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Review

Marine and estuarine natural microbial biofilms: ecological and biogeochemical dimensions

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Abstract: Marine and estuarine microbial biofilms are ubiquitously distributed worldwide and are increasingly of interest in basic and applied sciences because of their unique structural and functional features that make them remarkably different from the biota in the plankton. This is a review of some current scientific knowledge of naturally occurring microbial marine and estuarine biofilms including prokaryotic and microeukaryotic biota, but excluding research specifically on engineering and applied aspects of biofilms such as biofouling. Because the microbial communities including bacteria and protists are integral to the fundamental ecological and biogeochemical processes that support biofilm communities, particular attention is given to the structural and ecological aspects of microbial biofilm formation, succession, and maturation, as well as the dynamics of the interactions of the microbiota in biofilms. The intent is to highlight current state of scientific knowledge and possible avenues of future productive research, especially focusing on the ecological and biogeochemical dimensions.

Keywords: C-cycle; marine food webs; microbial ecology; N-cycle; microbial trophic relations

1. Introduction

This is a review of current scientific knowledge of marine and estuarine natural microbial biofilms focusing on prokaryotes and eukaryotic microbes, including biofilm formation, structure,

ecological function, and likely role in major biogeochemical cycles. Considerable research attention has been given to the fundamental properties of aquatic biofilms, especially freshwater aquatic biofilms [1–5]. However, less basic research attention appears to have been given to naturally occurring marine biofilms, but considerable more attention has been given to biotechnological and biofouling aspects. Although biofouling is not treated here, because the emphasis is on naturally occurring biofilms of basic scientific interest, some useful reviews and perspectives on biofouling and technology are available for readers who may be interested in these topics [6–11]. Some of the biofouling research has provided valuable insights into essential aspects of naturally occurring marine biofilm formation, structure and biological functions, and relevant research results from some of the biofouling studies are reviewed here where they provide insights about biofilms in the natural environment. In general, research articles reviewed have been selected to emphasize particular aspects of current knowledge related to marine prokaryotic and microeukaryotic biofilms especially as a foundation for, and subsequent emphasis on, the role of marine biofilms in biogeochemical cycles.

While this review of necessity is focused on major topics of marine and estuarine microbial biofilms with particular attention to environmental and biogeochemical themes, other published sources provide perspectives that include the interaction of marine biofilms with larger plankton (such as settling of metazoan larvae, invertebrates and larger algae) that are typically late colonizers on microbial-established biofilms and contribute substantially to the biofilm mass [12]. The subsequent sections of this review address the following topics: 2. Marine Microbial Biofilm Initiation, 3. Biofilm Development and Biotic Successions, 4. Biofilm Structure and Microbial Organization (including archaeal, bacterial, and mixed microbiota biofilms), 5. Perspectives on Biogeochemical Cycles and Marine Biofilms, and 6. Conclusions and Future Research Opportunities. Major topics addressed in this review and relevant citations to published literature are listed in Table 1.

2. Marine Microbial Biofilm Initiation

The general events in aquatic biofilm initiation, including microbe settling from the plankton, surface attachment, and their interactions during early biofilm development have been reported extensively [13], particularly the conditions favoring initial settling [14]. Marine planktonic bacteria are among the first microbes to settle and colonize submerged surfaces. Chemical and physical properties of the surface, however, affect the rate and selectivity of attachment by initial colonizers, and this topic is reviewed more fully below.

2.1. Surface properties favoring bacterial sorption

The surface chemistry and surface energy are important properties of submerged surfaces that influence initial microbial attachment [15,16]. In general, a range of studies indicate that minimal long-term adhesion of microbiota is associated with surfaces having initial "low energy" that is surface tensions between 20 and 30 dynes cm^{-1} [15], although the surface properties may change due to subsequent adhering microbiota, thus influencing what further biota attach to the surface. For

many organisms, however, minimal adhesion is related to low surface energy. An initial and spontaneous set of events, occurring within minutes of immersion of a clean surface in seawater, produces a "conditioning film" by adsorption of organic molecules, and possibly some inorganic material such as metal oxides or fine clay mineral particles from the overlying bulk water phase [17].

Table 1. Summary of major topics and cited published sources.

1. Introduction
General properties of aquatic biofilms [1–5]; Specific topics of possible interest related to biofouling and antifouling, not addressed here [6–11].
2. Marine microbial biofilm initiation
General aspects [13,14]; 2.1 Surface properties favoring bacterial sorption [15–17]; 2.2 Surface properties and adhesion [16–19]; 2.3 Bacterial two-phase attachment processes and surface motility [20–24]; 2.4 Surface substratum composition effects (glass, ceramic, etc.) [25], including effect on total biofilm C [26].
3. Biofilm development and biotic successions
3.1 Initiation, bacterial physiological changes [27]; 3.2 Successional stages of bacterial biofilm development [28,29], and of mixed biotic biofilms [30–34], including picoeukaryotes [35–38]; 3.3 Cell-to-cell signaling during biofilm initiation and proliferation during succession [39–44], and during subsequent modifications including disruption [45,46].
4. Biofilm structure and microbial organization
4.1 Overview and general aspects [47–55], including bacterial [50,51], and mixed biota with diatoms [52–55]; 4.2 Archael biofilms [56–63]; 4.3 Bacterial biofilms [49, 64–66]; 4.4 Microalgal-dominate biofilms [67–72], and physiological adaptations [70–73].
5. Perspectives on biogeochemical cycles and marine biofilms
Overview [1,73,74]; 5.1 Carbon cycle: C primary fixation [75–85], and link to heterotrophy and respiration [79–83], biofilms and C dynamics on water column particulates [84,85,97–103]; 5.2 Nitrogen cycle: cyanobacteria and N fixation [86–88], Anammox reactions and release of N_2 [89–92].

2.2. Surface properties and adhesion

Some first principles of thermodynamics have been applied to explain the physical forces promoting initial passive adhesion of particles such as bacteria to submerged aquatic surfaces. The tendency, or driving force, for a microorganism to attach to a substrate surface is given by the free

energy of adhesion (ΔG^{adh}), which can be expressed as a thermodynamic energy balance among the interfacial energies; especially between the substratum, the organism, and the surrounding liquid as follows: $\Delta G^{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL}$, where γ_{BS} is the interfacial tension between the organism (e.g. a bacterium) and substratum, γ_{BL} is the interfacial tension between the organism and the liquid, and γ_{SL} is the interfacial tension between the substratum and the liquid. The interfacial energies are typically estimated by surface contact angles (θ). To more accurately estimate the values of each of the contributing interfacial tension (γ) terms in the equation, Ista et al. [16] varied the chemical composition of self-assembled, artificial mixed monolayers (SAMS) under carefully controlled conditions. Two series of mixed monolayers were produced, consisting of: (1) methyl- and hydroxyl-terminated and (2) methyl- and carboxylic acid-terminated SAMs with identical series of stepped (decreasing) contact angles in an aqueous medium. The attachment of bacterial cells and algal spores was tested on each series to better establish the physico-chemical forces that may account for the initial passive attachment of microbiota. The results for bacteria are summarized here first because bacteria are among the most common early colonizers. The gram-negative bacterium *Cobetia marina* was used as a model organism, previously isolated from a marine biofilm. *C. marina* attached in increasing numbers to SAMs with decreasing advancing water contact angles (θ_{AW}), which is in accordance with equation-of-state models of non-living colloidal attachment. Consistent with previous studies, *C. marina* attached preferentially to hydrophobic surfaces, and given that the cell surface of *C. marina* is also relatively hydrophobic, these results agree with the predictions of the equation-of-state models of bacterial attachment published by Absolom et al. [18]. Zoospores of the alga *Ulva*, that sometimes settle subsequent to microbial biofilm formation also showed a similar correlation between substratum θ_{AW} and attachment as was found for the bacterium; but when the hydrophilic component of the SAMs was changed by incorporating a more polar carboxylate terminal group, the profile attachment by Ulva zoospores was significantly different, suggesting that a more complex model of interfacial energetics is required other than the equation of state based solely on interfacial tensions, including polar and other charge-based possible interactions.

In addition to the non-biotic conditioning due to sorption by particulates that alter the surface properties of immersed substrates, early colonizing bacteria also alter the physico-chemical properties of the surface. Grasland et al. [19] characterized the hydrophilic, electrostatic and Lewis acid-base cell surface properties of marine bacteria while they were adhering to a hydrophilic support immersed for 3 and 6 h in seawater. Eleven strains possessed a hydrophilic surface and five a hydrophobic surface. Although the majority of the bacteria presented an electron-donating character, some could not generate Lewis acid-base interactions with the underlying support. However, all strains possessed an isoelectric point ranging from 2.2 to 3.4 and were negatively charged at the pH of seawater. Overall, hydrophilicity was a preponderant feature governing their surface altering properties among these bacteria, but other properties should not be ignored. For example, regardless of the hydrophilic or hydrophobic character of the cell surfaces, the majority of the bacteria possessed an electron-donating character, an isoelectric point (IEP) value ranging from 2.2 to 3.4, and a negative net charge at alkaline pH values. Thus, surface properties of early bacterial biofilms may have selective effects for further settlement and colonization of the biofilm by subsequent microbes recruited from the overlying water.

2.3. Bacterial Two-phase Attachment Processes

There is substantial evidence that adhesion of bacteria from seawater is selective in a two-phase process: (1) reversible attachment, and (2) irreversible attachment. Dalton et al. [20] reported that microbes initiating biofilm formation on a surface are capable of independent movement during a stage of reversible adhesion before they begin to exude exopolysaccharides (EPS) and adhere irreversibly. Thus, some of the random patterns of distribution observed by Marshall et al. may have resulted partially from this temporary motile phase. Marshall et al. [21] noted that during the 24-h study, there were no specialized attachment appendages (pili, or holdfasts), but some bacteria produced extracellular polymeric strands that might be of significance in the irreversible sorption on the surfaces. Although no evidence of attachment appendages were observed in the study by Marshall et al. [21], twitching motility, mediated by flagella and type IV pili in biofilms of *Pseudomonas aeruginosa*, has been reported to play important roles in surface aggregation [22]. Indeed, increasing evidence points to a critical mechanosensing role of bacterial flagella, among other surface sensing mechanisms in some bacterial species, during initial substrate surface contact and settling of bacteria prior to sorption and full attachment on the surface [23]. Although current models indicate a variety of response mechanisms for the transduction of the mechanosensory signals at surfaces varying among species, typically contact with a surface impedes flagellar rotation and induces mechanical changes in the membrane-bound, flagellar molecular rotating motor, thus initiating a cascade of molecular signals that result in physiological adaptations of the bacterium to enhance surface attachment, including secretion of biofilm forming substances [23].

Marshall et al. in other studies [24] examined in greater detail the sorption of two marine bacteria to submerged surfaces during the instantaneous reversible phase, and the time-dependent irreversible phase. The reversible sorption of the nonmotile bacterium *Achromobacter* strain R8 decreased to zero as the electrolyte concentration decreased, or as the thickness of the electrical double-layer increased. Bacteria were reversibly sorbed at lower concentrations of a divalent electrolyte ($MgSO_4$) than of the monovalent electrolyte (NaCl), an effect likely related to the greater compression of the double-layer in the divalent system at comparable electrolyte concentrations. The concentration of the electrolyte at which all bacteria were repelled from the glass surface depended on the valency of the cation. Thus, the reversible phase is interpreted in terms of the balance between the electrical double-layer repulsion energies at different electrolyte concentrations and the Van der Waals attractive energies that promoted binding of the bacterium to the surface. Even with electrolyte concentrations typical of seawater, the bacteria are probably held at a small distance from the glass surface by a repulsion barrier. Marshall et al. [20] using microscopic observations, examined the sequence of sorption of five different bacterial types on glass slides and electron microscope coated grids immersed in seawater for periods of up to 24 h. The five types of bacteria were: (1) small rods (< 0.8 μm), large rods (> 0.8 μm), (3) cocco-bacilli, (4) curved rods (*Vibrio* and spiral forms), and (5) stalked bacteria (caulobacters and hyphomicrobia). Electron microscopic examination showed that bacteria were observed on grids after 1 h immersion, and numbers of bacteria increased with longer immersion times. The predominant cell type was conventional, rod-shaped bacteria. Observation of immersed glass slides showed that some rod-shaped bacteria were sorbed within one h, while coccoidal and spiral forms generally were observed after 6 to 8 h.

Moreover, some stalked bacteria were found after 24 h and constituted a substantial fraction of the population after several days. Diatoms and heterotrophic protists were observed in small numbers after five days immersion in seawater. Overall, selective irreversible sorption favored persistence of short rods. Curved rods and cocco-bacilli were more randomly spread at various points on the slides, suggesting that any selective sorption of such types probably varied with the actual species present and the prevailing conditions in the particular seawater sample used in the experiments.

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2.4. Surface substratum composition

Given the significant effects of the surface substratum, Witt et al. [25] investigated the differences in bacterial communities when different settlement substrates (i.e. glass slides, ceramic tiles, coral skeletons and reef sediments) were used. The communities were grown in situ for 48 days at two locations in the Whitsunday Island Group (Central Great Barrier Reef) during two sampling times. Bacterial composition of the biofilm was analyzed using terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of 16S rRNA genes. The results showed that substrate type had little influence on bacterial community composition. Of particular relevance, glass slides and coral skeletons exhibited very similar communities during both sampling times, suggesting the suitability of standardized glass slides for long-term biofilm indicator studies in marine environments of this kind.

Further studies on the dynamics of biofilm formation during the first 24 h found that total organic carbon (TOC) reached the highest level (~ 270 μg cm^{-2}) at two to four h following deployment of unglazed ceramic tiles in the Gulf of Eilat [26], possibly due to adhesion of organics such as sugars, proteins and humic substances from the water column. This peak in TOC and its subsequent decline suggests that the bacteria, while still in the reversible stage of adhesion, utilized the conditioning film as a source of nutrition thus causing the subsequent decrease in TOC.

In addition to the physico-chemical properties of the clean surface and conditioned characteristics due to initial adsorption of non-biological particulates, physical features such as smoothness or roughness of the surface, and biological factors such as deposition of extracellular polymer substances (EPS) by initial colonizing microbes also influence subsequent attachment and

development of the biofilm. In some cases additional biota settle leading to mixed biofilms containing communities of bacteria and eukaryotic microbes, including autotrophs (e.g. diatoms) and heterotrophs (e.g. amoebae, ciliates and flagellates) as examined more fully in the next subsection.

3. Biofilm Development and Biotic Successions

3.1. Biofilm initiation and bacterial physiological changes

Major physiological changes occur when bacteria switch from a planktonic stage of growth to surface-associated biofilm growth. For example, biofilm growth of the marine bacterial isolate, *Pseudoalteromonas* sp. 1398 results in major changes in its cytostolic and extracellular proteomes, a response that is influenced by calcium concentration, including the amount of surface-associated biomass and extracellular matrix material produced by the isolate [27]. Four extracellular proteins, characterized by N-terminal sequencing, showed increased abundances, while one protein, flagellin, showed reduced abundance at higher Ca^{2+} concentrations. Microscopic observations concurrently showed that higher **[**Ca^{2+}**]** associated with surface growth resulted in the repression of flagella production. Approximately 22% of the total cytosolic proteins had differing abundances in response to surface-associated growth when the cells were cultivated in 1 mM Ca^{2+}. At higher [Ca^{2+}], the number increased to 38%. Fifteen other cellular proteins that were differentially expressed in response to biofilm growth and/or Ca^{2+} were identified as factors involved in cellular metabolic functions. These included putative proteases and transport proteins, although there were several proteins not previously characterized. Overall, this evidence indicates that Ca^{2+} causes global changes in matrix material, as well as in cellular and extracellular protein profiles of *Pseudoalteromonas* sp. 1398.

3.2. Successional stages of bacterial biofilm development

In a study of initial bacterial colonization of immersed surfaces in temperate coastal waters by Dang et al. [28], multivariate statistical cluster analyses indicated that the succession of early surface-colonizing bacterial assemblages followed sequential steps on all types of test surfaces (glass, Plexiglas, and polyvinyl chloride). The Rhodobacterales, especially members of the marine *Roseobacter* clade (including the genera *Leisingera*, *Loktanella*, *Nereida*, *Octadecabacter*, *Roseobacter*, *Roseovarius*, and *Sulfitobacter*), formed the most common and dominant primary surface-colonizing bacterial group. The results of this study, combined with previous studies of the Atlantic coast, indicate that the Rhodobacterales are the dominant and ubiquitous primary bacterial surface colonizers in temperate coastal waters of the world. Moreover, the microbial surface colonization followed a successional sequence largely composed of members of the *α–proteobacteria*. The *γ-Proteobacteria* were also abundant, with 42 molecular operational taxonomic units accounting for 33.1% of the 426 clones analyzed. They were quite diverse and were related to *Alteromonas*, *Glaciecola*, *Halomonas*, *Methylococcus*, *Oceanospirillum*, *Oleispira*, *Pseudoalteromonas*, *Psychrobacter*, *Vibrio*, some other unidentified γ-*Proteobacteria*, and bacterial symbionts of marine invertebrates.

Using a more finely adjusted temporal analysis of marine biofilms, Lee et al. [29] examined the succession of bacterial communities during the first 36 h using three kinds of surfaces (acryl, glass, and steel) at Sachon harbor, Korea. Samples were taken at 3–15 h intervals. While slight differences in the bacterial communities were observed with different substrata, major differences were observed between 9 and 24 h for all substrata. Using 16S rRNA gene analyses, major populations of γ-*Proteobacteria* were predominant in the biofilm community during 0 to 9 h; whereas, the ratio of *α–proteobacteria* increased 2.6 to 4.8 fold during 24 to 36 h of biofilm formation, eventually emerging as the most dominant group. Thus, this study indicates that *γ-Proteobacteria* may be more important as pioneering microbes during the earliest temporal phases of biofilm formation than previously realized.

Initial conditioning of the substrate surface and the subsequent formation of a mixed bacterial and diatom community during the first 24 h were studied by Satheesh and Wesley [30]. Acrylic surfaces (coupons) were submersed in a tropical coastal environment. Adsorption of carbohydrates, proteins, calcium, magnesium, nitrite, nitrate and phosphate were monitored along with microbes, although other molecular constituents may also be important, and further research is needed on this topic. Carbohydrate, protein, nitrite and nitrate were adsorbed on the coupons within one h of exposure. Carbohydrates showed a maximum value of 0.28 mg cm^{-2} after 24 h, and concurrently protein concentration reached a maximum of 0.41 mg cm^{-2}. Adsorption of calcium and magnesium was observed after three h. The presence of bacteria was also observed on coupons within one h and diatoms were observed after 15 h. Diatoms such as *Navicula* and *Nitzschia* were the dominant colonizers during the early stages of the biofilm development followed by *Pleurosigma* and *Bacillaria*.

Additional studies have examined changes in marine biofilms over time in relation to variables relevant to environmental change such as increasing temperature and ocean acidification. For example, Rao [31] examined the effects of increasing temperatures on biofilms in coastal waters using the changes in temperature near a nuclear power plant, and reported that increase in water temperature (~ 5 ^{o}C) enhanced the metabolism and influenced most of the biofilm parameters assayed at the warmer station, namely the biofilm was very thick (113 μm) compared to the biofilms produced at the cooler station (22 μm). Overall, the pattern of growth parameters such as biofilm thickness, biomass, chlorophyll *a*, particulate organic carbon, hexose sugar and diatom counts showed a similar trend (i.e. a sharp increase after 96 h of biofilm growth) in the biofilm formed at the warmer station. The potential effects of ocean acidification due to rising atmospheric CO_2 concentrations were studied by Witt et al. [32] based on experimental analysis of biofilm formation under three scenarios: A) pre-industrial (~ 300 ppm), B) present-day (~ 400 ppm), C) mid century (~ 560 ppm), and D) late century (~ 1140 ppm). Based on 16S rRNA gene analyses, CO_2-correlated bacterial community shifts among treatments A, B and D were driven by decreases in the relative abundance of *α–proteobacteria* and increases of Flavobacteriales (Bacteroidetes) at increased CO_2 concentrations, indicating pH sensitivity of specific bacterial groups. Moreover, elevated pCO_2 (C + D) shifted biofilm algal and diatom communities with a significant increase in C and N contents, yet O_2 fluxes, measured using light and dark incubations, remained unchanged, suggesting that the biofilm community adjusted to maintain a steady state of oxygen production. Changing climate patterns associated with global warming and rising sea levels are expected to have profound effects

on coastal environments. Hudon and Bourget [33] examined the effects of natural tidal fluctuations producing periodical immersion on biofilm communities grown on plastic panels in the St. Lawrence Estuary during May to November (1978). In the intertidal zone, bacteria settled after 8–12 weeks, while *Achnanthes brevipes* var. *parvula* appeared after 20 weeks, the only diatom species able to resist semi-diurnal emersion. The ability of the panels to retain water through detritus and irregularities in their surface was probably the main factor allowing the development of this mixed bacteria and diatom community. Panels immersed only at spring tides (monthly) were rapidly colonized by bacteria, and subsequently heavy diatom settlement occurred within 4 weeks. Successive monthly emersions eliminated or strongly reduced diatom populations, which were replaced by filamentous (Ectocayaceae) algae.

The interaction dynamics between initial bacterial colonizers and diatoms using the representative bacteria *Pseudoalteromonas* sp. (strain 3J6) and the benthic diatoms *Amphora coffaeformis* and *Cylindrotheca closterium* during growth for 22 days was studied by Doiron et al. [34]. Images obtained using confocal microscopy showed a difference of surface adhesion between *Pseudoalteromonas* 3J6 and diatoms. In fact, a stronger surface adhesion was found with *C. closterium* suggesting cohabitation between *Pseudoalteromonas* 3J6 and *C. closterium* compared to an adaptation for bacteria and *A. coffeaeformis*. Exponential growth of bacteria depended on the growth of the co-cultivated diatom strain. Diatom strains varied in their growth responses, either growing faster or slower in co-culture with *Pseudoalteromonas* 3J6. The diatom strain that showed the best growth in the presence of P*seudoalteromonas* 3J6 was *C. closterium*. Its population increased at the same rate as the bacterial population. In contrast, the bacterial population decreased when the microalgal population of *A. coffeaeformis* increased. Overall, these data suggest that the cellular attachment and the growth dynamics of bacteria during biofilm formation and succession depend dynamically on each species of diatoms coexisting with the bacteria in the biofilm.

In comparison to diatoms and larger microalgae, picoplankton (< 3 μm) are major primary producers in oceanic and coastal marine environments [e.g. 35,36], and therefore may contribute substantially to marine biofilms [37]. A study of picoplankton communities in tropical marine biofilms (Dona Paula Bay) established on glass slides after immersion for up to 192 h, showed the presence of three groups of picophytoplankton: (1) two prokaryotes that were *Prochlorococcus*-like organisms (PRO), (2) *Synechococcus* (SYN), and (3) picoeukaryotes (PEUK) [38]. Nanoeukaryotes, largely represented by diatoms, were also monitored, and appeared much later in the biofilm succession than the picoeukaryotes. In the total biofilm community, total detectable prokaryotes were dominant throughout the study period. Contribution of SYN was highest (50%) in the earlier stages, which were later overtaken by PRO-like cells. At the end of the incubation period, heterotrophic bacterial numbers were the highest (1.7×10^5 cells cm^{-2}) followed by densities of PRO-like cells (4×10^2 cells cm^{-2}), SYN (4.5×10^3 cells cm^{-2}) and PEUK (1.2×10^3 cells cm^{-2}). The contribution of PEUK and nanoeukaryotes was always below 20%. Picophytoplankton contribution to the total photosynthetic biomass was > 60% in the initial period of biofilm formation, both in terms of numbers and biomass with PEUK as the major contributors. However, after 2 days of incubation, their contribution to total chlorophyll declined, thus revealing that although their numbers were increasing, picophytoplankton were succeeded by nanoeukaryotes in terms of biomass.

3.3. Cell-to-cell signaling in biofilms

A variety of cell-to-cell signaling molecules are involved in communication among bacteria and with cohabiting microeukaryotes in biofilm formation and its subsequent changes over time [39]. In general, bacteria adhering to surfaces produce diffusible molecules that have the capacity to serve as signaling molecules among the community of bacteria in the film. These molecules, generally described as autoinducer (AI) molecules, induce changes in the physiological adaptations of biofilm dwelling bacteria by altering gene expression in a process known as "quorum sensing" (QS). The inductive effects only accrue when the concentrations of the AI molecules reach a particular threshold level. Among the changes induced by QS are a switch from planktonic adaptive modes of life to surface-attached adaptations, including deposition of EPS and other chemically complex secretory products that promote adhesion of the bacteria to the substratum and contribute to the matrix surrounding the bacteria in the biofilm. AI molecules also may be important in algal biofilm formation as explained more fully below.

Among the AI signaling molecules during QS, N-acyl homoserine lactones (AHLs), have been identified in freshwater and marine biofilm microbial communities as candidates, with some strong experimental evidence [40]. They are present in bacterial and mixed (prokaryote and microbial eukaryote) biofilms and may be an important signaling molecule in cross-kingdom signaling in marine bacterial and diatom biofilms [41–43]. For example, AHLs have been identified as possible signal molecules in the formation of diatom (*Cylindrotheca* sp.) biofilms [44]. Biofilm cultures of *Cylindrotheca* were treated with AHLs. After 15 days, the AHLs promoted an increase in Chlorophyll *a* (Chl. *a*) concentration and higher concentrations of extracellular polymeric substance (EPS) contents in the diatom-biofilm compared to controls without the AHLs. When AHL inhibitors were added to control for interference by AHLs secreted from biofilm bacteria, the experimentally added AHLs still increased the Chl. *a* and EPS contents. Scanning electron microscope and confocal laser scanning microscope analyses demonstrated that AHLs promoted the formation of the diatom-biofilm. A non-invasive micro-test technique provided evidence that AHLs promoted Ca^{2+} efflux in *Cylindrotheca* sp., which implied that Ca^{2+} ions could be correlated with the AHLs' induced positive effect on the formation of diatom-biofilms.

In addition to the positive effects of signaling molecules on marine biofilm formation, there is also evidence that they can produce biofilm structural modifications in mixed biofilms. The effects on mixed biofilm formation by low concentrations of three diffusible molecules known to be cell-to-cell signaling molecules; i.e., nitric oxide (NO), *cis*-2-decenoic acid (CDA) and patulin (a mycotoxin) were studied by Walker and Keevil in marine biofilms [45]. Both NO (from a sodium nitroprusside donor) and patulin reduced biofilm formation by more than 90% at the highest concentrations studied (5 mM and 50 μM, respectively). However, colony counts indicated that the effect of patulin is likely due to toxicity as opposed to the signaling mechanisms for NO and CDA. The biofilm communities were also analyzed taxonomically using denaturing gradient gel electrophoresis (DGGE) molecular genetic techniques to determine whether there was any variation in the effects of each molecule on different bacterial taxa. No differential effects were seen on the communities and the biofilms appeared to form according to a neutral community model.

Bacterial secreted proteins also have been identified as possible biofilm disrupting agents, thus

altering the biofilm structure. A protein isolated from the tropical marine strain of an epibiotic bacterium (*Bacillus licheniformis*) has been shown to be a biofilm disruptive agent [46]. The protein was effective against the surface fouling bacterium *Bacillus pumilus* TiO1 cultures, among other pathogenic bacteria. To determine the relative disruptive effects on the growth of biofilm forming bacteria, the minimum inhibitory concentration (MIC) was obtained for three biofilm forming bacteria: *Candida albicans*, *Pseudomonas aeruginosa,* and *Bacillus pumilus*. MIC was defined as the lowest concentration that inhibited growth of each of the three bacterial cultures. The MIC was 1.6 mg ml^{-1} against *C. albicans*. For *P. aeruginosa* and *B. pumilus,* the MIC was 3.12 mg ml^{-1}. As a point of reference, the MIC for tetracycline is in the range 0.06–32 μg ml^{-1} for a broad group of sensitive bacteria. The protein inhibited microbial growth, decreased biofilm formation, and dispersed pre-formed biofilms of the representative cultures in polystyrene microtiter plates and on glass surfaces. Further research is needed to more fully explore how biofilm altering agents affect the structure and composition of biofilms in the natural environment as they mature.

4. Biofilm Structure and Microbial Organization

4.1. Overview and general aspects

Considerable research interest has been devoted to microbial organization and physical structure of freshwater [4,47,48] and marine biofilms [49–55]; especially the latter, because of possible insights into methods of ameliorating biofouling. Some representative examples of research on the structure and functional ecology of naturally occurring marine biofilms are summarized here beginning with Archaeal biofilms followed by prokaryote (bacterial), and finally eukaryotic microbial dominate biofilms.

4.2. Archaeal biofilms

The architecture and diversity of archaeal biofilms in mixed communities with bacteria have been studied in diverse marine or saline environments [56], including methane-bearing sea floor sediments [57], deep-sea black smokers and hydrothermal vents [58,59], Antarctic seawater [60], and under water springs of the Dead Sea [61]. Biofilm-forming archaea include strains encompassing both major archaeal domains: the Euryarchaeota and Crenarchaeota. The multicellular structures they formed exhibit a broad spectrum of architectures, reaching from surface-attached patches to dense biofilms with flat or tower-like structures, unattached cell aggregates, or filamentous streamers. Some Haloarchaea adhere on abiotic surfaces; however, the biofilm architectures can differ as exemplified by differences among the genera. For example, *Halobacterium salinarum* DSM 3754T, strain R1 and a novel Antarctic isolate, t-ADL DL24, form carpet-like multilayered biofilms containing micro- and macro-colonies. Biofilms formed by *Haloferax volcanii* DSM 3757T and *Halorubrum lacusprofundi* DL28 produce large aggregates of cells adhering to the surface [62]. Furthermore, the internal arrangement of species in mixed biofilms can develop a specialized organization, apparently depending on the microbial physiological characteristics. In one case, at a deep-sea active sulfide chimney where the biofilms were analyzed by 16S rDNA analysis and FISH,

four different horizontal zones were found. The exterior zones consisted of a mixed population formed by bacteria and archaea dominated by euryarchaeal species. This composition changed considerably toward the interior zone. This zone was dominated by uncultured crenarchaeal species related to other 16S rDNA isolates derived from deep subsurface hydrothermal environments, probably driven by an improved adaptation to temperatures above 100 ^{o}C [63].

4.3. Bacterial biofilms

Bacterial biofilm community structure was examined in an estuary using stainless steel and polycarbonate surfaces immersed in seawater from the Delaware Bay [49]. Although the authors hypothesized that bacterial communities on the two different surfaces would initially differ substantially and become more similar over time, to the contrary they found the composition was initially the same, but ultimately differed after approximately one week of growth. A possible explanation is that the initial conditioning by nonbiotic material deposited on the two surfaces produced a masking layer that obscured the surface properties of the underlying substratum, and thus the initial colonizing bacteria may not have detected the specific properties of the underlying surface. Later, as the bacterial communities developed and interacted, peculiar interspecies associations may have occurred, thus accounting for the changes in biofilm composition over time.

Based on confocal microscopy data, the three-D architecture of bacterial biofilms from two different surfaces of the French Atlantic coast (intertidal mud flat and a harbor-situated carbon steel structure) were described by Doghri et al. [64]. Out of 25 strains capable of depositing a biofilm in laboratory microtiter plates, only four also formed thick and stable biofilms on glass surfaces under dynamic conditions. These strains developed biofilms with four different three-dimensional architectures when observed by confocal laser scanning microscopy. That is, *Flavobacterium* sp. II2003 biofilms produced mushroom-like structures, *Roseobacter* sp. IV3009 biofilms were quite homogeneous, *Shewanella* sp. IV3014 displayed hairy biofilms with horizontal fibers, whereas *Roseovarius* sp. VA014 developed heterogeneous and tousled biofilms.

Using 16S rRNA partial gene sequence analysis, Narvaez-Tapata et al. [65] examined the molecular diversity of epilithic biofilms along a subtropical intertidal rocky shore in the Southern Gulf of Mexico. Triplicate 2 cm × 2 cm biofilm samples per site were taken randomly from 0.6 m × 0.6 m quadrats located in two sampling sites that were established 20 m apart within a homogeneous calcareous intertidal platform. All-totaled, twenty-two partial rRNA sequences, belonging to four bacterial divisions (Cyanobacteria, Bacteroidetes, Actinobacteria and Proteobacteria), were recovered from these biofilms; of these, Cyanobacteria were the most abundant (41%), followed by Bacteroidetes group (27%), Proteobacteria (18%) and Actinobacteria (9%). There was considerable diversity between the two sites, although they were only 20 m apart. Major groups identified at site 1 corresponded to organisms affiliated within the division Proteobacteria (*Rhodovulum strictum* and *Pseudomonas* spp.) and Actinobacteria (*Rubrobacter radiotolerans* and *Frankia* spp.). In contrast at Site 2, the main groups appeared to be certain Bacteroidetes (organisms related to *Lewinella persicus* and *Flexibacter tractuosus*) and a different population of *Xenococcus* (AB074510). In addition, spatial variability was reported in the biofilm community structure within the same site. For example, biofilm samples from site 1 grouped together only at the 80% level of similarity, indicating that

significant differences in bacterial community structure also occurred on medium-scale analysis between biofilm samples taken at a distance of tens of centimeters from each other. Of particular interest was evidence of aerobic, bacteriochlorophyll-containing bacteria (*Rhodovulum* and *Porphyrobacter*) that in addition to commonly observed cyanobacteria in marine biofilms, may provide additional sources of photosynthetically fixed C compounds for the heterotrophic members of the bacterial biofilm community.

Biofilm bacterial growth in laboratory experiments using glass slides as a substrate has been estimated when influenced by temperature and salinity using seawater from the Black Sea (Eforie Nord) [66]. Bacterial densities (number mm^{-2}) were determined by direct microscopic observation of stained slides. Variations in salinity were assessed first at the lowest level approximating those in the Danube tributary (5 $^{o}/_{oo}$), which produced minimal growth reaching 0.96×10^3 cells mm^{-2} after 2 h; but eventually at 72 h, the density was 6.67×10^3 cells mm^{-2}. The linear equation of growth was $D = 0.4756\ T + 0.823$ ($R^2 = 0.96$), where D = density (cells mm^{-2}) and T is time (h). At more optimal salinities closer to that of the Black Sea of 15 $^{o}/_{oo}$, growth at 2 h reached 1.05×10^3 cells mm^{-2}, with a maximum at 48 h of 7.32×10^3 cells mm^{-2}; and the growth equation was $D = 0.5917\ T + 1.1858$ ($R^2 = 0.94$). Temperature variations ranging from 6 ^{o}C to 23 ^{o}C also produced pronounced effect on biofilm bacterial growth. After 2 h at 6 ^{o}C, densities were 0.67×10^3 cells mm^{-2} and thereafter reached a peak of 5.13×10^3 at 48 h; with a growth equation of $D = 0.3755\ T + 0.9202$ ($R^2 = 0.80$). In a more optimal temperature (23 ^{o}C) after 2 h the density was 1.85×10^3 cells mm^{-2}, and the peak at 48 h was 7.45×10^3 cells mm^{-2}. The equation for growth was $D = 0.4992\ T + 2.4367$ ($R^2 = 0.89$). As reported in other biofilm research studies, the distribution of the bacteria on the substratum initially formed microcolonies in relatively low numbers that subsequently enlarged forming larger macrocolonies over a total time of 72 h.

4.4. Microalgal-dominate biofilms

Composition and biomass of mixed algal and bacterial biofilms were also studied as a function of seasonal differences in temperature and salinity by Chiu et al. [67]. Laboratory grown biofilms, using natural seawater collected from the field during summer 2003 and winter 2004 were allowed to develop for 20 days. Overall, total biofilm biomass was greater in summer than in winter (ranging from 10 to 46 μg dry weight cm^{-2}), and greater at 34‰ than at 20‰. The biofilm chl. *a* content was affected by all three factors (season, temperature, and salinity), with significant interactive effects among the three factors. C/N ratios were affected by season (winter > summer) and temperature (30 ^{o}C > 16 ^{o}C in summer), but not by differences in salinity. However, in summer, both bacterial and diatom community compositions were significantly affected by salinity. Distinctive communities developed at 34‰ and 20‰. In contrast, temperature differences exerted a major influence on community composition in winter, that is distinct communities developed at 30 ^{o}C compared to 16 ^{o}C. In addition to natural variations in localized biofilm environments, increasing evidence points to major effects on marine biofilm structure and function due to pollution and eutrophication. The structure and function of microbial biofilms colonizing stones and sediments in eutrophied portions of Boddens in the Baltic Sea exhibited major changes in trophic status due to eutrophication [68]. With increasing eutrophication, the ratio of autotrophic to heterotrophic microbial processes became

greatly reduced [68] .

The structure of microphytobenthic biofilm microbial mats (consisting largely of benthic microalgae) growing on soft sediment surfaces such as in intertidal mud flats was studied by Underwood [69] and summarized in a rather comprehensive review. Microphytobenthic biofilms are dominated by motile diatoms, and other microalgal groups, including some green algae, dinoflagellates, euglenophytes, as well as cyanobacteria. However, diatoms dominate the biomass in the majority of habitats. Because the intertidal zones are typically high energy sites, resuspension and deposition of sediments caused by the constant tidal and wave movement selects for motile taxa (e.g. biraphid pennate diatoms) able to cope with intermittent burial and with an ability to move back to the illuminated sediment surface. Dense populations of benthic diatoms (exceeding 10^4 cells cm^{-2}) produce large quantities of EPS within the sediment matrix and also on the sediment surfaces. In microphytobenthic biofilms, diatoms and cyanobacteria are major producers of EPS, resulting in biofilms up to 3–4 mm thick, forming sediment-stabilizing "skins" on the surface of intertidal sediments. Concentrations of EPS, and other carbohydrate fractions, can be as high as 16,000 μg glucose equivalents (6.4 mg C) g^{-1} dry weight sediment, although this may be somewhat of an underestimate due to the limitations of the quantitative techniques used for sediment extracts. In situ visualization of EPS in natural sediments using low temperature microscopy usually shows a honeycomb structure of cells and EPS on top of the sediment surface, which is probably more normal than the "strands and filaments" visualized by electron microscopy, that may be artifacts. EPS is most likely present in sediments as an unstructured, unformed gel-solution matrix surrounding and binding sediment particles and cells.

In some marine environments, secreted EPS may help to protect diatoms in biofilms from salinity stress [70]. For example, the diatom *Cylindrotheca closterium* grown in a xanthum gum matrix at salinities of 35, 50, 70 and 90‰ had significantly increased cell viability, growth rate and population density by up to 300, 2,300 and 200%, respectively. Moreover, diatoms grown in 0.75% w/v xanthan, subjected to acute salinity shock treatments (salinities up to 90‰), maintained photosynthetic capacity within 4% of pre-shock values, whereas cells grown without xanthan declined by up to 64% during hypersaline shock. EPS has also been shown to be present in very high concentrations in the brine channels of melting arctic sea ice, and it appears to play important buffering and cryoprotectant roles for the biofilm microorganisms, especially diatoms, by ameliorating harsh winter conditions of high salinity and potential ice-crystal damage [71].

In addition to vertical movements of diatoms to prevent damage in high energy intertidal biofilms, vertical locomotive patterns also occur in response to light intensity. In experimental studies, Perkins et al [72] examined physiological regulation and vertical migration patterns of diatoms during a six h immersion period using chemical inhibitors under two light treatments (ambient interval and constant light at 300 μmol m^{-2} s^{-1}). Overall, there were two main conclusions: (1) the primary response to accumulated light dose was vertical movement, which when experimentally inhibited resulted in severe down-regulation, and/or photoinhibition; (2) diatoms down-regulated their photosynthetic activity in response to accumulated light dose (e.g. over an entire immersion period) using a combination of vertical migration and physiological mechanisms that may contribute to their diel and/or tidal patterns in productivity. Particularly, optimization of photosynthetic activity in response to an increasing exposure to light (i.e. an accumulated light dose

response rather than the dose intensity, per se) is largely due to vertical cell migration. The diatoms take a position in the sediment surface layer such that the attenuation of light provides an optimal light environment for their photochemistry. In general, photosynthetic light response curves appear to saturate at 400 to 800 μmol m^{-2} s^{-1}, and it is likely that the diatoms situate themselves beneath the sediment surface where the light intensity is closer to 800 μmol m^{-2} s^{-2} or lower, rather than the much higher values on the sediment surface during sunny days. Moreover, in terms of adaptive efficiency, cell migration may well be more energetically favorable than physiological down-regulation processes such as non-photochemical quenching induction (e.g. down-regulation through the xanthophyll cycle induction). However, the results suggest that a probable combination of vertical migration and physiological mechanisms result in a diel and/or tidal pattern of down-regulation. Based on a physiological ecology perspective, it is reasonable that after adequate light exposure for photosynthate production, cells would down-regulate their photosynthetic activity when tidal or diel light cycles were less favorable for primary production.

5. Perspectives on Biogeochemical Cycles and Marine Biofilms

Given the substantial microbial density and complexity of marine biofilm communities, including closely linked metabolic networks, and complex coordination through shared signaling molecules such as quorum sensing [73], it is very likely that marine biofilms can contribute substantially to some major biogeochemical cycles. Moreover, based on their substantial biomass globally [1], their role in biogeochemical chemical cycles would seem to be very plausible and of immense interest [74].

Overall, a search of the literature revealed rather limited research attention that specifically falls within the realm of the role of marine microbial biofilms in biogeochemical cycles. Some examples are reviewed here focusing particularly on the carbon biogeochemical cycle and the nitrogen cycle where there is good evidence of emerging interest. Especially given the significance of atmospheric CO_2 in climate change, and the possible role of the marine environment in modulating sources and sinks of CO_2, additional research on the role of biofilms in the carbon biogeochemical cycle would seem to be of great importance. Therefore, some examples of research on the role of marine microbial biofilms in processes related to C geochemistry are summarized first.

5.1. Carbon cycle

Carbon fixation by photosynthetic microbial biofilms can vary substantially based on the thickness of the biofilm and of course the geographic and environmental locale. Lugomela et al. [75] reported carbon fixation rates of 0.05, 0.3 and 0.5 kg C m^{-2} y^{-1} for thin (~ 0.5 mm), medium (~ 1 mm) and thick (~ 2 mm) biofilms, respectively. They estimated an overall primary production rate of 0.14 kg C m^{-2} y^{-1} at depths in the water column of about 5 m in sub-tidal biofilms near Zanzibar, Tanzania. The results they report for thin and thick biofilms are lower compared to those reported for microbial mats from Mellum Island, North Sea and Solar Lake, Sinai, of 1.83 and 2.26 kg C m^{-2} y^{-1}, respectively [76]. The differences may be attributed to the low biomass of the biofilms in the Zanzibar locales as compared to the well-developed microbial mats in the other sites. The overall

productivity estimate, however, of 0.14 kg C m^{-2} y^{-1} is comparable to the gross primary production values ranging from 0.09 to 0.16 kg C m^{-2} y^{-1} reported for fine and coarse sediments, respectively, from the Great Barrier Reef lagoon, Australia [77]. Furthermore, net primary production values of 0.17 kg C m^{-2} y^{-1} were reported in microphytobenthic communities, also from the Great Barrier Reef [78].

Intertidal biofilms on rocky substrata can be major centers of carbon nutrient dynamics as exemplified by studies in the Douro lower estuary of Portugal [79]. Hourly net primary production (389 ± 168 mg O_2 m^{-2} h^{-1}) substantially exceeded hourly net respiration rates (50 ± 18 mg O_2 m^{-2} h^{-1}) by five to 11 times, suggesting a net positive balance toward C fixation. These results were comparable to those of adjacent muddy and sandy sediments. The mean value for total organic matter was 13.4 ± 8%. A positive linear relationship was reported between Chl. *a* and total organic matter ($r = 0.84$, $p < 0.001$, $n = 24$), suggesting that the majority of the organic matter in the biofilms was attributed to algae.

Organic C incorporated in estuarine biofilm microalgal EPS has been traced using isotopic labeling techniques from the algal primary producers into the heterotrophic members of the biofilm food web, including bacteria [80], a phenomenon more widely documented in ocean environments [81]. Coupled pulse-chase isotopic labeling and simultaneous tracking of ^{13}C in EPS polysaccharides and phospholipid fatty acids (PLFAs) directly demonstrated flow of C between biofilm autotrophs and heterotrophic bacteria, and also established the significance of diatom-derived carbohydrates in this exchange. There was a significant isotopic enrichment of glucan residues indicating that a large proportion of fixed C was initially sequestered into intracellular chrysolaminaran. Moreover, labeled C was also quickly incorporated in diatom PLFA 20:5ω3 and EPS. The saccharides in the EPS fractions were enriched rapidly, followed by labeling of distinct bacterial PLFAs through heterotrophic utilization of EPS within four h. Maximal isotopic enrichment of diatom and Gram-negative bacterial PLFAs, and evidence in the EPS fractions, occurred after four h. ^{13}C-labeling increased in Gram-positive bacterial PLFAs throughout the study period. Further evidence indicated persistence of assimilated C in the organisms within the biofilms through time and indicated rapid turnover of polysaccharide pools within the biofilms. After 48 h, PLFAs remained highly labeled relative to diatom-derived polysaccharides. Overall, the evidence of major C flow from EPS of biofilm primary producers to heterotrophs is consistent with broader evidence of the importance of microbial EPS in marine C dynamics and biogeochemical processes [82].

Carbon fixation by microbial communities inhabiting deepsea basalt microbial biofilms using ^{13}C-labeled bicarbonate indicated incorporation of ^{13}C into organic matter within the rock microbial communities, but the amount varied with the location of the basalt [83]. The incorporation of ^{13}C into seafloor-exposed rocks was most significant, but inconclusive for subseafloor basalts. Potential rates of carbon fixation in seafloor-exposed sites were estimated to be as much as 0.1–10 nmol C g^{-1} day^{-1}, and when scaled to the global production of oceanic crust, the total C fixation rates could be as much as 10^9–10^{12} g C y^{-1}, an estimate consistent with earlier predictions based on thermodynamic calculations. This study was one of the first to provide strong evidence that basalt-hosted autotrophy could be a significant contributor to seafloor organic matter across the broad geographic expanse of the deep ocean seafloor.

Sinking particulates in the ocean water column and other particles with adhering microbial

biofilms designated as "Marine snow" support bacterial biofilms that are potentially active loci for C dynamics and remineralization [84]. Bacteria attached to sinking particulates encounter unique environmental constraints and demands that may promote group signaling through quorum sensing resulting in adaptive responses to better exploit the particulate surface. Among other responses, increased efficiency is realized when the cost of producing exoenzymes is shared among the community of particle-attached bacteria. A decrease in advective and diffusive losses of exoenzymes in biofilms can greatly increase particle-attached bacterial production and would likely offset the additional energetic cost associated with producing the biofilms. From a biogeochemical perspective, conditions in the natural environment (e.g. advection, particle sinking, and chemoattraction) are likely to increase the number of bacteria contacting a particle [85]. Consequently, variation in bacterial colonization rates due to these environmental factors may influence the length scales of C remineralization and the dynamics of the mesopelagic food web by enhancing the ability of bacteria to utilize organic C in particles. For example, if colonization is rapid, particle-attached bacteria working in coordinated groups can solubilize and consume large, fast-sinking particles at time scales that are sufficiently rapid to be comparable to effects of predation by zooplankton. The bacteria also compete with zooplankton predation by rapidly solubilizing particles into molecules too small for larger zooplankton to ingest [84]. Overall, biofilms on particles settling into the deep ocean provide novel opportunities to more fully explore the diverse roles that biofilm communities perform in the oceanic C biogeochemical cycle.

5.2. Nitrogen cycle

Sufficiently illuminated marine biofilms containing mixed communities including cyanobacteria, heterotrophic bacteria, and microphytoplankton, are actively involved in the N cycle as well as the C cycle. Cyanobacteria are commonly observed in marine biofilms in near-surface and deeper locations in the water column and are significant contributors to nitrogen fixation. Magalhães et al. [86] examined flux rates of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O) as well as nitrogen (N) fixation, nitrification, and denitrification at two intertidal sites widely studied (muddy and sandy sediments) in comparison to two sites representing the rocky intertidal zone, where far less biogeochemical research has been done. All sites were located at the Douro River Estuary (Portugal). During daylight, NH_4^+ and NO_3 uptake rates together with ammonification could supply the requisite N requirements of the primary producer communities at all four sites. Dissolved inorganic nitrogen (DIN) removal was largely mediated through N assimilation by benthic or epilithic primary producers. N fixation, nitrification, and denitrification were minor processes in the overall light DIN cycle. At night, distinct DIN cycling processes took place in the four environments. Denitrification rates ranged from 9 ± 2 to 360 ± 30 μmol N_2 m^{-2} h^{-1}, accounting for 10–48% of the water column NO_3^- uptake; nitrification rates varied from 0 to 1,712 ± 666 μmol NH_4^+ m^{-2} h^{-1}. Overall, mean rates of DIN net fluxes and the processes involved were higher in the rocky biofilms than in the sediments. More particularly, the intertidal rocky biofilms released 10 times the amount of N_2O that was produced in intertidal sediments (up to 17 ± 6 μmol N_2O m^{-2} h^{-1}), representing one of the highest N_2O release rates recorded for marine systems.

Additional evidence of salinity and inorganic nitrogen concentrations on nitrification and

denitrification rates in intertidal sediments and rocky biofilms, also in the Douro River estuary, was obtained in field studies and laboratory enrichment experiments during a period of 12 months [87]. There were no salinity effects on denitrification in either study site, suggesting that halotolerant bacteria dominated the denitrifier communities. By contrast, nitrification rates were stimulated when salinity increased from 0 to 15 $^{o}/_{oo}$. When NO_3^- was added in laboratory experiments, denitrification rates were elevated in sandy sediments, but not in rocky biofilms. However, in rocky biofilms a positive and linear relationship (r = 0.92) was observed between denitrification rates and water column NO_3^- concentrations during the monthly surveys. N_2O:N_2 ratios increased rapidly when NO_3^- increased from 63 to 363 μM; however, data from monthly surveys indicated that environmental parameters other than NO_3^- availability may be important in controlling the variation in N_2O production via denitrification. When ammonium was added in sandy sediments, nitrification rates were increased by 35% for the 20 μM NH_4^+ addition, but NH_4^+ appeared to inhibit nitrification at high concentrations (200 μM NH_4^+). In contrast, rocky biofilm nitrification was stimulated by 65% when 200 μM NH_4^+ was added. The different responses observed between the two sites suggest that the rocky biofilm nitrifier community is more tolerant of higher NH_4^+ concentrations.

Temporal variations in the physical and chemical properties (with an emphasis on nitrate reduction) were studied in biofilms established on plexiglass panels placed in the Edaiyur-Sadras estuary in India [88]. The biofilm thickness ranged from 65 to 270 μm over a period of seven days. Significant correlations were observed between biofilm thickness and the following variables: biomass (r = 0.95; p = 0.0001), diatom counts and hexose sugar levels (r = 0.95; p < 0.0001), and nitrate reducers and ammonia production (r = 0.97; p = 0.0001). After 24 h of biofilm growth, facultative anaerobic bacteria such as nitrate reducers were observed and remained up to 96 h, but by the end of 168 h the estuarine biofilm showed significant levels of nitrate. Ammonia oxidizing bacteria were present after 96 h of biofilm growth. The biofilm was initially dominated by bacteria, followed by diatoms, and finally after 168 h, it was largely dominated by heterotrophic bacteria. The denitrifying bacteria observed during the course of this study were *Alcaligens* sp., *Bacillus* sp., *Micrococcus* sp., *Pseudomonas* sp. and *Vibrio* sp. This was one of the first reports that nitrate reduction and ammonia oxidation occur in a seven-day-old marine biofilm. This process may have happened due to diffusion of ammonia from the anaerobic zone to the oxygen rich surficial regions of the biofilm, where aerobic microbial oxidation of ammonia resulted in nitrate formation and subsequently its diffusion back to the anaerobic zone.

One of the more recently explored aspects of the nitrogen cycle in marine systems is the anammox reaction releasing N_2; i.e., biological anaerobic oxidation of NH_4^+ to N_2 with NO_2^- reduction [89]. Although the initial discovery of anammox as a source of N_2 in the sea was as early as 1941 [90], it has received less attention in marine environments compared to other aquatic environments. A variety of methods for detection of anaerobic ammonium oxidizing bacteria driving the anammox reaction have been developed including N isotopic labeling, lipid assays specific to anammox bacterial cells, and molecular genetic techniques. These include fluorescent in-situ hybridization (FISH) based techniques, PCR-based methods, and quantitative PCR (qPCR) [91].

Fluorescent in-situ hybridization was used by Awata et al. [92] to examine the effects of temperature and salinity on the microbial structure of marine anammox biofilm bacteria under controlled laboratory conditions in an up-flow, fixed-bed column reactor. Anammox activity was

observed at 20 and 30 ^{o}C, but not at 10 ^{o}C. The maximum nitrogen removal rate was 0.32 kg TN m^{-3} day^{-1} when the ammonium and nitrite removal efficiencies were 61.4 and 89.7%, respectively. Variations in salinity from ~ 0.9 to 3.5% had no discernible affect on the anammox reaction. Based on the FISH analysis, two different species of *Candidatus* Scalindua were present, very likely closely related to *Candidatus* Scalindua wagneri (husup-a7-Iike Organisms) and *Candidatus* Scalindua marina (husup-a2-Iike organisms). Only the husup-a7 bacteria contributed to nitrogen removal as N_2 at 30 ^{o}C. At 20 ^{o}C, both groups of bacteria were present, possibly because the operational conditions at 20 ^{o}C were suitable for the additional growth of the husup-a2-like organisms. These results suggested that among marine anammox species, the husup-a7-like organisms have a wide optimum range of temperature and husup-a2-like organisms have a very narrow temperature growth range.

6. Conclusions and Future Research Opportunities

6.1. General conclusions

Given their substantial biomass and geographically wide global distribution, much more research is warranted on naturally occurring marine biofilms, beyond the significant emphasis currently placed on practical aspects such as marine biofouling. Marine biofilms have been researched in a wide variety of geographic locales, including coastal, estuarine, deep ocean, and marine rock and sediment microbial communities at worldwide locations. The research reviewed here indicates that marine biofilm structure, and development during microbial succession is largely similar to that in naturally occurring freshwater biofilms [2,48], with modifications due to salinity and nutrient conditions of the marine environment. Initial conditioning of the substrate by surface binding of inorganic and organic ions and molecules affects the microbe's initial reversible attachment that ultimately leads to adhesion and the first stages of colony formation. Surface energy and the concentration of ions interact with settling microbes and determine reversible adsorption of the microbes to the surface, which at very low ionic concentrations approaches zero. Initial adsorption of bacteria by Van der Waals attraction to the surface can be modulated by the presence of a surface ionic double layer. After microbial adsorption, microcolonies expand and eventually produce macrocolonies of varied architecture that are partially specific to the microbial species, but also modified by water currents and in some cases predation by bacterivorous protists.

Bacterial biofilms typically precede mixed biofilms containing diatoms and picoplankton that settle later and enhance phototrophic fixation of C. Motile diatoms adjust their vertical position in the biofilm to achieve optimal irradiation dose, in addition to physiological responses whereby they adapt to diel light cycles and tidal rhythms. There is some evidence that that photosynthetic C fixation in some biofilms exceeds loss by respiration, thus possibly leading to a net C gain in biomass. Organic C fixed by diatoms and other EPS-secreting photosynthetic microbes in the biofilm can be transferred to heterotrophic microbes that digest EPS and assimilate the released organics. Carbon fixation rates in seafloor, exposed basalts may be as much as 10^{9}–10^{12} g C y^{-1}. Carbon fixation in subtidal biofilms may vary with biofilm thickness; i.e., 0.05, 0.3 and 0.5 kg C m^{-2} y^{-1} for thin (~ 0.5 mm), medium (~ 1 mm) and thick (~ 2 mm) biofilms, respectively. Estimated overall primary production rates of 0.14 kg C m^{-2} y^{-1} have been reported. This production rate is

comparable to the gross primary production values ranging from 0.09 to 0.16 kg C m^{-2} y^{-1} reported for fine and coarse sediments, respectively, from the Great Barrier Reef lagoon.

The presence of biofilm cyanobacteria and heterotrophic bacteria involved in reactions of the N cycle suggests that these widely geographically distributed microbial communities may have a significant role in the global N biogeochemical cycle, including the role of anammox bacteria that mediate release of gaseous N_2 through ammonium oxidation in seawater environments. Differences in nitrogen metabolism in rocky and sandy sediment biofilms have been reported. NO_3^- addition in laboratory experiments, enhanced denitrification rates in sandy sediments, but not in rocky biofilms. However, in rocky biofilms a strong positive linear relationship was observed between denitrification rates and water column NO_3^- concentrations. When ammonium was added to sandy sediment, nitrification rates were increased by 35% with 20 μM NH_4^+, but NH_4^+ appeared to inhibit nitrification at higher concentrations (e.g. 200 μM NH_4^+). In contrast, rocky biofilm nitrification was stimulated by 65% when 200 μM NH_4^+ was added.

Laboratory studies of anammox bacteria in biofilms indicated that a maximum nitrogen removal rate of 0.32 kg TN m^{-3} day^{-1} is possible when the ammonium and nitrite removal efficiencies were 61.4 and 89.7%, respectively. The reaction rates were highly dependent on temperature, but less so on salinity. In laboratory studies of ammonia oxidizing bacteria from estuaries, anammox activity has been observed at 20 and 30 ^oC, but not at 10 ^oC, indicating a possible strong temperature dependency of anammox release of N_2 from biofilms in marine environments.

6.2. Future research prospects

In general, it appears more is known about the composition and structure of marine biofilms than their role in ecology and biogeochemical processes. Some suggestions are presented for future avenues of research using modern analytical techniques that may help to better elucidate the dynamics of biofilm function and their role in global biogeochemical cycles. Particular attention is needed for more comparative analyses of biofilm structure and function across different biogeographical locales and in relation to hydrology (especially physical and chemical properties). Much more detailed information can be obtained by a more systematic analysis of changes in biofilm community composition and physiological ecology during initial stages of formation and subsequent succession of biofilm development, especially using modern techniques such as transcriptome analyses to identify what genes are being transcribed and what proteins are being produced during each of the major stages of biofilm development. There is good developing evidence that transcriptome analyses of biofilms can provide important insights into the dynamics of microbial physiological ecology especially related to the effects of QS, group signaling, and the induction of gene activity [93–95]. For a more comprehensive account focused on bacteria see a recent review by Dang and Lovell [96].

While many of these studies provide proof of concept for application to natural marine microbial biofilms, some of the published studies concern either disease-producing microbes or laboratory investigations that are not focused on the natural environment. Combining molecular genetic microbial techniques (e.g. PCR-DGGE) to more fully account for the taxonomic composition

and diversity of developing biofilm microbial consortia along with transcriptome analyses to document gene activation may provide a more complete account of the dynamics that occur during natural marine biofilm development. In this regard, combining the above analytic techniques with the use of fluorescent in-situ hybridization (FISH) may identify more clearly the pattern of microbial taxa activated during different phases of biofilm development and/or across different environmental settings and water column features.

Although considerable attention has been given to microbes associated with open ocean floc (marine snow), less attention has been given to the ecology and biogeochemistry of surface microbial communities associated with floc and particulates in estuaries, though it is known that they can be highly abundant (exceeding 100 per liter in some cases) [97]. Moreover, estuarine floc supports a diverse association of surface-dwelling bacteria [98] and microeukaryotes, including evidence of heterotrophic microflagellates [99–103]. Current techniques are available to size partition and separate suspended floc with minimum artifacts, thus allowing a more detailed examination of the floc-dwelling microbial communities [102]. Some promising lines of research include the following. What taxa are characteristic of estuary particulates in different locales, along salinity gradients, and in different strata in the water column using microscopic observations and molecular genetic techniques? How does the total biomass and C content of floc-dwelling microbiota vary with major variations in water column properties? What are the trophic relations among the microbiota, including the differences in biomass and trophic flow of C among the biofilm taxa? To what extent do particle-bound (floc) microbes contribute to respiratory CO_2 emission compared to freely suspended microbes in the plankton? What is the available source of organic nutrients assimilated by the floc-dwelling microbes using isotopic tracer techniques including ^{14}C, ^{13}C, ^{15}N as has been applied more generally in other water column studies? Do seasonal cycles in temperate locales influence the microbial community composition on particulates, and what are the trophic relations, sources and fate of nutrients, as well as variations in emission of respiratory CO_2?

Further research, beyond particulates in the estuarine water column, is also warranted on the role of biofilms in estuarine and coastal tidal sediments, including mud flats [e.g. 104], where substantial sources of nutrients and favorable surfaces for microbial attachment and biofilm development may support significant communities of prokaryotic and eukaryotic microbes serving important roles in the trophic and biogeochemical dynamics of shallow water environments. Moreover, the dynamics of resuspension of biofilms into the overlying water column, and possible contribution to the suspended particulate biofilm load, is worthy of further study [e.g. 103,105,106，107]. Research on shallow-water mudflat biofilms is a topic that could be pursued profitably using combined field-based methods and laboratory microcosm experimental approaches, especially incorporating some of the modern analytical techniques cited above.

Turning attention more generally, one of the more productive and possibly increasingly important areas of research is the role of marine biofilms in biogeochemical cycles. It is clear from some of the few studies reviewed here that there is substantial potential in pursuing more fully how microbial communities in marine biofilms may serve as major links in the C and N biogeochemical cycles. While some research has focused on other mineral elements important in biogeochemical cycles such as silica and phosphorus [86], the importance of C as a major element of life and its increasing significance in climate change (as sources of atmospheric CO_2 and CH_4) warrants more

systematic research on C dynamics in marine biofilms. Modern high sensitivity microelectrodes can be used in field-based and laboratory experimental studies to more fully document the sources and sinks of CO_2 in biofilms, particularly mixed biofilms where consortia of phototrophic and heterotrophic microbes provide a more substantial source of evidence for phototrophic C fixation and its transfer among heterotrophic members of the microbial community. The substantial deposition of EPS organic matter in biofilms may also be a significant link in the C biogeochemical cycle and much more information on the composition and abundance of EPS in varying geographic environments and under different nutrient regimes is needed. For example, we are only beginning to explore more fully biofilm communities in high latitude environments where there is increasing evidence that marine biofilms in sea ice, for example, may contribute substantially to the C cycle. Areal C content has been reported as high as 3.3–4.0 g C m^{-2} in arctic sea ice brine channels when the ice was thickest [71]. Further research is needed documenting how much of the C fixed by primary producers (e.g. bacteria and diatoms) is converted to EPS in brine channel sea ice biofilms. The role of archaea in marine biofilms, especially their community composition and role in metabolic transformation of C compounds and production of CH_4 is worthy of much more attention.

Heavy isotopes such as ^{13}C and ^{15}N have been used to some extent in mapping C and N dynamics in marine biofilms (examples reviewed above), but much additional research is needed to more fully document these processes for biofilms in different stages of development, and across varied marine environments, including estuarine and oceanic sites; especially focusing on effects of physical and chemical environmental variables on the sources and fate of C and N in biofilm microbial foodwebs. There is good evidence that the quantity and quality of autochthonous and allochthonous sources of organic C nutrients vary across different environments, especially in relation to the turbidity of the water column and the geographic locale. More turbid water, where primary production is limited, e.g. near highly productive land margins, may receive a larger proportion of allochthonous organic nutrients; while clearer water, with sufficient mineral nutrients, is more likely to include greater contributions of autochthonous C sources due to in-situ primary production. In general, how these variations contribute to differences in biofilm structure and function need more intensive investigation across broad biogeographic regimes.

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Conflicts of interest

The author declares no conflict of interest in this research.

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