Chapter 4
Extracellular Vesicles in the Environment

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Abstract Extracellular vesicles are small membrane-bound structures released by cells from all domains of life. Diverse populations of vesicles are found in many natural ecosystems, where they mediate complex networks of interactions between microbes and their local environment. Vesicles can serve numerous functions, including transporting and delivering compounds such as lipids, proteins, nucleic acids, and small molecules, between organisms and across both spatial and temporal dimensions. In this review I consider extracellular vesicles from an ecological perspective, exploring their influence on both the biotic and abiotic environment. I summarize our current understanding of vesicle contents and distributions in various microbial habitats, their potential contributions to nutrient pools and food webs, and the many ways in which vesicles can influence the physiology, ecology, and evolution of microbial communities. While many questions concerning the ecological impact of extracellular vesicles remain to be answered, it is becoming increasingly evident that these particles play important roles in the global ecosystem.

4.1 Introduction

Bacteria are found in essentially every habitat on Earth. Due to their remarkable abundance, small size, and fast metabolisms, microbes have the ability to profoundly impact both their local environment and, ultimately, drive the major biogeochemical cycles that sustain life on the planet. Bacteria evolve and function within complex ecological communities, and the physiology and behavior of these globally important microbial consortia can be affected by interactions with other cells and the surrounding abiotic environment (Azam and Malfatti 2007; Hibbing et al. 2010). In recent years, our understanding of the mechanisms underlying microbial interactions has been expanded by the discovery of extracellular vesicles (EVs). EVs are small,

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membrane-bound structures released by cells from all domains of life, with numerous studies documenting vesicle release by archaea, bacteria, and eukaryotes (Deatherage and Cookson 2012). These structures are now known to be prevalent in a variety of environments, raising questions about their roles within natural ecosystems.

This chapter discusses extracellular vesicles from an ecological and environmental standpoint, examining vesicle contents, distributions, contributions to global nutrient pools, and putative functions within microbial ecosystems. An ecological perspective is important when considering the roles of EVs produced by any organism and within any environment, whether in the ocean or within the human body; however, this chapter will focus primarily on bacterial membrane vesicles (MVs) within nonhost-associated communities and in nonpathogenic contexts. The emerging body of research on extracellular vesicles indicates that these submicron particles likely influence many aspects of microbial community function and evolution, ultimately affecting the global ecosystem in ways that we are just now beginning to identify.

4.2 Formation and Contents of Membrane Vesicles

Extracellular vesicles can be formed through a variety of processes in prokaryotic and eukaryotic cells, but all vesicles share a common set of basic properties: they are small, spherical structures, typically between 20 and 250 nm in diameter, which are bounded by a lipid bilayer membrane (Schwechheimer and Kuehn 2015). In bacteria, MVs are primarily thought to derive from local regions of the cell's outermost membrane that begin to protrude away, eventually expanding (i.e., "blebbing" out) until they separate from the rest of the cell (Schertzer and Whiteley 2012). The putative mechanisms of vesicle formation in bacteria have been reviewed extensively (Brown et al. 2015; Schwechheimer and Kuehn 2015; Pathirana and Kaparakis-Liaskos 2016; Toyofuku et al. 2019), including elsewhere in this book (see also Chaps. 2 and 3), and will not be detailed here.

The biological impacts of vesicles are largely attributable to the fact that they serve as a versatile secretion, transport, and delivery mechanism for cells (Kulp and Kuehn 2010; Guerrero-Mandujano et al. 2017). As MVs are formed and released by a bacterium, they can take a variety of compounds with them into the extracellular milieu (Brown et al. 2015; Schwechheimer and Kuehn 2015). Besides the lipids that comprise the vesicle membrane, MVs can contain an array of proteins originating from all compartments of a bacterial cell, though the degree to which specific proteins are preferentially "packaged" as cargo into vesicles remains an open question (Bonnington and Kuehn 2014). MVs carry a variety of small molecules as well, and vesicles may be a particularly useful vehicle for exporting hydrophobic

compounds such as quorum-sensing quinolones into an aqueous extracellular environment (Mashburn-Warren and Whiteley 2006).

A remarkable diversity of nucleic acids can be found in EVs, including chromosomal DNA fragments (ranging from hundreds to many thousands of bp long), plasmids, viral genomes, and a variety of messenger, transfer, ribosomal, and small RNAs (Soler et al. 2008; Biller et al. 2014; Gaudin et al. 2014; Sjöström et al. 2015; Blenkiron et al. 2016). In bacterial and archaeal vesicles, these nucleic acids are found either within MVs and/or associated with their outer surface (Renelli et al. 2004; Bitto et al. 2017). The mechanisms responsible for moving nucleic acids from the cytosol into a MV remain unclear. In the Gram-positive bacterium *Streptococcus mutans*, the protein secretion machinery may play a role in moving DNA into MVs (Liao et al. 2014). In Gram-negative bacteria, the discovery that some outer membrane vesicles can contain both outer and inner membranes provides an alternate explanation for DNA export based on cellular topology (Perez-Cruz et al. 2013): if both inner and outer membranes were to simultaneously "bleb" out of the cell and form a vesicle, then a sub-compartment within the vesicle would originate directly from the cytosol, avoiding the need for DNA to cross the inner membrane. It is not yet clear, however, whether such "outer-inner membrane vesicles" are the only ones to contain DNA, nor how the chromosomal DNA fragments found within vesicles are generated in the first place.

The ability of bacteria, archaea, and eukaryotes to export portions of their genetic information via extracellular vesicles has a number of functional implications (discussed in more detail below), and also provides a means to identify which cells in a given environment produce these structures. Based on the assumption that the majority of DNA contained within a vesicle likely originated from the cell that produced it, metagenomic sequencing of vesicle-enclosed DNA collected from both coastal and open-ocean water samples has shown that organisms from all three domains, including representatives of at least 33 different phyla, produce vesicles in marine environments (Biller et al. 2014). These results further support the notion that most, if not all, microbes release extracellular vesicles, and indicate that vesicle production is a common occurrence in the natural environment—not just in laboratory cultures (Fig. 4.1a).

4.3 Variation Among Vesicles

Studies of MVs from *Pseudomonas aeruginosa* and *Escherichia coli* have long noted that these model organisms produce heterogeneous vesicle populations varying in physical properties including particle size (Kadurugamuwa and Beveridge 1995) and buoyant density (Kesty and Kuehn 2004; Renelli et al. 2004) in culture. Such heterogeneity has been observed in vesicles from the marine cyanobacteria *Prochlorococcus* and *Synechococcus* as well (Biller et al. 2014). The concept of vesicle heterogeneity further applies to their contents. For example, one investigation into the DNA distribution among vesicles isolated from various marine

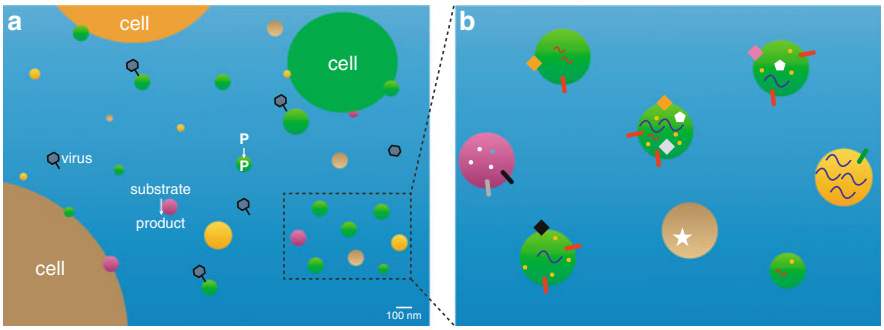

Fig. 4.1 Extracellular vesicles in the marine environment. (**a**) Vesicles (small circles) originating from different cells (larger circles, distinguished by colors) can freely diffuse and interact with cells, viruses, or other components of the environment. Vesicles can impact the local ecosystem through activities such as mediating extracellular enzymatic reactions, gathering nutrients like phosphorus (P), or delivering materials among cells. Cells and vesicles are shown at a greater relative abundance (and closer proximity) than would be expected at this scale for illustrative purposes. Vesicles are colored to match the cell that produced them; cells and vesicles associated with particle surfaces are not depicted. (**b**) Individual extracellular vesicles, whether produced by the same or different microbes, can vary in size and the composition of contents such as DNA, RNA, proteins and metabolites (depicted by different symbols on the surface or within the vesicles)

microbes showed that long (>5 kb) DNA fragments were found in fewer than 1% of vesicles, with a larger (but unknown) fraction of vesicles presumably containing shorter DNA fragments (Biller et al. 2017). Similarly, most DNA in *P. aeruginosa* MVs is associated with only the smallest vesicles (Bitto et al. 2017). Following from these data, it seems reasonable to speculate that the distribution of proteins, RNAs, and other components will almost certainly vary among individual vesicles as well (Kulp and Kuehn 2010). Thus, while vesicles can collectively contain a huge diversity of compounds, it is important to consider vesicles as a population of heterogeneous individual particles, each with potentially distinct functional capabilities (Fig. 4.1b). Further complicating the picture is that the vesicles produced by a single strain can also vary with changes in growth phase and environmental conditions (Orench-Rivera and Kuehn 2016; Zavan et al. 2019) or due to different mechanisms of vesicle formation (Turnbull et al. 2016).

4.4 Distribution of Vesicles in the Environment

Where are vesicles found, and how many are there? Diverse microbes isolated from both aquatic and terrestrial environments release MVs in culture, implying that vesicles are likely to be widespread throughout nature. Recent studies have started to shed some light on the abundance and distribution of vesicles in the field, highlighting the need to consider how the physical context of different habitats may influence MV functions.

4.4.1 Vesicles in Aquatic Systems

Extracellular vesicle abundances in the environment are best understood in the oceans. Bacteria are a widespread and essential part of the marine ecosystem, where they can be found at densities on the order of 10^5–10^6 cells mL^{-1} in surface waters; archaea and eukaryotes are important components of ocean microbiomes as well (Moran 2015). Membrane vesicle release has been observed in laboratory cultures of numerous marine bacteria, ranging from globally abundant cyanobacteria to heterotrophs isolated from both mid-latitude and polar regions (Frias et al. 2010; Biller et al. 2014, 2017; Li et al. 2016). The presence of vesicles in seawater has also been directly confirmed by electron microscopy in samples from both coastal and open-ocean environments (Biller et al. 2014). Though measurements of vesicle abundances have been traditionally based on measurements of lipid abundance or vesicle protein content, newer technologies for nanoparticle characterization are beginning to provide more quantitative estimates of vesicle numbers. Measurements based on nanoparticle tracking analysis revealed that in surface waters, EVs are found at concentrations comparable to that of bacterial cells, with ~10^6 vesicles mL^{-1} identified in seawater samples taken from the coast of Massachusetts and ~10^5 vesicles mL^{-1} in samples collected in the Sargasso Sea (Biller et al. 2014). In the Sargasso, vesicle abundance gradually decreased with depth, mirroring the overall change in bacterial abundance. Since vesicles were almost certainly lost during many of the sample collection and processing steps used to complete those measurements, it is important to note that those concentrations should be considered lower bounds—the actual vesicle concentration in these environments is likely greater. While direct measurements of vesicle abundance have only been made in samples from the mid-latitudes to date, vesicle release has also been noted in cultures of Antarctic microbes, suggesting that vesicles are a cosmopolitan feature of marine environments globally (Frias et al. 2010).

Vesicles are components of freshwater ecosystems as well. Electron microscopy evidence indicates that microbes naturally produce MVs within a variety of tropical freshwater habitats (Silva et al. 2014), and vesicles are found in cultures of diverse freshwater bacteria such as *Cylindrospermopsis raciborskii* (Zarantonello et al. 2018), *Shewanella* spp. (Gorby et al. 2008), and *Synechocystis* PCC6803 (Pardo et al. 2015). The abundance and distribution of vesicles in fresh or brackish water samples remains to be characterized. The presence of endotoxins in drinking water has been suggested as additional indirect evidence for vesicles in freshwater (Toyofuku et al. 2015), though whether this material is truly associated with vesicles is unclear. Vesicles, or vesicle-like liposomes, have also been implicated as a potential source of material clogging filtration membranes used in water treatment processes (Barry et al. 2014).

Vesicles are generally assumed to diffuse throughout a well-mixed aqueous system, allowing them to randomly encounter other components of the environment (Fig. 4.1). There are, however, potential conditions under which EVs may not be able to freely move away from the cells that produced them, resulting in microscale "patchiness" of vesicle distributions. *Shewanella oneidensis* vesicles, for example,

can be tethered to cells by thin nanowires (Gorby et al. 2008), which may provide some restraint on the ability of a vesicle to move within low-shear settings. More broadly, considerations of microscale physics show that bacteria live in a world of low Reynold's numbers and high viscosity, where flows around a cell can be laminar instead of generally turbulent (Lauga 2016). This may impact the ability of diffusion to separate vesicles from cell surfaces under some conditions, where vesicle movement will be subject to constraints of the local diffusion boundary layer surrounding a cell (Stocker and Seymour 2012). Over time, and in a relatively turbulent aqueous environment, this boundary layer may not provide much of an impediment to vesicle release away from a microbial cell; by contrast, in a low flow, poorly mixed environment, vesicles may have greater difficulty moving away from the cell surface. Such conditions may lead to vesicles remaining locally restricted near the cell, potentially influencing their ecological function(s).

4.4.2 Vesicles in Surface-Associated Communities

Many microbes do not live free-floating in a liquid environment, but are instead attached to surfaces. Such communities are frequently found in the form of biofilms: collections of bacteria bound to each other and fastened to a surface by secreted extracellular polymeric substances. Electron microscopy surveys have demonstrated that vesicles are an abundant feature of natural biofilms isolated from such diverse sources as domestic water drains, sewage and water treatment plants, pulp and paper manufacturers, freshwater fish aquariums, water storage tanks, and riverbed sediments (Schooling and Beveridge 2006). Large organic particles (μm to cm scale), such as "marine snow" and transparent exopolymer particles, are also plentiful in marine (and freshwater) environments (Simon et al. 2002). These aggregates are colonized by diverse microbial biofilm communities, and presumably should contain vesicles as well. Marine and freshwater sediments also harbor complex communities of bacteria (Nealson 1997), but the concentration and distribution of vesicles on sediments are not yet known. Most vesicles released within a biofilm or other aggregate will likely remain confined within the biofilm matrix and thus would not be included in measurements of planktonic vesicles, which typically remove particles of this larger size class. These considerations suggest that vesicles in aquatic environments may be even more abundant, and perhaps more spatially structured, than is reflected in current data. Abundant bacteria are also associated with particles in the atmosphere (Barberán et al. 2015), and work on indoor dust particles has revealed data consistent with the idea that particle-associated vesicles are found in the air (Kim et al. 2013).

Terrestrial bacteria, such as the model organism *Bacillus subtilis*, release MVs in liquid cultures grown in the lab (Brown et al. 2014). While these organisms presumably also produce vesicles in their natural environment, vesicle concentrations in soils are unknown. The physical properties of soil particles impose a distinct set of constraints on vesicle movements and interactions as compared to aqueous environments. Soil composition can vary across numerous dimensions, including the

composition of the soil particles (organics, sand, clay, etc.), the charge of these particles, and their hydration levels. Water films surrounding soil particles can be less than 10 nm thick (Or et al. 2007); since this is markedly thinner than a typical MV, vesicles would not be expected to freely diffuse in the same way that they could in a well-mixed, turbulent aquatic habitat (Shetty et al. 2011). Vesicle movement in soils may be further restricted by charge interactions between vesicles and soil particles.

Since dry or nearly desiccated particle surfaces are not necessarily conducive to vesicle diffusion, biofilms represent a likely ecological context for many vesicle activities in soils. Additionally, terrestrial microbes can utilize other mechanisms to overcome the physical constraints associated with soil particles and enable vesicle-based interactions. *B. subtilis*, for instance, can produce "nanotubes" that extend between cells and mediate intercellular exchanges (Dubey and Ben-Yehuda 2011), possibly via vesicles. Another related mechanism was identified in the soil microbe *Delftia*, which forms tubular surface structures termed "nanopods" that can extend more than 6 μm away from the cell surface (Shetty et al. 2011). These nanopods are composed of surface layer proteins and membrane vesicles, providing a means for vesicles to be exchanged directly over long distances. Vesicles have also been seen to form long "chains" reaching away from the surface of *Myxococcus xanthus* (Remis et al. 2014). The potential use of intercellular tubes to exchange vesicles may not be limited to soil microbes, as the human pathogen *Francisella tularensis* also produces tubular outer membrane vesicles from its surface when grown in liquid culture and on agar plates (McCaig et al. 2013).

4.4.3 What Modulates Vesicle Distributions in the Environment?

The abundance of vesicles at any given time and place represents the net balance between the rate of vesicle production by the community and the rate of vesicle loss. The relevant rates on both sides of this equation are currently poorly constrained. Vesicle production in a given environment will fluctuate as a function of many factors: first, MV release varies among bacterial strains as measured on a per-cell, per-generation basis (Biller et al. 2014), so community composition and growth rates of individual taxa will affect vesicle abundance. MV production can be further influenced by environmental conditions such as nutrient availability (Prados-Rosales et al. 2014a; Orench-Rivera and Kuehn 2016; Sampath et al. 2018; Gerritzen et al. 2019), temperature (Frias et al. 2010; MacDonald and Kuehn 2013), UV exposure (Gamalier et al. 2017, Zarantonello et al. 2018), and oxidative stress (MacDonald and Kuehn 2013). The rotation of sheathed flagella has also been linked to vesicle release (Aschtgen et al. 2016a), suggesting that conditions increasing flagellar activity may also lead to higher local MV concentrations. There is evidence that vesicle production is under at least some degree of genetic control as well, since mutations have been identified in multiple Gram-negative bacteria that either

increase or decrease the rate of vesicle formation (McBroom et al. 2006; Rath et al. 2013; Kulp et al. 2015; Nakayama-Imaohji et al. 2016; Resch et al. 2016). It is not yet clear how many of these genetic mechanisms specifically regulate vesicle production as opposed to indirectly influencing vesicle release through impacts on cellular envelope structure and/or other physiological factors, including oxidative stress levels or lipid production (Gerritzen et al. 2019). Regardless, these data indicate that the specific genetic composition of a microbial community will also affect overall vesicle production rates.

Even less is known about the rates and mechanisms of vesicle loss. Studies of cyanobacterial extracellular vesicles showed that average vesicle size and abundance did not significantly change over the span of 2 weeks when kept in sterile seawater (Biller et al. 2014). Though these data cannot rule out microscale changes occurring among individual vesicles, they indicate that vesicles are inherently stable structures—at least in a high salinity environment where lipids will be most thermodynamically stable in a spherical form. Dispersal rates, whether through random diffusion or active mixing, will further influence vesicle concentrations within a given region of an aqueous environment. Other processes surely contribute to vesicle loss, including breakdown by microbes or extracellular enzymes, consumption of vesicles, uptake/fusion of vesicles into the surfaces of other cells, abiotic degradation, or adsorption and subsequent sequestering of vesicles onto surfaces. The relative contributions of these different vesicle removal mechanisms are not yet known, but together such factors will affect not only the number of vesicles in a given place and time but also the average vesicle half-life and distance a vesicle might be able to travel.

4.5 Vesicles as Discrete, Structured Packets of "Dissolved" Nutrients

At a basic level, extracellular vesicles can be viewed as simply secreted packets of organic molecules. Given that vesicles can contain lipids, proteins, small molecules, and nucleic acids, these structures are therefore a potential source of biologically important nutrients including organic carbon, nitrogen, oxygen, phosphorous, sulfur, and trace metals. Vesicles thereby represent components of global nutrient pools and microbial food webs.

4.5.1 Vesicles Are an Investment of Cellular Resources

The bulk of our current understanding of MVs comes from laboratory studies of relatively large, fast-growing bacteria grown in nutrient-rich media, where the release of proteins and other material into vesicles may represent a negligible loss to the cell. By contrast, most bacteria in the environment grow slowly, with doubling times on the order of days to weeks or even longer (Kirchman 2016). The oligotrophic oceans, for example, are extremely nutrient-poor and dilute environments,

where essential nutrients are found at picomolar concentrations and bacterial cells are at least 100–200 body lengths away from each other on average (Biller et al. 2015; Moran 2015). These conditions impose a distinct set of selective pressures on marine microbes as compared to those experienced within other environments such as the human gut, where organisms generally live at much higher local densities and experience "feast and famine" type regimes. Oligotrophic microbes have a number of adaptations that appear to help them survive in a world of limited nutrients. One evolutionary approach is to reduce cell size: *Prochlorococcus* and *Pelagibacter*—the most abundant autotrophic and heterotrophic bacteria, respectively, in the oceans—are both tiny cells, with diameters less than 1 μm (Chisholm et al. 1988; Rappé et al. 2002). This means that the production of a single 100 nm diameter vesicle by these organisms represents a proportionally greater amount of cellular resources than it would in a larger bacterium such as *E. coli*. *Prochlorococcus* has evolved numerous ways to reduce its nutrient requirements, such as using sulfolipids instead of phospholipids in its membrane to conserve phosphorous, which is a limiting nutrient in some ocean regions (Van Mooy et al. 2009). Given all of this, it is therefore perhaps surprising that *Prochlorococcus* cells would release potentially critical limiting nutrients within a vesicle. For bacteria producing on the order of 1–10 MVs per cell per generation (Biller et al. 2014), this might represent a nontrivial amount of material to export—particularly when considering the nutrients that are lost are in the form of energetically and chemically "expensive" materials like lipids, proteins, and nucleic acids.

Why, then, might cells—especially those growing in nutrient-poor environments—release extracellular vesicles in the first place? Do EVs simply perform sufficient beneficial functions to justify the investment of resources, meaning vesicle production is maintained through natural selection? Or are other factors at play? One hypothesis for the apparent ubiquity of EV production is that vesicle release reflects a vestige of the earliest forms of life on Earth, wherein lipid vesicles could have provided an environment for metabolic reactions to occur and facilitated exchange of RNA and other compounds among ancient cells (Gill and Forterre 2016). Vesicles could also be, at least to some degree, an unavoidable consequence of having a lipid bilayer membrane, since bits of membrane protruding from the cell will be thermodynamically favored to self-assemble into a spherical vesicle under various conditions (Huang et al. 2017). Structuring the cell envelope in such a way as to prevent membrane "blebs" from forming, such as by increasing membrane crosslinking, may have too many other deleterious consequences for the cell, making vesicle production essentially an accepted loss term. Consistent with this idea is the observation that while some genetic mutations either increase or decrease vesicle production in *E. coli*, none have yet been found that can completely abolish vesicle release (McBroom et al. 2006; Kulp et al. 2015). Evidence from *Salmonella enterica* also indicates that vesicle release may be tied, at least in part, to the processes required to remodel bacterial outer membranes in response to environmental changes (Elhenawy et al. 2016). Regardless of the original evolutionary and mechanistic origins of vesicle release, the secretion of these particles and their associated contents represents a means through which bacteria release potentially valuable nutrients and energy.

4.5.2 How Much "Dissolved" Material in the Environment Is Enclosed Within Vesicles?

In aquatic sciences, measurements of "dissolved" versus "particulate" nutrients have historically been based on operational criteria wherein materials are considered "dissolved" if they pass through a 0.2 μm diameter filter, and "particulate" if they are >0.2 μm (Azam and Malfatti 2007). Within this "dissolved" fraction are found not only truly dissolved monomeric molecules, but also an entire size continuum of particles—ranging from nm-scale inorganic colloids to larger, biologically-derived organic structures like viruses and vesicles (Azam and Malfatti 2007). Of perhaps particular relevance in the open ocean is the fact that vesicles represent a discrete, colloidal structure that is relatively concentrated with nutrients within an otherwise dilute environment. Though many common methods for determining the concentration of important nutrients like nitrite, nitrate, and organic carbon in seawater likely already include vesicle materials, it is not yet known what fraction of these nutrients are truly "dissolved" versus associated with vesicles.

The realization that seawater contains abundant extracellular vesicles may partly explain a number of previous oceanographic observations. For instance, early proteomic studies of seawater revealed that some of the most abundant "dissolved" peptides were membrane proteins (Tanoue et al. 1995), and it is quite likely that this includes membrane proteins associated with extracellular vesicles. Similarly, lipids were found to comprise a notable component of total dissolved organic carbon (DOC) in seawater (Aluwihare et al. 1997), and lipid membrane-bound extracellular vesicles likely represent a fraction of this. Extracellular DNA has been repeatedly observed in the oceans (DeFlaun et al. 1987; Brum 2005), which is somewhat surprising given that microbes can rapidly utilize free DNA as a nutrient source (Jørgensen and Jacobsen 1996; Lennon 2007). Given what we now know, at least some of this dissolved DNA may not be truly "free" but instead afforded a degree of protection inside vesicles. As a final example, ATP has been identified in bacterial MVs (Pérez-Cruz et al. 2015); ATP is also found within the <0.2 μm size fraction of seawater, where it is utilized by microbes as a source of either phosphorous or purines for biosynthesis (Azam and Hodson 1977). Chemical analyses of this "dissolved" ATP have shown that it likely comes from grazing and/or cellular excretion (Nawrocki and Karl 1989; Björkman and Karl 2001), consistent with the hypothesis that at least some fraction of seawater ATP is associated with MVs.

4.5.3 Vesicles as a Component of Global Dissolved Organic Carbon Pools

From a food web perspective, vesicles represent a "snack pack"—a discrete, locally concentrated bundle of bioavailable nutrients that could be utilized by single-celled

or multicellular organisms. It is therefore worth considering the place of vesicles within global food webs, focusing here again on the ocean ecosystem. Marine primary producers (phytoplankton) use sunlight to fix inorganic carbon into organic molecules, which are later released and then utilized by the heterotrophic community for either energy or biosynthesis. These organic molecules are typically thought to be released by phytoplankton through direct excretion of the compounds, leakage across membranes, or as a consequence of cell lysis (Azam and Malfatti 2007). Recent findings indicate that vesicle secretion should be considered in this context as well. Experiments with the common marine heterotroph *Alteromonas* demonstrated that they can utilize purified extracellular vesicles from *Prochlorococcus* as their sole organic carbon source (Biller et al. 2014). This result highlights the potential for vesicles to contribute to marine food web interactions as part of "dissolved" organic carbon pools. The identity of the vesicle-associated biomolecule(s) consumed by *Alteromonas* or other microbes is not yet known, but could potentially include the membrane lipids as well as vesicle-associated proteins and/or small molecules. Extracellular DNA can serve as a nutrient source in planktonic environments and marine sediments (Jørgensen and Jacobsen 1996; Dell'Anno and Danovaro 2005), leading to the hypothesis that some cells might utilize vesicle-associated DNA for food.

Dissolved organic carbon pools in the global oceans are massive, with the deep ocean containing roughly as much carbon as there is CO_2 in the atmosphere (Hansell and Carlson 2015). DOC can generally be divided into two fractions: "labile" carbon, which is quickly utilized by microbes within the ocean food web, and "refractory" carbon which is less accessible. While any individual extracellular vesicle likely contains only 50–100 $\times$ 10^{-18} g of carbon (Biller et al. 2014), their apparent ubiquity in the marine environment and ability to be consumed by other bacteria indicates that they may have a large combined impact on labile DOC pools. A back-of-the-envelope calculation shows that if an average marine bacterium releases 1 vesicle per day, with each vesicle containing 0.1–1% of a typical marine bacterial cell's mass (Fukuda et al. 1998; Biller et al. 2014; Bar-On et al. 2018), then the ~1.3 $\times$ 10^{15} g of bacterial carbon biomass in the pelagic oceans (Bar-On et al. 2018) would produce on the order of ~4.7 $\times$ 10^{14}–4.7 $\times$ 10^{15} g of "dissolved" vesicle C biomass each year. Assuming that vesicles are composed of primarily labile material, this suggests that bacterial vesicle biomass represents a potentially notable fraction of the estimated 15–25 $\times$ 10^{15} g of total marine labile DOC produced annually (Hansell 2013). There is, of course, considerable uncertainty in these estimates, but the exercise emphasizes the potential for vesicle-associated carbon to be an important part of organic carbon flux in the oceans. Future work will hopefully permit better estimates of the contribution of vesicles to global DOC pools and fluxes, and improve our understanding of the variation in both vesicle production and consumption among different bacterial, archaeal, and eukaryotic taxa. In addition to the role of vesicles in carbon flux, the protein and DNA within vesicles implies that vesicles may also comprise part of the dissolved nitrogen, sulfur, and phosphorous pools (Lynch and Alegado 2017).

4.5.4 *Vesicles as Reservoirs and Scavengers of Inorganic Nutrients*

Nutrient-binding proteins and transporters have been identified within vesicles, raising the possibility that some vesicles might contain materials they have "scavenged" extracellularly, in addition to any nutrients carried out of the producing cell (Schwechheimer and Kuehn 2015). For example, MVs released by the marine cyanobacterium *Prochlorococcus* contain putative phosphate-binding proteins (Biller et al. 2014), suggesting that these vesicles might be able to bind extracellular phosphate as they diffuse through the ocean and shuttle those concentrated materials to another cell (Fig. 4.1a). Binding and transport of trace metals represent another potential function for vesicles; to date these mechanisms have been primarily studied in pathogens and host-associated microbes, but the concepts are likely broadly relevant. For instance, the proteome of *Neisseria meningitidis* vesicles contains both iron- and zinc-binding proteins (Lappann et al. 2013). When *Mycobacterium tuberculosis* is grown under low-iron conditions, it releases extracellular vesicles containing mycobactin, a compound that can bind iron and help these organisms obtain this nutrient (Prados-Rosales et al. 2014b). In *P. aeruginosa*, the *Pseudomonas* quinolone signal (PQS) is both a quorum-sensing molecule trafficked within MVs (Mashburn-Warren et al. 2008; Schertzer and Whiteley 2012) as well as an iron-binding compound (Bredenbruch et al. 2006; Schertzer et al. 2009), providing yet another avenue for vesicles to contribute to metal uptake. From an evolutionary perspective, the ultimate benefit of releasing vesicles into the environment where they can serve as a nutrient "snack pack" for others may depend on a complicated set of tradeoffs that considers the amount of cellular resources invested into each vesicle, the rates of vesicle–cell encounters, the identity of the limiting resource(s) in that environment, any mutualistic interactions between organisms, and the degree of specificity with which that vesicle will interact with closely or distantly related cells.

4.6 Ecological Roles of Vesicles

The variety of biological compounds that can be transported by extracellular vesicles underlies the remarkable diversity of their potential functional abilities. Studies have revealed contributions of vesicles to an ever-increasing number of processes, suggesting that vesicles may play many varied ecological roles within natural communities. While the exact impacts of any individual vesicle interaction may be small and heavily context dependent, collectively such vesicle-mediated processes could have a marked impact on the structure, function, and evolution of microbial communities globally.

4.6.1 Manipulators of the Local Environment

Studies of MVs released by cultured bacteria have repeatedly shown that vesicles can be associated with a variety of proteins, including functional enzymes (Kadurugamuwa and Beveridge 1995). Extracellular vesicles thus provide a mechanism for cells to affect their local environment through enzymatic activity, facilitating microbial niche construction. Exporting proteins in or on a vesicle as opposed to directly releasing an exoenzyme into the extracellular milieu could provide benefits including affording the enzyme protection from environmental damage and allowing for simultaneous co-secretion of multiple proteins involved in a complex or pathway (Borch and Kirchman 1999; Lee et al. 2013). Vesicles could also provide a local structure wherein substrates are kept in close proximity to enzymes that act on them, avoiding issues with diffusion in aqueous extracellular environments (Bonnington and Kuehn 2014).

Vesicle proteomes have revealed not only the diversity of vesicle-associated proteins, but also the fascinating observation that these proteins can originate from a variety of cellular compartments. For instance, MVs from Gram-negative bacteria contain not just outer membrane and periplasmic proteins—as would be expected based on the common models of vesicle formation—but also cytoplasmic proteins (Pérez-Cruz et al. 2015; Yun et al. 2017; Zakharzhevskaya et al. 2017). These repeated observations, across distantly related bacteria, suggest that the export of cytoplasmic proteins is a common biological feature of vesicles and not simply a technical artifact. Extracellular vesicles, therefore, provide a mechanism through which enzyme-mediated activities can occur away from the cell, perhaps allowing these proteins access to substrates that they might not otherwise encounter in their typical subcellular location (Ebner and Götz 2019).

Vesicle-associated enzymes are responsible for many of the functional roles currently described for these structures (Schwechheimer and Kuehn 2015). Pioneering studies in *P. aeruginosa* noted that enzymes contributing to pathogenesis were found in vesicles (Kadurugamuwa and Beveridge 1995). Vesicles were also implicated as agents of microbial "warfare," based on experimental demonstrations that vesicle-associated hydrolases and endopeptidases could lyse other microbes (Kadurugamuwa and Beveridge 1996; Li et al. 1998; Vasilyeva et al. 2008). Vesicles are also capable of catalyzing reactions that enable cells to broadly manipulate their local chemical environment. For example, *S. oneidensis* vesicles contain active reductases that facilitate electron transfer in these cells, allowing them to reduce terminal electron accepters located away from the cell surface (Gorby et al. 2008). *Staphylococcus aureus* vesicles contain functional β-lactamases that can degrade the antibiotic ampicillin (Lee et al. 2013). Other findings indicate that MV-associated enzymes can contribute to nutrient acquisition. Vesicles released by human gut *Bacteroides* contain active hydrolases that can break down extracellular substrates, releasing soluble nutrients that can then be utilized by the cells (Rakoff-Nahoum et al. 2014; Li et al. 2016). Relatedly, the rumen-associated microbe *Fibrobacter succinogenes* produces vesicles containing carbohydrate-degrading enzymes that

can depolymerize various plant polysaccharides, likely facilitating further degradation and utilization of these nutrients (Arntzen et al. 2017). MVs from coral-associated *Vibrio* strains also contain active proteases, glucosidases, lipases, and chitinases, which could contribute to pathogenesis or nutrient acquisition as well (Li et al. 2016).

These enzymatic data raise additional questions concerning how much of total bacterial exoenzyme activity is associated with individually secreted, truly "dissolved" proteins as compared to enzymes associated with vesicles. For instance, alkaline phosphatase is an exoenzyme long known to play important roles in the marine phosphorous cycle (Hoppe 2003). The majority of marine alkaline phosphatase activity is typically found in the "dissolved" seawater fraction, but at least some of this activity may come from vesicle-associated enzymes, as suggested by the finding that MVs from a marine *Vibrio* contain active alkaline phosphatase activity (Li et al. 2016). Bacteria from other environments, such as *P. aeruginosa* and the soil bacterium *Myxococcus xanthus*, also secrete active alkaline phosphatase in vesicles (Kadurugamuwa and Beveridge 1995; Evans et al. 2012). While these data lead to a number of compelling hypotheses concerning potential vesicle functions, to date they are only proof of concept. We still lack quantitative information about the degree to which vesicle-associated enzymes contribute to processes within natural microbial ecosystems, either extracellularly or within cells that interact with vesicles.

4.6.2 Vectors of Intercellular Exchange and Signaling

Extracellular vesicles can carry a diverse suite of compounds released by one cell across spatial and temporal distances to another cell. Such intercellular exchanges may lead to a variety of potential outcomes, the exact nature of which will depend on the organisms involved as well as the contents of those vesicles. Some examples of vesicle-mediated intercellular exchange include previously discussed roles in nutrient exchange and protein delivery. In addition, vesicles can serve as vehicles for transferring signaling molecules. For instance, MVs from *P. aeruginosa* have been shown to traffic the hydrophobic bacterial quorum-sensing signal PQS; interestingly, PQS can also directly contribute to vesicle formation and signal packaging in this bacterium (Mashburn and Whiteley 2005; Schertzer and Whiteley 2012). By enclosing a relatively high local concentration of such molecules, vesicles likely prevent secreted signaling molecules from diluting to the point where the signal concentration would be insufficient to elicit a response. This can, however, change the nature of these signaling interactions. Individual vesicles from *Paracoccus denitrificans* carry sufficient quorum signal to elicit a response in recipient cells, suggesting that vesicle-mediated signaling may lead to a binary response that differs from the more canonical density-dependent quorum sensing (Toyofuku et al. 2017). MV-mediated signaling contributes to cross-domain interactions as well. In one example, the marine Bacteroidetes *Algoriphagus machipongonensis* was found to induce multicellular colony development of a choanoflagellate, *Salpingoeca rosetta*,

via sulfonolipids trafficked via MVs (Alegado et al. 2012; Lynch and Alegado 2017). MVs also participate in the mutualistic symbiosis between *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*. Here, *Vibrio* MVs convey developmental signals to the squid, helping to drive host developmental changes required for successful colonization (Aschtgen et al. 2016b).

Vesicle-based intercellular delivery could occur through various mechanisms. Some models of vesicle delivery implicate the fusion of vesicles with the outer membrane of a cell, releasing all of the vesicle's contents into the recipient (Kadurugamuwa and Beveridge 1996, 1999). Alternatively, vesicles either attached to or held in stable proximity to the surface of a cell through charge interactions (Kadurugamuwa and Beveridge 1996) could deliver material via "flipping" of molecules from the vesicle membrane into the cell (Remis et al. 2014). Cells could perhaps also use enzymes to degrade a vesicle extracellularly and subsequently acquire specifically desired components through standard import pathways. The uptake of vesicle material into eukaryotic cells can occur via any one of multiple endocytic processes, which have been reviewed elsewhere (Mulcahy et al. 2014).

The factors that determine whether a given vesicle will associate with and deliver material to another cell remain unclear. Vesicles can clearly mediate transfer between different microbes (Yaron et al. 2000), but experimental data is emerging indicating that species/strain boundaries can exist between vesicles and cells in some cases (MacDonald and Beveridge 2002; Toyofuku et al. 2017; Tashiro et al. 2017). The factors mediating such strain specificity could include surface protein interactions, zeta potential (Tashiro et al. 2017), hydrophobicity (MacDonald and Beveridge 2002), envelope and boundary layer structure of the cells, or other properties yet to be identified.

4.6.3 Reservoirs of Genetic Information and Vectors of Horizontal Gene Transfer

Transformation, transduction, and conjugation have historically been viewed as the primary mechanisms of bacterial horizontal gene transfer (HGT), but extracellular vesicles can mediate HGT as well. Bacterial and archaeal vesicles can enclose DNA ranging in size from entire plasmids to both short (<100 bp) and long (anywhere from hundreds of bp to >20 kb) fragments of chromosomal DNA (Hagemann et al. 2013; Biller et al. 2014, 2017; Gaudin et al. 2014; Erdmann et al. 2017). Multiple studies have shown that bacterial MVs can successfully deliver this DNA cargo into other bacterial or eukaryotic cells, supporting the likely contribution of vesicle-mediated delivery to HGT in natural systems (Dorward et al. 1989; Kolling and Matthews 1999; Yaron et al. 2000; Chatterjee et al. 2017; Bitto et al. 2017; Grüll et al. 2018).

The extensive diversity of DNA associated with vesicles in the marine environment (Biller et al. 2014) highlights not only the vast potential for vesicles to mediate

cross-species HGT, but also points to vesicles as an environmental genetic "reservoir" from which any cell could sample. Vesicles provide a degree of protection to DNA in the environment that a free-floating DNA molecule, which could be consumed or degraded, would not receive. In one study, vesicle DNA could still be transformed into cells following nearly 2 years of storage at 4 °C—even with nuclease enzymes present in the same solution (Blesa and Berenguer 2015). Thus, the "dissolved information" contained within vesicles represents an expansive and possibly long-lived pool, with the vesicle-associated DNA potentially outlasting the strain that released it. The transfer of RNA, including small RNAs, among cells also suggests potential roles for vesicle-mediated exchange impacting genetic regulation within microbial communities (Dauros-Singorenko et al. 2018; Tsatsaronis et al. 2018; Cai et al. 2018).

Some DNA may be able to facilitate its own transfer via vesicles in a quasi-viral manner. The plasmid pR1SE, identified in the Antarctic haloarchaeon *Halorubrum lacusprofundi* R1S1, encodes proteins that facilitate the formation of plasmid-containing extracellular vesicles. These vesicles can then "infect" cells lacking pR1SE, causing those hosts to produce plasmid-containing vesicles themselves (Erdmann et al. 2017). This finding mirrors previous observations of "viral-like particles" (likely membrane vesicles) in *E. coli* that can move DNA between cells and induce the recipients to produce more of these DNA-bearing structures; whether a similar mechanism mediates this phenomenon remains unclear (Chiura et al. 2011; Velimirov and Ranftler 2018).

The relative contribution of vesicle-mediated transfer, conjugation, transformation, and transduction to overall rates of HGT in different environments, or among different strains, is not known, though each mechanism has unique tradeoffs that likely influences the rate of successful HGT under different conditions (Nazarian et al. 2018). In laboratory cultures, rates of vesicle-mediated plasmid transfer between different Gram-negative strains have been shown to vary as a function of the specific donor strain, recipient strain, plasmid characteristics, and the genes being transferred; these rates were not correlated with the genetic relatedness of the donor and recipient (Tran and Boedicker 2017, 2019). Vesicle-mediated HGT is also subject to the previously discussed factors possibly influencing vesicle–cell interaction rates, combined with a consideration of the heterogeneity of DNA contained within any one individual vesicle (Biller et al. 2017). While the extent of vesicle HGT still needs to be elucidated, it is exciting to consider that vesicles could provide cells with a means to acquire DNA from a broader diversity of sources than transduction or conjugation. Many viruses, particularly the tailed viruses, exhibit quite specific and narrow host ranges (Kauffman et al. 2018), whereas the breadth of vesicle transfers between disparate cell types demonstrated to date hint that there may be fewer potential barriers for EVs. Ultimately, the amount of HGT mediated by vesicles as compared to viruses will depend not only on the relative abundance of these structures in a given environment, but also the fraction of each particle that contains host DNA, and the differences between their encounter dynamics, host specificity, and delivery efficiency (Nazarian et al. 2018).

4.6.4 Impacts on Cell–Surface Interactions

Production of extracellular vesicles affects the physical interaction of cells with surfaces, thus influencing cellular distributions and motility. In some instances, membrane vesicles promote attachment of bacteria by contributing to the formation of biofilms (Schooling and Beveridge 2006; Yonezawa et al. 2009; Grande et al. 2015), possibly through a structural role for MVs and their associated DNA. Vesicles can influence the physical attachment of microbes to other cells, as has been noted in studies of the oral cavity where vesicles can promote bacterial attachment to both host epithelial cells (Meyer and Fives-Taylor 1994; Inagaki et al. 2006) as well as to other microbes (Kamaguchi et al. 2003). In different contexts, vesicles inhibit cell–surface interactions. For example, vesicle production by *Xylella fastidiosa* facilitates the ability of this plant pathogen to move throughout the plant by preventing bacteria from sticking to surfaces (Ionescu et al. 2014).

4.6.5 Defensive Roles and Vesicle–Virus Interactions

Vesicles can play a variety of defensive and protective roles for cells. Some bacterial MVs bind toxic compounds such as antimicrobial peptides or hemin, thereby reducing the local concentration of that molecule and promoting cell survival (Manning and Kuehn 2011; Roden et al. 2012). MVs have also been proposed to provide a means for cells to remove damaged molecules (Schwechheimer and Kuehn 2015). Vesicles are further able to contribute to bacterial defenses against viral infections. Viruses and vesicles have a complex and fascinating set of interrelationships, and EVs are known to impact viral infection dynamics in multiple systems. Phage recognizes potential target cells through specific interactions with molecules on cell surfaces; since bacterial MVs contain material from the outermost membrane, any vesicle with the appropriate phage receptor molecule could be bound by that virus (Manning and Kuehn 2011). In this way, vesicles can serve as a "decoy" of sorts for the cell, which would lead to nonproductive infections and a reduction of the infectious viral population. Phage can bind vesicles released by marine cyanobacteria (Biller et al. 2014), and the presence of vesicles has been shown to reduce phage infection of both *E. coli* and *Vibrio cholerae* (Manning and Kuehn 2011; Reyes-Robles et al. 2018).

Vesicles do not, however, only act to inhibit viral infection. In the marine alga *Emiliania huxleyi*, viruses instead appear to use EVs to promote their own infection cycle (Schatz et al. 2017). Infected *E. huxleyi* cells release many EVs containing both small RNAs as well as a putative small signaling molecule; when these vesicles are taken up by uninfected *E. huxleyi* cells, the vesicle contents induce some currently unknown changes in the recipient cells that speed up subsequent viral infection cycles. In this system, the presence of EVs also led to a marked increase in viral half-life through an unknown mechanism (Schatz et al. 2017).

The fact that vesicles can transport surface molecules between cells provides a means for MVs to expand the host range of a phage. In *B. subtilis*, pre-treatment of a strain resistant to a particular phage with MVs released by a phage-sensitive strain led to the infection of the previously resistant strain, mediated by vesicle delivery of the phage receptor onto resistant bacterial cells (Tzipilevich et al. 2017). Changes in the phage host range could also occur via vesicles which contain viral genomes (either from cellular sources or perhaps an extracellular phage infection) and then deliver that DNA into cells (Yaron et al. 2000; Gaudin et al. 2014). In this way, vesicles containing viral DNA might facilitate viral "infection" in a manner that is not subject to the same barriers experienced by the virus itself. Since viruses and vesicles co-occur in marine (Biller et al. 2017) and other environments, future work will be required to untangle the many ways in which EVs impact phage dynamics—and vice versa.

4.7 The Future of Vesicle Research: Challenges and Opportunities

Despite the many advances we have made in understanding extracellular vesicles from bacteria, archaea, and eukaryotes, the extracellular vesicle field is, in many ways, still in its infancy. While there are countless questions to address, much of EV research is currently hindered by the simple fact that these structures are extremely difficult to work with. Nanoparticle analysis and imaging technologies are rapidly improving, but isolating and quantifying vesicles, particularly from the environment, remains a particular challenge. EVs from many types of natural samples can be found at concentrations close to or below some instrument detection limits, necessitating extensive sample concentration and processing; in addition, different isolation protocols can greatly influence study results (Singorenko et al. 2017). Separating extracellular vesicles from other types of small particles like viruses and inorganic colloids is still an inexact science, dependent on differences in charge, density, and other properties that do not always sufficiently discriminate among particle types. Whereas studies of eukaryotic extracellular vesicles (exosomes) frequently utilize antibodies to isolate or identify specific exosome populations of interest, there does not currently appear to be anything close to a universal epitope shared among all bacterial MVs produced by diverse communities. These technical considerations are further complicated by one of the fascinating properties of vesicles, namely their heterogeneity. This diversity is likely an important contributor to the functional capabilities of vesicles, and raises questions concerning when and where it is appropriate to study EVs at the level of individual structures as compared to populations. Regardless, the field has made rapid progress over the last few years, and continued technical advances will undoubtedly help us to overcome some of these challenges.

It is now clear that vesicle production is widespread in the natural environment, and that they likely mediate a diverse network of microbial interactions. This is an exciting time in which we are beginning to unveil an entirely new dimension of complexity within natural ecosystems. Despite the technical and conceptual challenges that remain, I believe that the field is well poised to take on these challenges and develop a basic understanding of vesicle ecology—to study the processes that determine the abundance and distribution of vesicles, determine how vesicles interact with the biotic and abiotic components of the environment, and quantify the influence of vesicle-mediated processes within natural systems. To this end, we need to advance our knowledge of the basic "natural history" of vesicles in disparate habitats: How many are there? When are they produced? By which cells? How long do they last, and where do they go? On top of this, many questions remain to be answered concerning vesicle functions. For example, what is the relative rate of vesicle-mediated HGT in the environment as compared to other mechanisms such as phage transduction? When and where do vesicle-associated enzymes function, and how can we quantify their impact? How much of a role do vesicles play in organic carbon cycling? In other nutrient cycles?

Given the abundance of vesicles in the environment and the diversity of their cargo, it seems that exchange among bacteria, archaea, and eukaryotes could be much more frequent than is commonly appreciated; this, in turn, raises important questions as to how cells handle potentially frequent encounters with vesicles from either related or dissimilar organisms in the wild. What are the encounter dynamics between vesicles and cells in different environments, and how does this compare to cell–cell encounter rates? What factors influence the ability of a vesicle to interact with a cell? To what degree does an average bacterium contain some number of biomolecules produced by a different organism and delivered by vesicles? What are the consequences of this? The ability of cells to discriminate among vesicles, or not, will also influence the structure of microbial interaction networks and the degree to which extracellular vesicles should be considered a true 'public good' (Hasegawa et al. 2015). While many functions of extracellular vesicles have been described to date, it seems likely that we have only begun to uncover the ways in which EVs affect the global ecosystem.

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