been pointed out that marine algae and yeasts are promising hosts for future approaches in biofuel or biomass production due to their favorable properties for large-scale and low-cost cultivation; however, their full potential has yet to be fulfilled with the help of synthetic biology and metabolic engineering.[6] And this seems to hold true more generally: synthetic biology relying on marine microbes as hosts offers possibilities that cannot be realized with common terrestrial chassis organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*. This is due to intrinsic properties of marine microorganisms, such as their ability to grow at high salt concentrations, their adaptability to quickly changing environmental conditions, and their metabolic versatility that enables the utilization of unusual sources of carbon and energy. Despite these promising features, very few marine microbes have been developed as chassis organisms for synthetic biology so far. This is even more surprising since the potential fields of application for blue synthetic biology do not end at classical bioproduction approaches. A recent review on marine microorganisms in biotechnology mentioned the large potential for bioremediation and ecosystem restoration next to the more traditional goal of obtaining secondary metabolites that are relevant in medicine or cosmetics.[7] In fact, the investigation of marine natural products and the production of secondary metabolites by aquatic microbes constitute a thriving field of study, complete with its own journals and review series. Therefore, these topics will not be discussed here. In contrast, the applications of blue synthetic biology in bioproduction based on marine substrates as well as bioremediation have received much less scientific attention so far.

Consequently, this review aims to highlight promising approaches and established as well as emerging chassis organisms in blue synthetic biology (Table 1). It also outlines new areas of application for this young field, and describes the steps that are necessary to work towards these goals. When reading this overview of blue synthetic biology, some researchers will hopefully be inspired to consider marine chassis organisms for their future projects and contribute to the growth of the community. To speedily kick things off, the development of *Vibrio natriegens* as a fast-growing chassis for synthetic biology will be retraced.

2. *Vibrio natriegens*, the Fastest Bug in the Ocean

The facultatively anaerobic Gammaproteobacterium *V. natriegens* was first described more than 60 years ago,[20] and its claim to fame of being the fastest-growing bacterium known to date was established soon thereafter. An astounding generation time of less than 10 minutes when grown on rich medium was demonstrated;[21] nevertheless, this did not result in increased attention for this bacterium in the coming decades. Sporadic studies on *V. natriegens* physiology investigated the influence of sodium ion concentration on macromolecule synthesis,[22] the capability for N_2 fixation,[23] and the response to UV radiation[24]

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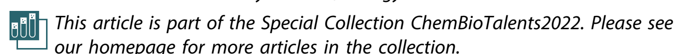
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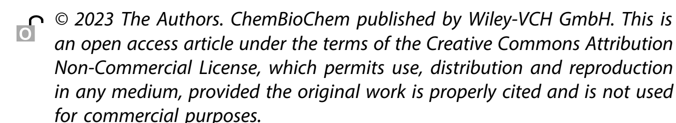
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as well as DNA repair by the *uvrAB* genes.[25] In accordance with its high growth rate, rRNA promoter activity was shown to be extremely high.[26] Furthermore, the draft genome sequences of two *V. natriegens* strains were published in 2013.[27] But by and large, research on this non-pathogenic *Vibrio* isolate was eclipsed by attention for its pathogenic relatives, such as *Vibrio cholerae* and *Vibrio parahaemolyticus*.

However, *V. natriegens* quickly moved to the center of attention of the global synthetic biology community when it was proposed as a fast-growing host for molecular biology.[28] The establishment of procedures for transformation and conjugation of plasmids and the development of the first genetic tools for this bacterium paved the way for a rapid succession of studies that raised *V. natriegens* to the status of an emerging chassis organism for biotechnology.[29] Several labs developed and benchmarked extensive genetic toolkits that include promoters, ribosome binding sites, and resistance markers,[8] numerous regulatory parts,[30] and synthetic promoters and 5′-UTRs.[31] Furthermore, *V. natriegens* was also at the center of the Grand Prize-winning project of the iGEM competition 2018,[32] which later resulted in a collection of 191 genetic parts for Golden Gate assembly.[33] To test novel genetic parts for this bacterium rapidly and efficiently, cell-free protein synthesis systems were developed by different groups.[34] Complementary to these approaches, the ability of *V. natriegens* to take up DNA from its environment was harnessed for multiplex genome editing by natural transformation[11] and also combined with CRISPR-Cas-based counterselection to enable more efficient protocols.[35]

The creation of genetic tools was complemented by a set of studies on *V. natriegens* physiology. ^{13}C metabolic flux analysis was applied to elucidate the metabolic network of the bacterium during growth on glucose,[36] and the substrate uptake rates under various conditions were determined.[37] The development of a CRISPRi-based functional genomics screen made it possible to identify a minimal set of genes required for rapid growth,[38] and it was discovered that extracellular electron transfer enhances anaerobic survival of *V. natriegens*.[39] Taken together, these findings generated valuable knowledge to adapt this bacterium as a synthetic biology chassis.

Consequently, the first studies that describe biotechnological production of value-added molecules using *V. natriegens* as a host have been conducted in the past years (Figure 1a). Recently, the production of 3,4-dihydroxyphenylalanine (L-DOPA) from catechol, pyruvate, and acetate,[40] the conversion of glycerol into propane-1,3-diol,[14] and the anaerobic production of succinate from glucose[41] were reported. More complex molecules, such as β-carotene and violacein, were also produced using engineered strains of this marine bacterium.[42] Furthermore, *V. natriegens* was transformed into an antibacterial platform by engineering an inducible toxin secretion system; this made it possible to study different effector activities and prey resistance mechanisms in a controllable system.[43]

It is likely that *V. natriegens* will be applied for other bioproduction processes in the near future due to its high growth rate, good protein expression capabilities, and ease of genetic manipulation.[44] A potential disadvantage might be the relatively high sensitivity of this bacterium to oxidative stress. But the crucial question for the future of the emerging *V. natriegens* field is whether this microorganism will be seen and used as a faster-growing version of *E. coli* in salt-rich medium, or whether its marine origin and additional metabolic capabilities compared to *E. coli* will help it to establish biotechnological applications that cannot be realized using its enterobacterial cousin. There are plenty of promising goals for future work, for example, bioproduction based on chitin or other polysaccharides that are abundant in marine settings, or the utilization of the natural N_2 fixation machinery of *V. natriegens* for (co)production of ammonia and derived nitrogen compounds. If creative bioengineers work towards achieving these goals, the full potential of this bacterium will be realized with the same rapidity as the division of its cells.

Lennart Schada von Borzyskowski received his Ph.D. degree in 2016 from ETH Zürich (Switzerland). During his postdoctoral stay at the Max Planck Institute for Terrestrial Microbiology (Marburg, Germany), he worked on the characterization and application of the β-hydroxyaspartate cycle, a metabolic pathway of global relevance that is found in marine bacteria. Lennart was appointed as an Assistant Professor at Leiden University (The Netherlands) in 2021. He leads a research group that investigates the metabolism of environmental bacteria and applies the findings towards novel sustainable approaches in biotechnology.

3. *Halomonas bluephagenesis*, the Prodigy of Bioplastic Production

The next microorganism that will be highlighted is also a Gammaproteobacterium boasting a long list of metabolic engineering feats that were achieved in a short span of time. *H. bluephagenesis* was isolated in 2011 from a salt lake in China[45] and is therefore not a marine, but a halophilic bacterium. Despite its relatively recent isolation, it is already one of the most successful chassis organisms in blue biotechnology, or more precisely blue polyhydroxyalkanoate (PHA) biosynthesis, as suggested by its name. This success story began with the report that *H. bluephagenesis* can be grown in non-sterile conditions on glucose, allowing for high cell density and polyhydroxybutyrate (PHB) content, which was then further increased by switching to a nitrogen-deficient medium.[45] Since the minimal medium used for cultivation of this fast-growing bacterium contains 6% NaCl and has a pH of 8.5 to 9.0, the risk of contamination is relatively low. As a prerequisite for further bioproduction studies, the strain's biosynthesis genes for PHA and the osmoprotectant ectoine were analyzed[46] and different genetic tools were developed. Genetic work with this organism is made more difficult by the fact that common transformation methods like heatshock or electroporation were not successful

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Table 1. Key achievements in the emerging field of blue synthetic biology.

Development of genetic tools
Comprehensive genetic toolset to engineer *V. natriegens*[8] Identification of T7-like expression systems for *Halomonas bluephagenesis* through phage genome mining[9] Genetic toolset suitable for diverse *Roseobacter* group bacteria[10]
Optimization of chassis organisms
Multiplex genome editing of *V. natriegens* by natural transformation[11] Increase of oxygen availability for *H. bluephagenesis* by using periplasmic hemoglobin[12] Physiological investigation of plasmid-cured *Phaeobacter inhibens* strains[13]
Bioproduction
Production of propane-1,3-diol from refined and crude glycerol using *V. natriegens*[14] Rational flux-tuning to produce PHB and ectoine using *H. bluephagenesis*[15] Conversion of alginate and mannitol into ethanol and butane-2,3-diol using *Vibrio* sp. dhg[16]
Bioremediation
Implementation of PETase in marine alga *Phaeodactylum tricornutum*[17] Engineering of three pollutant degradation routes into *V. natriegens*[18] Identification of key (marine) bioprocesses to help the planet[19]

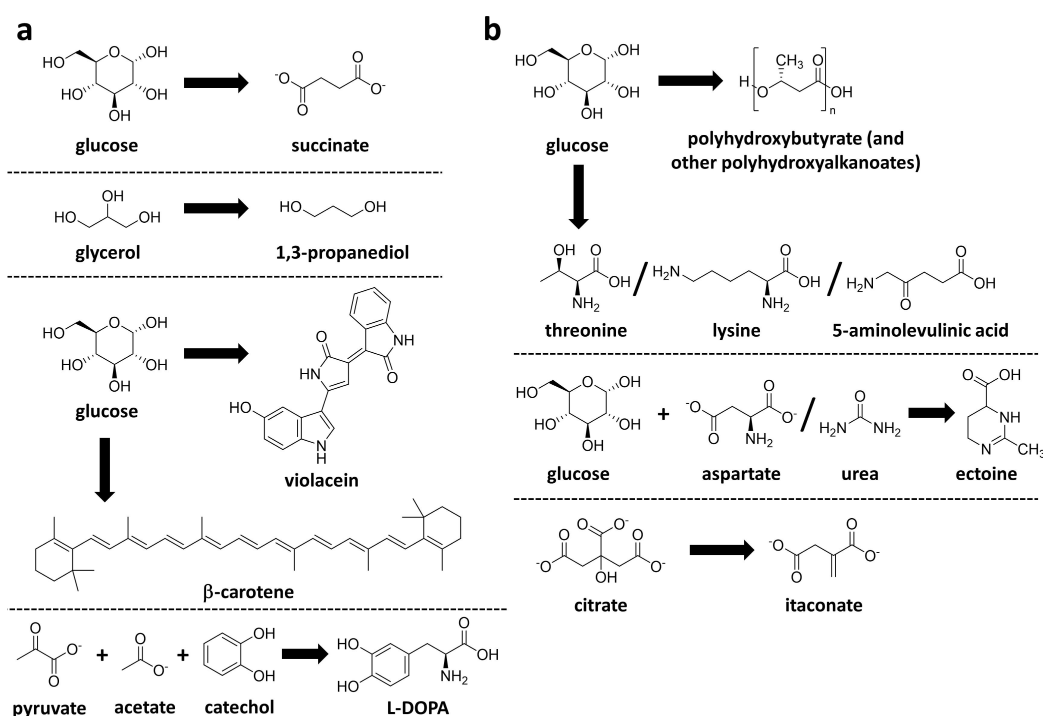

Figure 1. Biotechnological conversions of substrate(s) into product(s) realized with engineered strains of a) *V. natriegens* and b) *H. bluephagenesis.*

with the WT strain. However, after the inhibition of the native DNA restriction/methylation system, a stable plasmid with high copy number could be introduced by conjugation.[47]

Furthermore, the chromosomal expression of heterologous genes downstream of the porin gene could be realized.[48] Subsequently, a library of constitutive and inducible promoters based on the core promoter of this porin gene was developed[49] and later improved by saturation mutagenesis to span a larger expression range, which was demonstrated by the fine-tuned overexpression of a PHB biosynthesis gene.[50] In addition to this promoter set derived from a native promoter, several T7-like expression systems were identified by phage genome mining and successfully applied for controllable gene expression.[9] *H. bluephagenesis* is also amenable to CRISPR-Cas-based genome editing, as both Cas9-based editing tools[51] and Cas12a-based editing tools[52] were developed and successfully

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used. The broad toolset for gene expression and genome editing is complemented by a CRISPRi system that was developed and applied to realize enhanced PHA biosynthesis.[53]

The availability of these genetic tools enabled the improvement of some cellular properties of *H. bluephagenesis* to create an even more efficient chassis for PHA production. By expressing a hemoglobin-encoding gene and exporting the resulting hemoglobin to the periplasm via the Tat pathway, cellular oxygen availability could be increased. The same study reported the development of an oxygen-responsive promoter to express the PHB synthesis operon also under microaerobic conditions.[12] Additionally, a defective outer membrane of *H. bluephagenesis* was engineered by deletion of two genes involved in lipopolysaccharide biosynthesis. This resulted in improved oxygen uptake, better inducibility of promoters with IPTG, and allowed the possibility of electroporation of this strain for the first time.[54] The subsequent deletion of two further acyltransferases for lipid A production increased the outer membrane permeability further and allowed hyperproduction of a 3/4-hydroxybutyrate copolymer due to improved diffusion of the precursor molecule γ-butyrolactone into the cell.[55]

3.1. Many flavors of PHA can be produced from sugar with a pinch of salt

Adding to the previously mentioned examples of PHA biosynthesis as proof of principle for the functionality of different genetic tools, the full list of PHA bioproduction feats of *H. bluephagenesis* is long and impressive. Starting out with genetic manipulations to achieve improved production of a 3-hydroxybutyrate-3-hydroxyvalerate copolymer from glucose and propionate,[56] very high yields of PHAs (70–90 % of cell dry weight) based on different monomers were realized. This includes the production of a 3/4-hydroxybutyrate copolymer from glucose and γ-butyrolactone[57] or only glucose,[58] the biosynthesis of several functionalized copolymers from glucose and hex-5-enoic acid,[59] and the engineered overproduction of 3-hydroxypropionate from propane-1,3-diol, which enabled the biosynthesis of a 3-hydroxypropionate-3-hydroxybutyrate copolymer.[60] In similar approaches, the expression of genes encoding for TCA cycle enzymes was modulated to enable the production of a copolymer from 3-hydroxybutyrate and 3-hydroxyvalerate monomers from glucose as sole carbon source.[61] More complex copolymers, such as 3-hydroxybutyrate-4-hydroxybutyrate-5-hydroxyvalerate, could be produced when engineered strains of *H. bluephagenesis* were grown on glucose and suitable precursor diols (propane-1,3-diol, butane-1,4-diol, pentane-1,5-diol).[62] Furthermore, changes in the cellular redox state of *H. bluephagenesis* were achieved by deletion of the genes encoding for electron transfer flavoproteins and co-feeding of acetate and glucose. The resulting alterations of the NADH/NAD^+ ratio led to higher cell densities and increased PHA content.[63] Finally, a strong increase of PHA granule size was achieved by deletion of the *phaP* genes and overexpression of the *minC* and *minD* genes to block formation of cell fission rings. This approach resulted in large cell sizes (up to 10 μm), which were nearly completely filled by PHA granules.[64] The majority of these studies was conducted not only in shake flasks, but also in bioreactors with a volume of 7 L, demonstrating the scale-up potential of biotechnological PHA production with *H. bluephagenesis*.

While glucose was used as the standard carbon source for PHA bioproduction, high-throughput tests with Biolog plates revealed that 140 out of 190 tested compounds could be utilized for growth, and that acetate, lactate, ethanol, and glycerol could be used as alternative low-cost carbon sources.[65] Furthermore, *H. bluephagenesis* was recently engineered to secrete the enzyme α-amylase for starch degradation. This approach was successful, and PHA, ectoine, and threonine could subsequently be produced from corn starch.[66] However, less biomass was formed during growth on starch compared to growth on glucose, suggesting that polysaccharide degradation could still be improved further in future work.

Next to the strong focus on PHA bioproduction, other value-added compounds could also be produced by engineered strains of this halophile (Figure 1b). While the production of 5-aminolevulinic acid was successful, the achieved yield was lower than in comparable approaches using *E. coli* as production host.[67] The proteinogenic amino acids threonine[68] and lysine[69] could both be successfully produced from glucose. The osmoprotective compound ectoine, which is commonly produced by other *Halomonas* strains,[70] could also be generated by *H. bluephagenesis*. Ectoine yield during growth on glucose and aspartate or urea was increased by using two inducible systems to regulate expression of three gene clusters involved in ectoine biosynthesis.[15] Lastly, the high efficiency conversion of citrate to itaconate by an engineered strain of *H. bluephagenesis* was demonstrated as well.[71]

Its fast growth, possibility for non-sterile cultivation, and demonstrated capabilities for high yield production of PHA and other compounds make *H. bluephagenesis* a prime candidate for industrial-scale blue biotechnology in the coming years. In fact, the Chinese company Bluepha[72] is directly connected to the development and utilization of this emerging chassis organism. It will be interesting to see how the engineering work using *H. bluephagenesis* will be continued. The further diversification of the product spectrum can most likely be expected. Additionally, the use of C_1 feedstocks, such as methanol, formate, or CO_2,[73] by this bacterium would be a promising avenue to increase the sustainability of PHA bioproduction and move further towards a circular bioeconomy. Routes for the assimilation of these C_1 compounds have already been engineered into *E. coli*[74] and could potentially be transferred into *H. bluephagenesis* as well. Subsequent improvement of bioproduction from C_1 raw materials by directed evolution of the engineered strains would most likely result in efficient PHA accumulation without having to resort to glucose as growth substrate.

In summary, the impressive development of *H. bluephagenesis* from a newly isolated strain into a chassis organism mainly for non-sterile PHA production from glucose in just a little more than a decade shows how much progress can be achieved in blue biotechnology in a relatively short time frame, and may serve as an example for the development of other

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marine or halophilic bacteria for specific purposes in synthetic biology.

4. A thousand Routes Lead to Glycolysis: Degradation of Algal-Derived Polysaccharides

Another emerging area of interest in blue biotechnology and marine synthetic biology is not linked to a model strain, but rather to the use of a class of compounds, namely algal polysaccharides. These macromolecules might constitute a promising sustainable feedstock for the biotechnological production of ethanol or other value-added compounds. Algae photosynthetically convert carbon dioxide into biomass, but are capable of faster growth than most terrestrial plants and do not require land surface area for cultivation. In fact, it has been proposed that algae, more specifically kelp, could be grown in offshore aquafarms to realize the large-scale capture of carbon dioxide.[75] Subsequently, the algal biomass could be used as raw material for bioproduction or sequestered at the bottom of the ocean. However, the utilization of algal biomass, and specifically their polysaccharides, as substrates for microbial growth and production poses some unique challenges. While the main structural component of cell walls in terrestrial plants is cellulose, a linear chain of glucose monomers, there is a large variety of complex polysaccharides in the cell walls of marine algae. Seaweeds incorporate up to 40% agarose, porphyran, carrageenan, alginate, or ulvan into their cell walls.[76] Importantly, each of these polysaccharides consists of different monomers that are linked by a variety of glycosidic bonds, so that a multitude of enzymes is necessary for the microbial degradation and valorization of marine sugars.

Many marine microbes have evolved specialized enzymes and pathways to make use of these complex polysaccharides, and their investigation has progressed rapidly in the past decade. Some marine bacteria have even focused on a "vegetarian diet", that is, the exclusive utilization of algal-derived polysaccharides. As an example, while most *Vibrio* strains are generalists that thrive on diverse substrates, including animal-derived compounds, *Vibrio breoganii* was shown to be specialized to macroalgal substrates, including alginate and laminarin. In contrast, it cannot hydrolyze chitin and glycogen anymore.[77]

While the current knowledge on degradation pathways of algal sugars[78] and the progress in metabolic engineering for valorization of macroalgal biomass[79] have recently been reviewed, the following paragraphs will focus on selected engineering efforts in different microbial hosts that advance the possibility of sustainable bioproduction from marine polysaccharides. In addition, key studies on new enzymes and pathways will be highlighted in order to demonstrate that the complexity of this goal requires close collaboration between biochemists, microbiologists, and metabolic engineers in the coming years.

4.1. Valorization of marine sugars by genetically engineered microbes is increasingly successful

The efforts to valorize polysaccharides from brown macroalgae using optimized microbial strains started with the engineering of *E. coli* for depolymerization and conversion of alginate to ethanol. By overexpressing 20 genes for alginate depolymerization, transport, and metabolism from *Vibrio splendidus*, it was possible to generate a strain that produced 38 g/L ethanol over the course of 150 h in fed batch fermentations of macroalgal biomass.[80] A comparable engineering approach with a different product was applied by implementing the alginate catabolic pathway from *Vibrio algivorus* in *E. coli*, which resulted in the conversion of alginate into the amino acid lysine.[81]

A similar strategy was pursued using *S. cerevisiae* as chassis. The yeast was engineered to convert two sugars, 4-deoxy-L-erythro-5-hexoseulose urinate (DEHU) and mannitol, into ethanol, of which up to 36 g/L were produced in 90 h. DEHU and mannitol are downstream metabolites of the monomers of alginate, guluronate and mannuronate. Their valorization was enabled by overexpressing the genes encoding for a DEHU transporter and alginate catabolic enzymes. Additionally, the native mannitol catabolism pathway was deregulated to ensure more efficient conversion.[82] Another more recent study followed a comparable approach, but moreover added the goal to depolymerize alginate, which was achieved by the heterologous production of alginate lyase enzymes in *S. cerevisiae*. Subsequently, the engineered strain was shown to be capable of ethanol production during growth on alginate and mannitol.[83] However, the yield of 9 g/L after 168 h was substantially lower than the previously mentioned titer that was achieved by conversion of DEHU and mannitol, indicating that the depolymerization of alginate might be rate limiting in this case. Furthermore, the first steps to valorize another polysaccharide derived from brown algae by using engineered *S. cerevisiae* strains were undertaken as well. Laminarin, a branched polysaccharide comprised of linear glucose-based chains, can be cleaved by laminarinase enzymes. Heterologous production and secretion of different laminarinases resulted in successful degradation of the polymer into glucose, which was subsequently converted into ethanol (2 g/L after 120 h).[84]

Perhaps the most interesting study on the utilization of alginate by engineered microbial strains was performed using a nonstandard chassis organism. The isolate *Vibrio* sp. dhg was shown to be capable of fast growth on alginate ($\mu = 0.98\ h^{-1}$). Genome sequencing revealed its high similarity to the earlier discussed *V. natriegens*. Due to the similarly fast growth, but the additional capability of alginate assimilation, *Vibrio* sp. dhg was deemed a suitable chassis organism for bioproduction from alginate. Therefore, a genetic toolbox including constitutive and inducible promoters, 5'-UTRs, and basic genome editing tools was developed. Strain engineering resulted in the highly efficient production of ethanol (26 g/L in 24 h) and butane-2,3-diol (27 g/L in 24 h) from alginate and mannitol. Even the C40 carotenoid lycopene could be produced in this system faster than in engineered strains of *E. coli*. Notably, the production of ethanol directly from dried macroalgae was also possible (19 g/

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L in 24 h).[16] Taken together, this study demonstrates that high flux through the alginate catabolic pathway coupled to fast growth might be the key for fast and efficient production of value-added products from brown macroalgae.

Based on these and many other engineering efforts, three emerging strategies have recently been proposed: 1) the regulation of polysaccharide degradation pathways in their engineered hosts by dynamic circuits, 2) the strain engineering of halophilic microbes by applying recombinase-assisted genome engineering, and 3) the development of microbial consortia for conversion of algal polysaccharides.[79] Each of these strategies will have to deal with its own set of challenges, and will also require constant tweaks and updates to reflect the fact that different algae require different (sets of) microbial degraders, and that new polysaccharide-degrading pathways and strains are constantly being discovered.

4.2. The diversity of polysaccharide catabolic routes is ever-growing

The known diversity of metabolic routes for degradation of algal polysaccharides is already dazzling, and new enzymes and pathways are being discovered every year. In the following, just a few selected examples will be discussed to illustrate nature's ingenuity in degrading recalcitrant polymers as well as the challenge that awaits a metabolic engineer aiming to realize the upcycling of complex polysaccharides in a chassis organism. The Gammaproteobacterium *Pseudoalteromonas haloplanktis* ANT/505 was shown to encode for a pectin degradation pathway that was most likely acquired by horizontal gene transfer from terrestrial bacteria, but subsequently adapted to include secreted multi-modular pectate lyases, which might serve to reduce loss of substrate and enzymes by diffusion.[85] Other polysaccharides, such as agarose and porphyran, contain the monomer methylgalactose. To remove the methyl group from this compound and create galactose, which can then be assimilated, novel cytochrome P450 monooxygenases are used by *Formosa agariphila* and *Zobellia galactanivorans*, two species from the *Bacteroidota* phylum.[86]

The same bacteria are capable of degrading ulvan, the major polysaccharide of the fast-growing green seaweed *Ulva* spp. For this purpose, they employ a pathway comprised of at least 12 enzymes, including two polysaccharide lyases, three sulfatases, and seven glycoside hydrolases. Recently, the core pathway for ulvan degradation in *F. agariphila* was characterized.[87] In addition, further accessory enzymes are involved in the degradation of ulvan, such as a dehydratase that generates unsaturated uronic acid residues at the end of oligosaccharides.[88] Notably, the implementation of two *F. agariphila* enzymes (ulvan lyase and β-glucuronylhydrolase) in *Bacillus licheniformis* was sufficient to enable growth of this terrestrial host bacterium on ulvan, albeit with a rather low efficiency.[89]

While the ulvan degradation pathway is already quite intricate, the route for microbial utilization of the recalcitrant brown algal polysaccharide fucoidan is even more complex by an order of magnitude. The Verrucomicrobium "*Lentimonas*" sp. CC4 encodes for a pathway of about 100 enzymes to liberate the monomer fucose from fucoidan. Fucose must then be further metabolized in a bacterial microcompartment, since the reactive molecule lactaldehyde is produced during its breakdown.[90] The implementation of such a pathway into another microbial host, while theoretically possible, would far exceed the size of gene clusters or DNA fragments that have been engineered into any chassis so far. Therefore, the valorization of fucoidan and similarly complex algal polysaccharides might remain out of reach, unless genetic engineering tools for the original hosts of the respective degradation pathways would be developed.

5. The Quest for Synthetic Biology-Assisted Marine Bioremediation

In the previous paragraphs, different aspects of bioproduction based on marine strains or substrates were discussed. However, marine synthetic biology also offers another opportunity, which is certainly more challenging, but also more important for the future well-being of human societies and our planetary environment. This opportunity is the large-scale reversal of environmental problems by genetically enhanced microorganisms, for example, the bioremediation of aquatic pollutants or the decrease of atmospheric CO_2 concentrations.

5.1. From bioremediation to geoengineering and aquaforming

The discussion about these potential applications is not new; already in 1993, the bioremediation of oil spills by nitrogen fertilization of contaminated sites to stimulate growth of the native microbiota or addition of exogenous oil-degrading bacteria to speed up the breakdown of hydrocarbons was extensively reviewed.[91] Furthermore, research on hydrocarbon-degrading bacteria such as *Alcanivorax borkumensis* and their potential application in the bioremediation of oil spills reached a peak in the 2000s.[92] In the decades that have passed since then, it was pointed out several times that marine bacteria are the most suitable bioremediation agents in their native environment, since they are adapted to its high salinity and rapidly changing light and nutrient regimes. Their genetic manipulation with the goal of improved bioremediation properties was proposed as well,[93] but there are only very few cases in which marine microorganisms were engineered for enhanced pollutant degradation. Notable examples are the implementation of the plastic-degrading enzyme PETase in the marine microalga *P. tricornutum*[17] and the establishment of three different pollutant degradation pathways (for the plastic PET, the plastic additive hexabromocyclododecane, and the pesticide chlorpyrifos) in *V. natriegens*.[18]

In recent years, the idea of environmental bioremediation has experienced a renaissance, and concepts were developed

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that outline the deployment of (genetically engineered) microorganisms on a global scale. Going one step further than the traditional concept of degrading pollutants, these extended approaches also aim to tackle even larger environmental problems, such as the increasing concentrations of atmospheric and dissolved carbon dioxide that are driving the escalating climate crisis.[94] Within these frameworks, seven key microbial-based processes to address global environmental problems have been identified.[19] Notably, two of these processes are completely focused on the global oceans, namely the cleanup of plastic waste in marine ecosystems and the recovery of diluted phosphorus from marine ecosystems and sediments. Additionally, two other processes – decreasing the atmospheric levels of carbon dioxide and other greenhouse gases and eliminating pharmaceuticals and endocrine disruptors from trophic chains – are partly related to marine habitats. Such processes can be referred to as geoengineering or aquaforming (analogous to terraforming, the deliberate modification of land surface topography or ecology). To achieve these bold goals, it was proposed to apply not only natural, but also genetically enhanced bacteria. These engineered microbes might be equipped with suitable pathways to mineralize plastics, accumulate phosphate, capture carbon dioxide, or degrade pollutant molecules. Going one step further, these pathways could theoretically even be spread to environmental microorganisms by horizontal gene transfer or an engineered gene drive in order to realize fixation of a desired trait or pathway in a natural microbiome.[19]

The concept of intentionally spreading genetically enhanced microorganisms in the environment is obviously in stark contrast to the prevalent policy of strictly containing such strains within laboratories. Therefore, different ideas have been proposed to potentially realize the deployment of engineered microbes in the environment and still retain a high level of genetic safety.

5.2. Containment or spread of genetically enhanced microbes for bioremediation?

A fully contained in-situ approach for bioremediation in marine environments could be realized by employing floating bioreactors with engineered microbes. Polluted water could flow through these systems, allowing the contained microbes to degrade the pollutant in question without risking their release into the environment. This would constitute a safe option for synthetic biology-assisted marine bioremediation, but the efficiency of this approach would be limited by the maximum number of microorganisms that could be deployed and grown in such flow-through bioreactors.

Releasing engineered microbes into the environment could be made more safe by testing their deployment thoroughly in advance. Recently, it was proposed that such endeavors should be similar in design to the performance of multi-phase clinical trials before the approval of a new drug. In a stepwise scale-up, the efficacy and safety of engineered biological catalysts could first be tested in a laboratory before moving into mesocosms and finally contained ecosystems, before considering a deployment in the environment.[95] This tested deployment would aim to minimize the risks associated with releasing genetically enhanced microorganisms, while still enabling the spread of the bioremediation agents in the environment (Figure 2).

In a different approach, so-called kill switches for the biocontainment of engineered microorganisms have been designed and realized. These genetic circuits couple the expression of a gene encoding for a toxin to the absence of a survival signal, such as anhydrotetracycline.[96] The input signal can also be physical instead of chemical, for example a change in temperature.[97] Using these approaches, it was possible to limit the survival of engineered *E. coli* strains to defined conditions. More recently, these designs were improved using CRISPR-based kill switches that depend on the combined input of chemical and physical signals.[98] Similar results could be achieved by engineering strains with synthetic auxotrophies, meaning that released bacteria cannot survive in the absence of a given molecule. Furthermore, different types of ecological firewalls for the containment of engineered strains or communities have recently been proposed, and their efficiency has been calculated. These approaches rely on resource availability of pollutants as growth substrates, mutualistic dependency of ecological factors, or parasitic interactions of an engineered strain with the resident community.[99] However, no practical work on ecological firewalls has been performed so far, so that it is difficult to judge whether the effect that is predicted by differential equations on paper can be replicated in a complex real-world scenario. In summary, methods for safeguarded deployment would constitute a middle ground between containment and spread of genetically enhanced microbes in the environment; but it would be possible that the engineered safeguard mechanisms are disabled by evolutionary adapta-

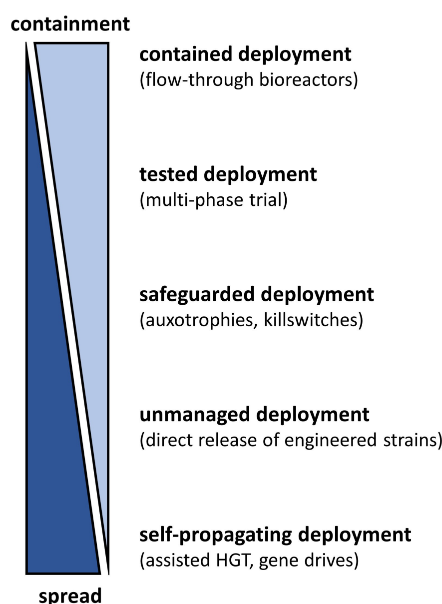

Figure 2. Different approaches to regulating the balance between containment and spread of engineered microorganisms during environmental deployment.

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tions or horizontal gene transfer between engineered strains and native microorganisms.

Moving closer towards the uncontrolled spread of genetically enhanced biocatalysts in the environment, the simplest option would be the direct release of engineered strains. In this scenario of unmanaged deployment, the only limit to the spread of engineered bioremediation agents would be their fitness in a natural habitat.

This limitation could theoretically be overcome by engineering assisted horizontal gene transfer from genetically enhanced bioremediation strains to the native microbiota. Large-scale gene drives in the environment, while theoretically possible, are obviously difficult to achieve and highly controversial from an ethical point of view.[100] Microbial gene drives have been tested in laboratory settings in the model organisms *E. coli*,[101] *S. cerevisiae*,[102] and *Schizosaccharomyces pombe*.[103] However, gene drive systems have not been established yet in any marine microorganisms. It might be possible to take the first steps in this direction by applying recently created vectors that are designed to propagate recombinant genes through microbiomes. Utilizing a minimized conjugation machinery, the effectiveness of these constructs was validated by transferring a gene encoding for green fluorescent protein from and to numerous donor and recipient strains, and notably even to consortia in the soil microbiome. There are tailored versions of this genetic tool for both gram-negative and gram-positive bacteria, as well as for eukaryotes.[104] If this system is shown to be efficient in the transmission of larger gene clusters as well, it might be a promising starting point to propagate pathways for bioremediation or greenhouse gas fixation through marine microbiomes. The deployment of self-propagating biologicals could be realized by using aerosols or wind-carried particles to reach a sufficient initial spread in a marine habitat.[95] However, the potential consequences of such actions would have to be studied thoroughly in theory and in practice before ever considering the release of self-propagating genetically enhanced microbes.

5.3. Novel marine chassis organisms are required for bioremediation

Clearly, additional work on self-propagating genetic modules and biocontainment of engineered organisms is urgently required. But even more necessary is the establishment of chassis organisms in marine synthetic biology that could potentially be deployed in situ for bioremediation or other purposes. Furthermore, the establishment of heterologous pathways for pollutant degradation, CO_2 fixation, or other aquaforming processes in microorganisms involve complex synthetic biology work, even more so since these genetically enhanced microbes should still be capable to grow as part of natural environmental communities. Therefore, a suitable chassis organism for aquaforming purposes should fulfill the following minimal requirements:

1) Efficient growth at environmental conditions;
2) Physiological adaptability and metabolic versatility;
3) Abundant and stable role in marine microbiomes;
4) Nonpathogenic towards humans and animals.

Additional features that might be of interest are the presence of native pathways for aquaforming processes or the frequent interaction with other marine microorganisms, in case the transfer of genetic information is desired. These requirements for a suitable microbial chassis organism for bioremediation are fundamentally different from the requirements for a good chassis for microbial bioproduction, as summarized in Table 2.

As outlined in previous chapters, there is a large set of genetic tools both for *V. natriegens* and for *H. bluephagenesis*. However, it is doubtful whether these bacteria would be suitable organisms for bioremediation and aquaforming. *H. bluephagenesis* was isolated from a salt lake and is therefore well adapted to harsh growth conditions, but did not evolve to handle the changing chemical, physical and biological conditions encountered in the oceans. *V. natriegens* is a marine bacterium, but its relatedness to both human and animal pathogens in the *Vibrio* genus make it less suitable for widespread in-situ applications, since the risk that virulence plasmids[105] could be transferred to (genetically enhanced) *V. natriegens* cells cannot be excluded. Previously, strains of *V. parahaemolyticus* and *V. crassostrea* have turned into lethal pathogens for marine animals after acquisition of a virulence plasmid,[106] demonstrating that this risk factor should not be neglected. Therefore, the use of *V. natriegens* and *H. bluephagenesis* as chassis organisms in marine synthetic biology will remain limited to roles in bioproduction.

Which other marine microorganisms could then be developed into a chassis for aquaforming? When looking at the requirements mentioned above, one quickly arrives at the fact that Alphaproteobacteria are the most abundant, adaptable, and versatile bacteria throughout the oceans.[3c] Among them, the most important group in terms of abundance and diversity are the *Roseobacter* group bacteria. This operational term does not denote a monophyletic group, but includes marine members of the *Rhodobacteraceae* family.[107] In fact, *Roseobacter* group bacteria have been proposed for geoengineering

Table 2. Selection criteria for suitable chassis strains in microbial bioproduction and microbial bioremediation.

Microbial bioproduction	Microbial bioremediation
fast growth	efficient growth at environmental conditions
high-density cultivation	adaptability and versatility
efficient conversion of substrate to product	abundant and stable role in microbiomes
genetic stability	nonpathogenic towards humans and animals
robustness	if desired: transfer of genetic information

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previously.[108] In the following, the key features of these abundant marine bacteria will be highlighted, and it will be discussed whether they are promising candidates to work towards the deployment and spread of engineered features in the marine microbiome.

5.4. Metabolic versatility and secondary metabolites make *Roseobacter* group bacteria ecologically successful

Roseobacter group bacteria are heterotrophs that have a flexible and versatile metabolism. The assimilation of common carbon substrates, such as amino acids and sugars, were investigated in detail,[109] and it was found that carbohydrate catabolism proceeds mainly via the Entner-Doudoroff pathway in the model strains *P. inhibens* and *Dinoroseobacter shibae*.[110] However, these bacteria are able to utilize a multitude of carbon sources for growth, among them glycolate,[111] ectoine,[112] DMSP,[113] benzoate and other aromatic compounds,[114] purine nucleotides,[115] and trimethylamine *N*-oxide,[116] to name just a few. Other compounds, such as carbon monoxide, are oxidized, but have no apparent effect on growth.[117] The metabolism of *Roseobacter* group bacteria can adapt to simultaneous assimilation of diverse substrates,[118] meaning that they can easily be grown in a complex medium.

The common mode of energy conservation of *Roseobacter* group bacteria is aerobic respiration. However, strains such as *D. shibae* can also perform denitrification under anaerobic conditions.[119] Furthermore, the same strain has served as model organism to investigate light response and aerobic anoxygenic photosynthesis in *Roseobacter* group bacteria.[120] Light enhances the survival of *D. shibae* during long-term starvation[121] and results in reduced respiration as well as increased anaplerotic CO_2 fixation via the ethylmalonyl-CoA pathway.[122] Interestingly, growth at high light intensity is linked to an increased electron transfer rate and increased assimilation of organic substrates.[123] In addition to these experimental findings, a genome-scale metabolic model for *D. shibae* was developed that also takes energy conservation by light-derived electrons into account[124] and could therefore be a valuable resource in future metabolic engineering approaches of this strain.

Furthermore, *Roseobacter* group bacteria are prolific producers of secondary metabolites, which they use to modulate their interactions with other marine organisms. A classic example is the finding that *P. inhibens* can promote the growth of the algae *Emiliana huxleyi*, but is also able to kill it with potent toxins, the roseobacticides, once it enters the later stages of the algal life cycle.[125] These bacterial-algal interactions are mediated by indole 3-acetate, a molecule that is synthesized by *P. inhibens* from algae-derived tryptophan.[126] *P. inhibens* is also capable of producing other secondary metabolites, such as tropodithietic acid, which serves both as an antibiotic and a global signaling molecule,[127] and its methylated analogue methyl troposulfenin.[128] Another model strain, *Ruegeria pomeroyi*, produces different lactones that are capable of killing algae[129] and bacteria.[130]

Due to their ability to produce compounds that are lethal to pathogenic microbes, wild-type strains of *Roseobacter* group bacteria are currently already in the focus for applications as biocontrol agents in marine aquaculture. It was initially found that strains of *Phaeobacter* and *Ruegeria* colonize separate niches in a fish aquaculture farm, both functioning to antagonize *Vibrio anguillarum*, a fish pathogen of economic relevance, under different growth conditions.[131] More specifically, *P. inhibens* inhibits growth of *V. anguillarum* on live fish feed, such as algae and copepods, thereby allowing improved growth of the cultured fish and their larvae.[132] Reviewing this field of application, it was recently concluded that *P. inhibens* can be considered a safe probiotic for application in aquaculture due to its antagonistic activity against pathogenic *Vibrio* strains, while having no negative effect on fish hosts and their microbiome. Furthermore, no virulent phenotypes of this species were found so far.[133]

However, genetic engineering of *Roseobacter* group bacteria will clearly be required to enable their potential application as aquaforming agents. In the following, the existing genetic tools and key chassis properties will be highlighted, and the next steps towards successful synthetic biology work with these microorganisms will be outlined.

5.5. Towards genetic engineering of *Roseobacter* group bacteria

Roseobacter group bacteria are generally genetically tractable, and a basic set of genetic tools for several species, including antibiotic markers, stably maintained plasmids, an oxygen-independent fluorescent protein, and protocols for transposon mutagenesis as well as targeted gene deletion, has already been established.[10,134] To extend this toolset, it might be suitable to use or adapt broad-host-range genetic tools that have been successfully applied in other Alphaproteobacteria, such as the MethyloBrick expression vector suite[135] or the pREDSIX/pTETSIX gene deletion systems.[136] Notably, both of these systems have already been used successfully in *Paracoccus denitrificans*, a terrestrial species of the *Rhodobacteraceae* family.[111,135] This makes it likely that they will also be functional in marine *Rhodobacteraceae* strains.

To further improve the range of genetic constructs for *Roseobacter* group bacteria and allow the stable maintenance of desired traits without antibiotic selection, it might be promising to create synthetic minichromosomes based on natural repABC replicons. This approach was already successfully applied for other Alphaproteobacteria, such as *Sinorhizobium meliloti*[137] and *Methylobacterium extorquens*.[138] Extrachromosomal plasmids play a large role in the genetic architecture of *Roseobacter* group bacteria,[139] so that there is no shortage of genetic parts that could serve as the basis for minichromosomes.

Together with the existing tools, these proposed strategies make it seem plausible that synthetic biology work using *Roseobacter* group bacteria as hosts will be possible in the near future. However, there is still a considerable amount of work to

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do before these microorganisms can catch up with more established systems. Notably, no CRISPR-Cas systems for *Roseobacter* group bacteria were established so far, let alone more advanced genome engineering tools such as MAGE or recombineering systems. However, so-called roseophages, viruses that are infecting strains of *Phaeobacter*, *Dinoroseobacter*, and *Ruegeria*, among others, were previously identified.[140] This might potentially enable the creation of advanced genetic tools that make use of the phage genetic machinery.

Many *Roseobacter* group bacteria are known for forming biofilms in their natural environment.[141] This trait can be relevant for the ecological success of a species, but is often undesired both in laboratory experiments and in biotechnology. An extrachromosomal replicon of 65 kb is required for biofilm formation and colonization of algae by *P. inhibens*; these traits were abolished after curing of this plasmid.[142] The deletion of rhamnose biosynthesis genes resulted in decreased biofilm formation as well.[141a] Therefore, it is possible to utilize these non-sticky strains for laboratory work. Furthermore, extrachromosomal replicons, especially a plasmid of 262 kb encoding for secondary metabolite biosynthesis, decrease the growth rate of *P. inhibens.* A strain that was cured of this plasmid was capable of faster growth and higher biomass yield.[13] Thus, several steps towards converting *P. inhibens* into a synthetic biology chassis[143] were already performed. However, it remains to be tested whether such altered or plasmid-cured strains are still able to thrive in a natural microbial community, which is a required trait for the application as bioremediation or aquaforming agent.

In summary, *Roseobacter* group bacteria are diverse and abundant, can make a living nearly everywhere due to their versatile physiology, and frequently interact with other marine microorganisms. Some genetic tools for this microbial group are already available, but additional work is clearly necessary to enable more ambitious synthetic biology projects with a *Roseobacter* chassis. Nevertheless, *Roseobacter* group bacteria seem to be the most promising candidates for developing genetically enhanced microbes for aquaforming in the future, also due to the fact that no human or animal pathogens from this group are known so far.

6. Summary and Outlook

Blue synthetic biology promises to be a growing field in the coming years. It can be expected that more and more researchers will work on novel biotechnological applications for the chassis organisms *V. natriegens* and *H. bluephagenesis*, and that technologies that permit the increased utilization of algal polysaccharides as a sustainable feedstock for bioproduction will be developed. Furthermore, the increased interest in developing synthetic biology approaches for large-scale bioremediation and geoengineering is likely to continue, and new concepts that have been proposed in theoretical studies will be translated to laboratory work. As the final part of this review, three key steps towards successful progress in blue synthetic biology will be summarized.

First, the chassis organisms described here need to be developed further in order to catch up with the accessibility and diversity of terrestrial hosts in synthetic biology. To this end, standardized genetic toolsets for these bacteria need to be developed and/or extended, and host properties should be modified according to defined criteria to convert environmental microbes into synthetic biology chassis.[143]

Second, more suitable enzymes for relevant bioproduction or aquaforming applications must be identified or created. As an example, the plastic-degrading enzyme PETase and its improved variants are mainly active at higher temperatures.[144] Therefore, the study of microorganisms degrading plastics in cold marine habitats[145] is of special interest in order to move towards enhanced bioremediation of plastics. Similarly, the discovery of new polysaccharide degradation pathways in marine bacteria should transition to the screening of the most suitable enzymes for assembling heterologous metabolic pathways for valorization of algal biomass in suitable hosts.

Third, conceptual and experimental work towards bioremediation in marine environments and aquaforming must be continued. This work should be accompanied by an open discussion about the risks and benefits of such approaches, involving the opinions and expertise of researchers (from biology and ecology as well as climate science and physics), public servants, and members of society. Given the steady progress of the climate crisis and the increase of environmental pollution, the advantages and disadvantages of all technologies that are available to mitigate these dire threats must urgently be considered.

When researchers orient themselves to these three goals, blue synthetic biology will sail towards an age of discovery that has the potential to create lasting sustainable solutions for the benefit of humanity and its home, the blue planet.

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Conflict of Interests

The author declares no conflict of interests.

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