# Bacterial extracellular vesicles exhibit distinct functional potential across the biogeographic provinces of the South Pacific Ocean

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**Keywords: bacterioplankton, membrane vesicles, horizontal gene transfer, siderophores, extracellular enzymes**

## Abstract

Bacterial extracellular vesicles (BEVs) are nanoscale membranous structures released by diverse types of bacteria. Laboratory model systems indicate that these nanoparticles may play several roles in the ecophysiology of marine bacteria. However, their actual functionality in the environment remains unclear. Here we describe the proteomic composition of marine BEVs over more than 5,000 nautical miles of surface waters in the South Pacific, linking BEV cargoes to the bacterial communities producing them. BEVs were consistently present across a range of biogeochemical conditions, with an overall abundance comparable to that of bacterial cells. However, the protein content of the BEVs varied significantly between different ocean regions. The BEVs were enriched in carbohydrate transporters under phytoplankton bloom conditions, and contained iron and phosphate uptake-related proteins in nutrient-limited waters. This suggests that BEVs could enable cells to perform key extracellular functions in the marine environment. Our observations further highlight the prevalence of BEVs and the biogeographic patterns of their functional potential across oceanic scales.

# Introduction

Bacterial extracellular vesicles (BEVs) are nanoscale, membrane-bound structures typically ranging in diameter from 20 to 250 nm, which are released by bacterial cells. They can result from 'blebbing' mechanisms in intact cells or be formed as a by-product of cell lysis. This can influence their contents and, consequently, their functional potential (Kulp & Kuehn, 2010). Since their discovery in human-associated *Escherichia coli* in the 1960s (Knox et al., 1966), most studies of BEVs have examined their roles within laboratory model organisms, particularly host-associated bacteria in animal and plant systems. BEVs can serve as a mechanism for removing harmful compounds from the cell, but can also transport various biological cargoes (e.g., nucleic acids and proteins) over long distances in a protected and locally concentrated manner (McMillan & Kuehn, 2021). One of the best-documented functions of BEVs in intercellular interactions is the transfer of DNA molecules (Bitto et al., 2017; Soler & Forterre, 2020), which can facilitate the exchange of antibiotic resistance genes between different bacterial lineages (Yaron et al., 2000), as well as the transfer of entire plasmids (Johnston et al., 2023; Renelli et al., 2004), recently termed 'vesiduction' (Soler & Forterre, 2020). BEVs can also play roles in bacterial quorum sensing (Mashburn & Whiteley, 2005; Toyofuku et al., 2017), biofilm formation (Yonezawa et al., 2009), and contribute to microbial "warfare" by delivering compounds that kill competing bacteria (Kadurugamuwa & Beveridge, 1996; Z. Li et al., 1998). As BEVs can contain a variety of components released by bacterial cells, including enzymes, receptors and transporters derived from cell membranes, they may interact with organic and inorganic chemicals in the environment (Orench-Rivera & Kuehn, 2016; Zlatkov et al., 2020). Such extracellular activities, including the enzymatic digestion or binding of various environmental compounds, are particularly important for bacteria living in aqueous environments (Toyofuku et al., 2023, 2015).

The ocean, where bacteria are among the most abundant organisms (Bar-On & Milo, 2019), is a highly dynamic environment both physically and biochemically. The surface ocean is characterised by a depletion of bioavailable nutrients required for life in some vast areas (e.g., oligotrophic oceanic gyres) (Browning et al., 2017) and a high availability in others (e.g., coastal upwelling regions) (Capone & Hutchins, 2013; Moore et al., 2013). To survive in such ecosystems, marine bacteria have developed a wide range of ecophysiological capabilities (Stocker, 2012; Worden et al., 2015; Zoccarato et al., 2022), and implement highly diverse nutrient and energy utilisation strategies (Kirchman, 1994; Reintjes et al., 2017; Tortell et al., 1999). In the interaction between marine bacteria and their oligotrophic environment, high cellular surface area to volume ratios facilitate efficient nutrient uptake (Gasol et al., 1995; Koch, 1996). In this sense, the release of marine BEVs by bacterial cells can be conceptualised as an expansion of the cell surface which disperses through the aqueous medium (Kulp & Kuehn, 2010), randomly encountering various components of the marine environment. Laboratory observations of several isolates of globally abundant marine bacteria suggest that BEVs may mediate specific extracellular functions in the ecophysiology of these bacteria, such as binding nutrients (e.g., iron or phosphate) and delivering them in concentrated form to bacterial cells (Biller et al., 2023; Biller, Lundeen, et al., 2022; Biller et al., 2014; Dürwald et al., 2021; Fadeev et al., 2023; Fischer et al., 2019). However, despite the potential importance of BEVs for marine bacteria, and ubiquity in the marine environment (Linney et al., 2022; Lücking et al., 2023), our understanding of their biogeographic diversity across large environmental gradients is unknown.

To address this knowledge gap, we conducted a spatially extensive sampling to examine the abundance and protein cargo diversity of BEVs across the South Pacific Ocean. We determined how the molecular cargo of marine BEV populations varies in context with their local microbial and biogeochemical environment, finding that BEVs may play distinct functions for different microbial populations. Our observations across large oceanic regions reinforce the emerging picture that important extracellular bacterial functions may be mediated by BEVs and can inform future efforts to test specific hypotheses about their ecological roles.

# Results and Discussion

**Distinct biogeochemical conditions across the South Pacific Ocean**

To investigate the potential ecophysiological functions of BEVs in marine bacterial communities we sampled across diverse environmental conditions, varying in nutrient limitations and phytoplankton growth conditions. We collected surface water (5-6 m depth) at 20 stations along a basin-scale transect that spanned the South Pacific Ocean (Fig. 1; Table S1). A detailed physicochemical characterisation of this transect showed that field sampling was carried out across four oceanic provinces (Liu et al., 2024; Yuan et al., 2025). Briefly, the easternmost region was the Chilean coastal upwelling zone (UP), characterized by elevated nitrate and phosphate concentrations, but depleted in dissolved iron. In this area there were elevated chlorophyll-a concentrations (ca. 3 mg $m^{-3}$; Fig. S1), with the phytoplankton community mostly composed of diatoms (Fig. 1). The transition zone between the upwelling and the subtropical gyre (TRAN) was characterized by elevated concentrations of phosphate and dissolved iron, but was depleted in nitrate (Fig. S1). This area also had relatively elevated chlorophyll-a concentrations (0.04-0.26 mg $m^{-3}$), with mixed phytoplankton communities. The centre of the subtropical gyre (GYRE) was characterized by very low nitrate concentrations and a declining spatial gradient from east to west of phosphate concentrations, with strongly depleted chlorophyll-a concentrations (< 0.03 mg $m^{-3}$; Fig. S1). The westernmost region (WEST) was defined as the area of the subtropical gyre with the lowest phosphate concentrations and higher (compared to GYRE) chlorophyll-a concentrations (0.03-0.14 mg $m^{-3}$; Fig. S1), with the phytoplankton comprising predominantly cyanobacteria.

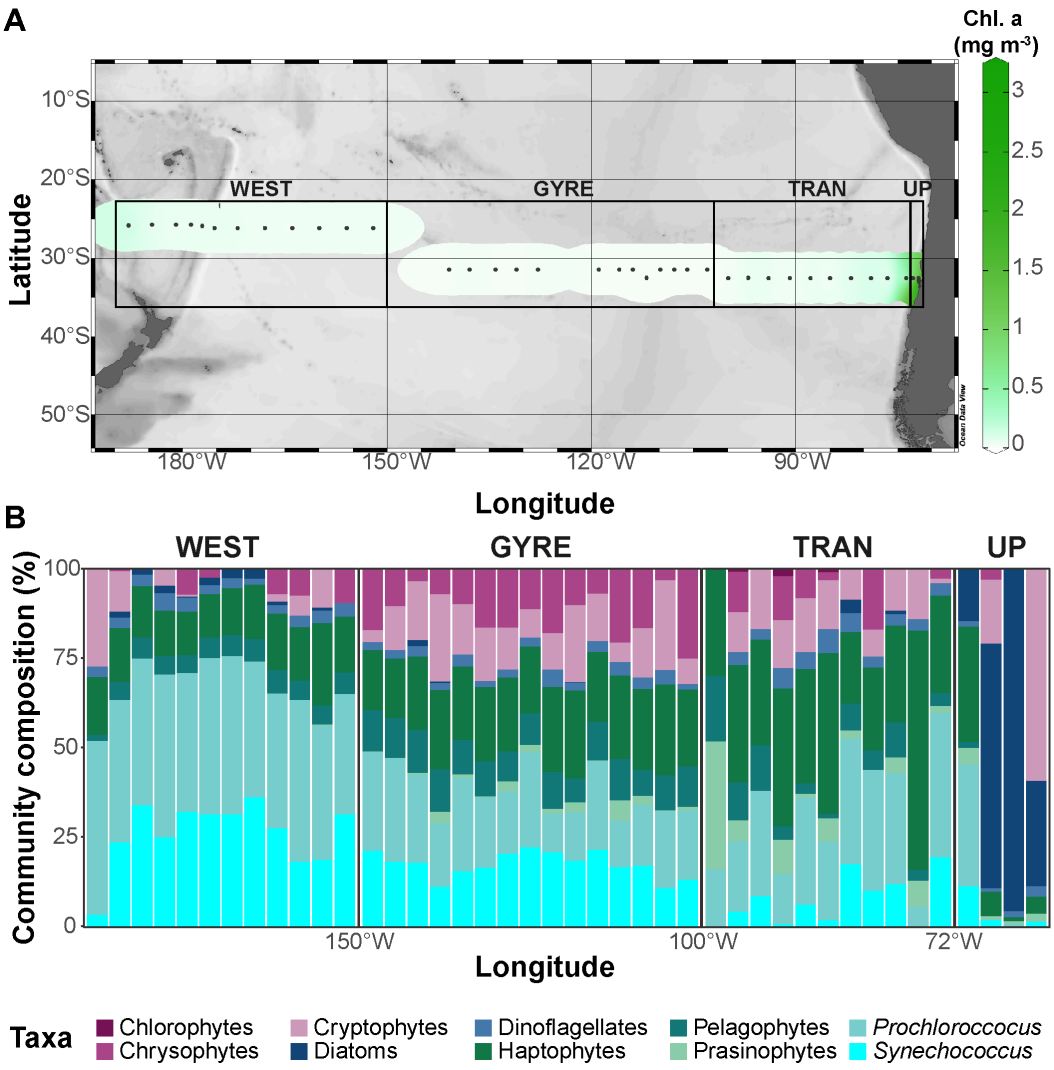



***Fig. 1 – Diverse biogeochemical regimes encountered across the South Pacific Ocean.*** *(A) Spatial grouping of the sampling stations according to four oceanic provinces: 'UP' - Chilean coast upwelling zone; 'TRAN' -*

*transition zone between the upwelling and the subtropical gyre; 'GYRE' - South Pacific subtropical gyre; 'WEST' - westernmost region. (B) The composition of the surface water phytoplankton at each station, estimated on the basis of diagnostic pigments.*

We found that bacterial abundance varied across the biogeochemical provinces (Table S1), and the taxonomic composition of the bacterial communities (Fig. 2) also differed significantly between them (PERMANOVA test; $F_{3,14}$=21.16, $R^2$=0.82, p=0.001). To investigate the physiological and metabolic processes expressed within the bacterial communities, we characterized the metaproteome of the bacterial cells, identifying between 4281-6200 cellular proteins at each station (Table S2). The composition of bacterial cellular proteins significantly varied between the four regions (PERMANOVA test; $F_{3,16}$=3.06, $R^2$=0.36, p=0.001; Fig. S2) and further differed between each pair of adjacent provinces (Table S3), indicating that these represented distinct functional ecosystems. Bacterial cell abundance was the highest in the upwelling zone (9-26 $\times 10^8$ cells $L^{-1}$), with the bacterial community composition showing a strong heterotrophic response to a diatom bloom (Fig. 2). This response was attributed mainly to members of the classes Gammaproteobacteria and Flavobacteria, which are typically active during elevated availability of organic carbon (Pontiller et al., 2022; Teeling et al., 2016). In the transition zone, bacterial abundance reached 0.7-3.5 $\times 10^8$ cells $L^{-1}$, with the cyanobacteria *Prochlorococcus* and *Synechococcus* comprising more than 50% of the bacterial community (Table S1). The elevated photosynthetic cyanobacterial activity in this zone was potentially stimulated by a nutrient influx from a returning lateral current (Liu et al., 2024). The subtropical gyre had the lowest bacterial cell abundances (0.2-0.9 $\times 10^8$ cells $L^{-1}$), with cyanobacteria comprising up to 50% of the bacterial community (Table S1). Metagenomic characterization of the bacterial community composition further suggested dominance of the oligotrophic Alphaproteobacterial order *Pelagibacterales* (Fig. 2)(R. M. Morris et al., 2002). This lineage has previously been shown to numerically dominate the bacterial communities of the ultra-oligotrophic South Pacific subtropical gyre (Oggerin et al., 2024; Reintjes et al., 2019). In the westernmost region, cell abundance was significantly higher than in the subtropical gyre (0.8-1.3 $\times 10^8$ cells $L^{-1}$; Student's t-test, p=0.002), with a predominance of *Prochlorococcus*. The photosynthetic cyanobacterial activity in this region may have been partly facilitated by input from a large volcanic eruption in the region two months prior to sampling (Zhang et al., 2024).

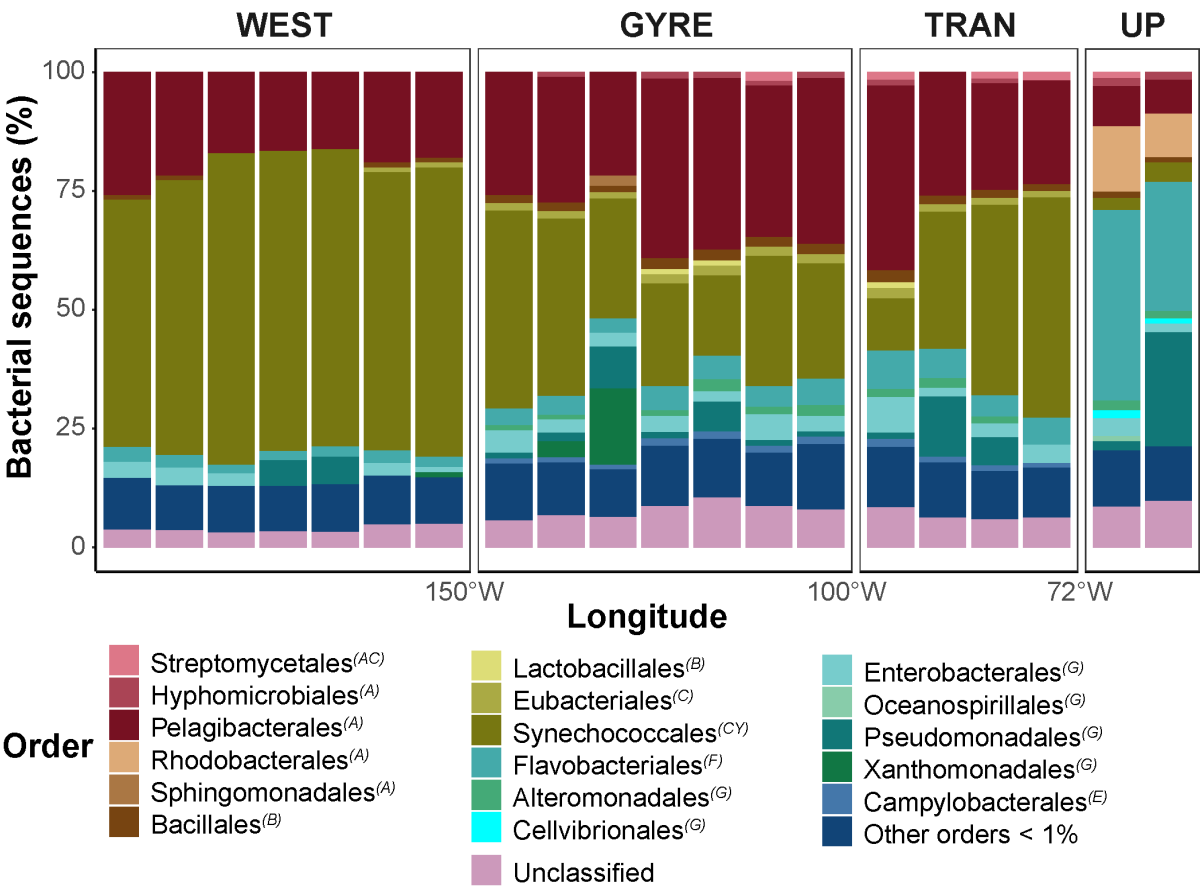



***Fig. 2 – Bacterial community composition based on taxonomic classification of metagenomic sequences.*** *The taxonomic class of each order is given in parentheses: 'AC' – Actinomycetes, 'A' – Alphaproteobacteria, 'B' – Bacilli, 'C' – Clostridia, 'CY' – Cyanophyceae, 'F' – Flavobacteriia, 'G' – Gammaproteobacteria, 'E' – Epsilonproteobacteria. 'Other orders < 1%' – refers to taxonomic orders that comprised less than 1% of the sequences; 'unclassified' – refers to bacterial sequences with unknown classification on an order level. All samples contained 2-5×10$^8$ classified sequences.*

**Abundance, characteristics, and main producers of marine BEVs**

To investigate the abundance and diversity of marine BEVs, we sampled the extracellular size fraction corresponding to BEV-like structures (100 kDa-0.22 µm) across all stations using size fractionated filtration. The size distribution of marine viruses overlaps with that of BEVs (Biller et al., 2017; Nekrouf et al., 2025; Soler et al., 2015), so we used density gradient separation to further enrich our samples for BEVs in the laboratory (see Materials and Methods). At least 50% of the purified BEVs were between 70-130 nm in diameter (Fig. 3), matching the size distributions previously observed in cultures of marine bacteria (Biller et al., 2023, 2014). Their abundance was overall comparable to the abundance of bacterial cells (Fig. 3), although this is likely a conservative estimate, since the density gradient separation led to a reduced yield of 6-52% of all nanoparticles (Fig. S3), and the storage of the samples on board at -20°C may also have resulted in a loss of BEV-like structures (Ahmadian et al., 2024).

We then characterised the bulk protein content in the BEV-designated density fraction (see Materials and Methods) and identified 1940-3394 proteins at each station (Table S1). Of these proteins only ca. 3% (58-123 proteins) were taxonomically or functionally (e.g., capsid or tail sheath) related to viruses, suggesting a strong enrichment of BEVs over other types of protein-containing marine nanoparticles in our samples. We found that 78-96% of proteins observed in the BEVs were also found in the corresponding cellular fraction at each station, but the composition of the proteins significantly varied between the two fractions (PERMANOVA test; $F_{1,38}$=15.01, $R^2$=0.28, p=0.001). This indicated that the BEV-associated proteins represented a distinct subset of the bacterial cellular proteome.

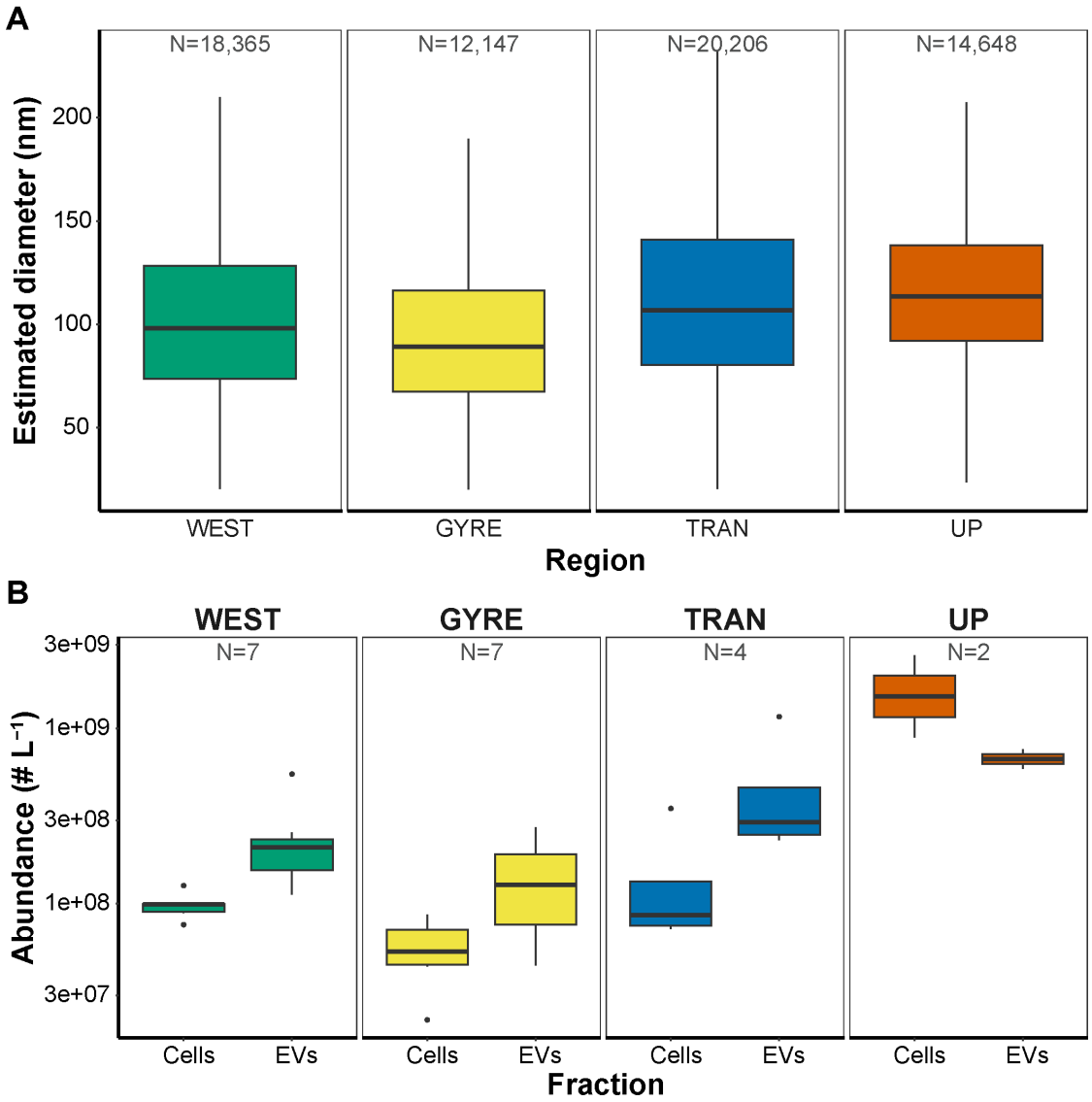
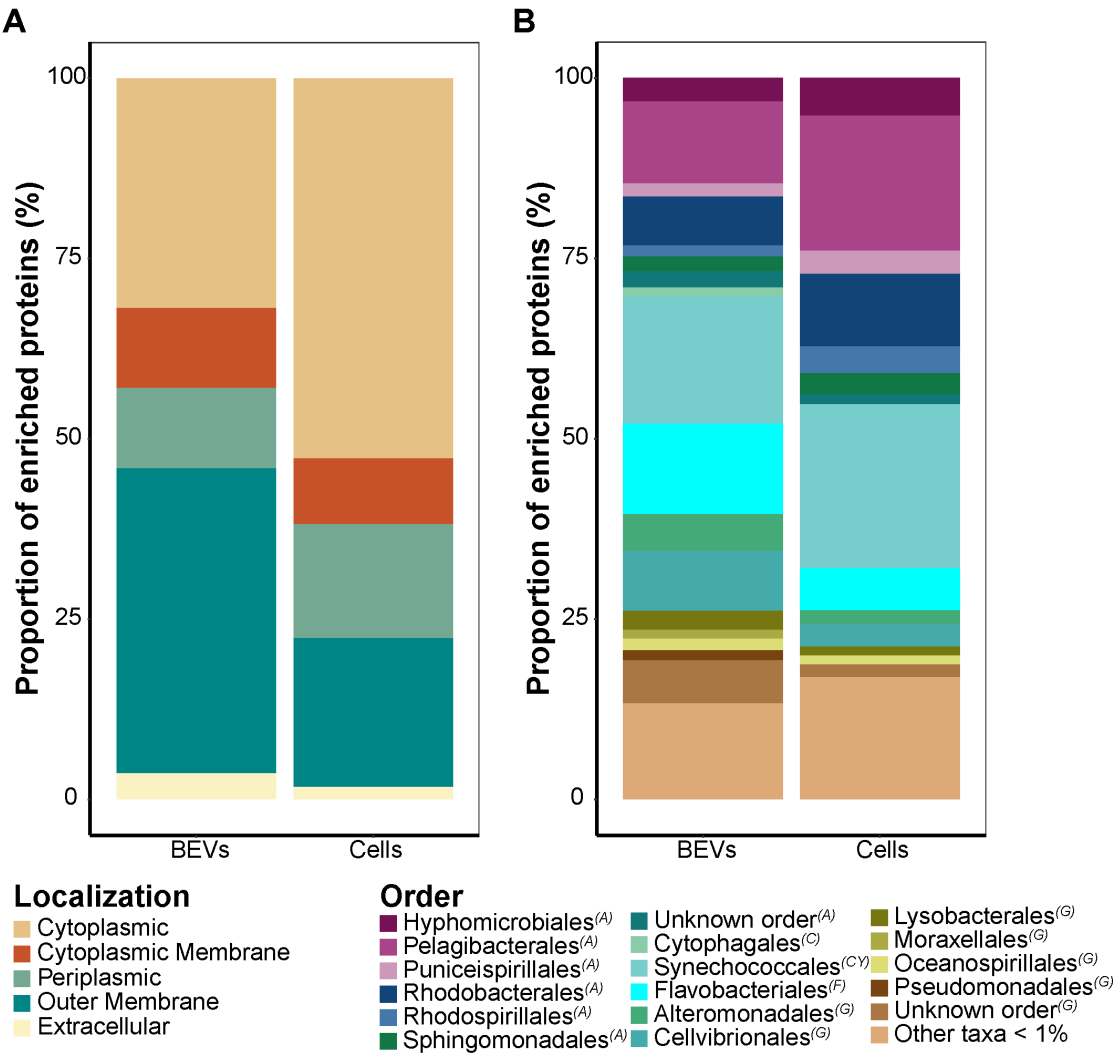



***Fig. 3 – Physical characteristics of marine BEV-like structures***. *(A) Estimated diameter distribution of the BEV-like structures. The error bars represent variation between NTA technical replicates. (B) Bacterial cell and BEVs abundances, estimated using flow cytometry and nanoparticle tracking analysis (NTA), respectively.*

Marine bacteria, which are mostly Gram-negative, can produce BEVs through blebbing of the outer membrane, leading to preferential incorporation of outer membrane and periplasmic material from the releasing cell (Kulp & Kuehn, 2010; Schwechheimer et al., 2013). External conditions have been shown to have an impact on the release of BEVs, potentially leading to some degree of preferential protein packaging (Gerritzen et al., 2018; Minhas & Greenman, 1989; Orench-Rivera & Kuehn, 2021; Sabra et al., 2003). Other BEVs may result from bacterial cell lysis, including due to bacteriophages, and thus represent a random assemblage of proteins from different subcellular origins (Pérez-Cruz et al., 2015; Toyofuku et al., 2023). To differentiate between these different origins of BEVs, we determined whether there is an enrichment in non-cytoplasmic proteins in the BEV fraction. We carried out a protein enrichment analysis on all samples in the BEV fraction as compared with the cellular fraction, and identified a total of 1065 proteins that were significantly enriched in the BEVs (absolute $\log_2$ fold difference > 1, adjusted p-value < 0.1), relative to their abundance in the cells. By comparison, 2017 proteins were significantly enriched in the cellular fraction (Table S4). Prediction of sub-cellular origins revealed that half of the BEV-enriched proteins were non-cytoplasmic, comprising mostly outer membrane and extracellular proteins, compared to ca. 25% of such proteins enriched in the cellular fraction (Fig. 4). Although we cannot exclude the presence of lysis-derived BEVs in our samples, the

178 enrichment of mostly non-cytoplasmic proteins suggests that the marine BEVs in our samples may have
179 derived primarily from membrane blebbing of bacterial cells (Toyofuku et al., 2023).



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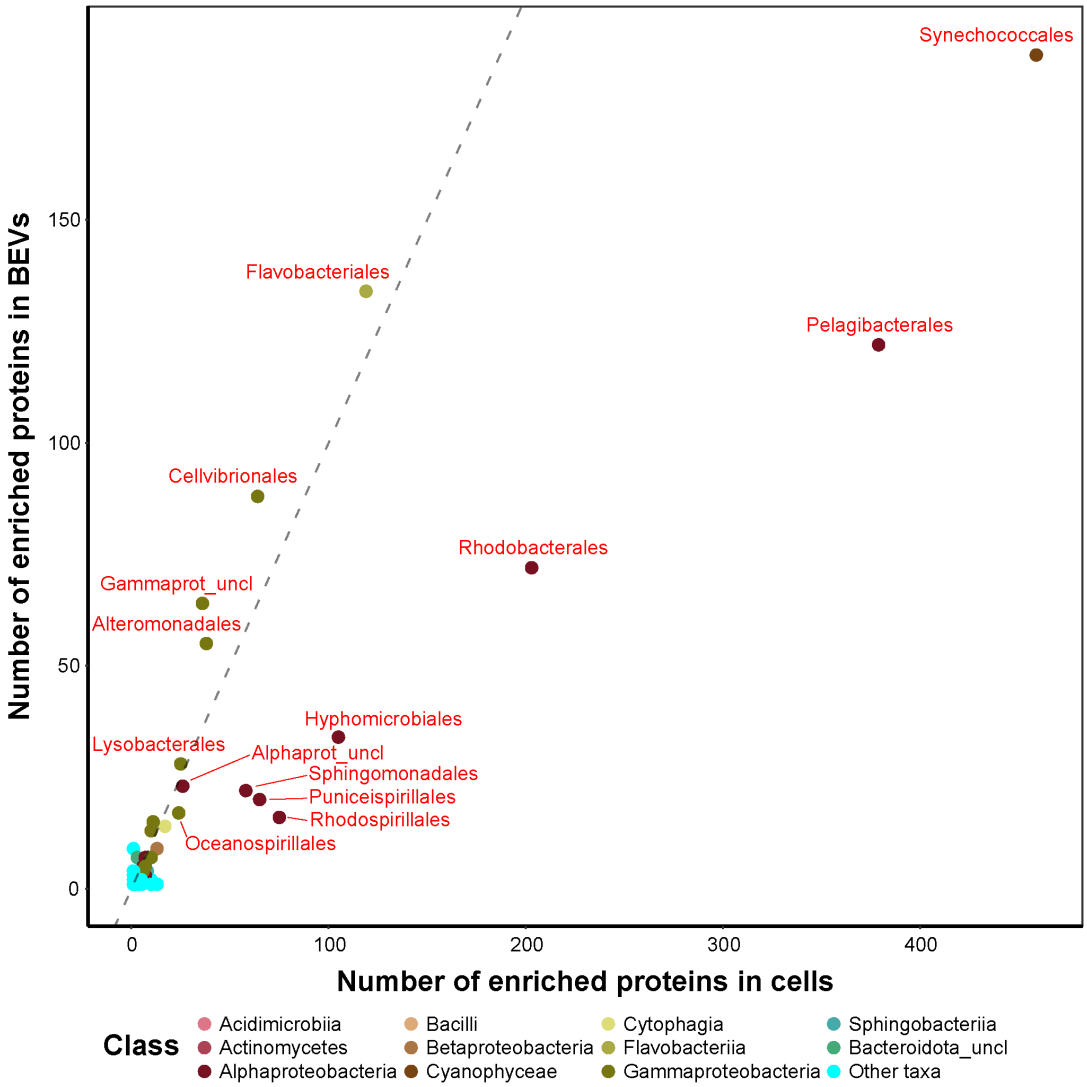
181 ***Fig. 4 – Overview of significantly enriched proteins in BEVs and cellular fractions.*** *The bar plots represent the*
182 *composition of all significantly enriched proteins according to their estimated sub-cellular localization (A) and*
183 *taxonomic origin (B). The total number of enriched proteins in the BEVs and the cellular fractions was 1121 and*
184 *2075, respectively. Taxonomic composition of enriched proteins in the cellular and BEVs fractions according to*
185 *different oceanic provinces. The taxonomic class of each order is given in parentheses: 'A' – Alphaproteobacteria,*
186 *'C' – Cytophagia, 'CY' – Cyanophyceae, 'F' – Flavobacteriia, 'G' – Gammaproteobacteria.*

187 Studies of laboratory cultures indicate that the major classes of marine bacteria exhibit significant
188 variations in BEV production rates (Biller et al., 2023). Our protein enrichment analyses from field
189 samples revealed that the two taxonomic lineages with the largest number of enriched proteins in both
190 the BEVs and in the cellular fractions were the orders *Synechococcales* (including *Prochlorococcus*
191 and *Synechococcus*) and the order *Pelagibacterales* (class Alphaproteobacteria). The ratio of enriched
192 proteins between the BEVs and cellular fractions of these lineages showed a clear tendency towards the
193 latter (Fig. 5). In contrast, enriched proteins associated with other bacterial orders, such as the
194 *Alteromonadales* and *Cellvibrionales* (class Gammaproteobacteria), and the order *Flavobacteriales*
195 (class Flavobacteriia), accounted for more than twice BEV-enriched proteins compared to the cellular
196 fraction (Fig. 5). These observations could indicate that BEVs from these lineages contain more protein
197 on average than those from Alphaproteobacteria and cyanobacteria. Alternatively, based on the

198 assumption that the number of enriched proteins reflects to some extent the abundance of BEVs, these
199 results are consistent with the previously raised hypothesis that fast-growing heterotrophic marine
200 bacteria contribute more to the BEV population than slow growing ones (Biller et al., 2023).




201

202 ***Fig. 5 – Ratio of significantly enriched proteins in BEVs and cellular fraction in each taxonomic order.*** *The*
203 *dashed line represents a theoretical ratio of 1:1 between the number of proteins enriched in each fraction. 'Other*
204 *taxa' represents classes with less than 3 enriched proteins in each of the fractions.*

205 The distinct biogeochemical conditions sampled in this study shape bacterial communities (Fig. 2),
206 which could potentially affect BEV populations and their functions (Biller et al., 2023; Orench-Rivera
207 & Kuehn, 2016). Therefore, in order to infer the potential functions of BEVs while accounting for these
208 differences, we examined the proteins enriched in BEVs separately for each oceanic province. As only
209 two stations sampled in the upwelling region, this region was excluded from further analysis. We found
210 a total of 1295 proteins (Table S5), which were associated with 77 different bacterial orders (Fig. S4),
211 that were significantly enriched in BEVs compared to bacterial cells. Different functional groups of
212 proteins were found enriched in BEVs in different oceanic provinces, suggesting that they may play a
213 role in multiple ecophysiological processes, the main ones of which are discussed below.

214 **Marine BEVs as mediators for extracellular electron transfer?**
215 The surface waters of the South Pacific receive high solar irradiance. High light levels with associated
216 enhanced UV radiation can induce oxidative stress in photosynthetic microorganisms (Mella-Flores et
217 al., 2012). Marine cyanobacteria (e.g., *Prochlorococcaceae* and *Synechococcaceae*) have developed

218 various strategies to cope with this environmental stressor, one of which is the transfer of excess
219 electrons to ferredoxins and flavoproteins (Scanlan et al., 2009). In our dataset, we observed that 10-
220 30% of all enriched cyanobacterial proteins in the BEV fraction were outer-membrane porins, compared
221 to 0.5-5% in the cellular fraction (Table S5). We also found ferredoxins and flavoproteins, which are
222 typically soluble, cytoplasmic proteins associated with the thylakoid membranes (Pierella Karlusich &
223 Carrillo, 2017), in cyanobacterial BEVs across all regions, with the strongest enrichment in the centre
224 of the subtropical gyre (Fig. 6). This enrichment under the nutrient limiting conditions may indicate that
225 such reduced electron carriers are exported in cyanobacterial BEVs as a cellular defence mechanism
226 against oxidative stress (Orench-Rivera & Kuehn, 2021).

227 Increasing light intensity, which can lead to oxidative stress in cyanobacteria, has been shown to
228 increase vesiculation rates in *Prochlorococcaceae* cultures (Biller et al., 2023; Biller, Lundeen, et al.,
229 2022). It has been also shown that marine heterotrophic bacteria, such as those belonging to the
230 *Alteromonadales*, support the growth of *Prochlorococcaceae* by scavenging excreted compounds
231 produced as a result of oxidative stress by the latter (J. J. Morris et al., 2011). Furthermore,
232 *Alteromonadales* are capable of taking up BEVs produced by *Prochlorococcaceae*, suggesting that
233 BEVs can serve as nutrient source for heterotrophic bacteria (Biller et al., 2014). Alternatively, based
234 on the demonstrated capability of various non-marine bacteria with anaerobic capabilities to acquire
235 energy from secreted flavoproteins (Marsili et al., 2008; Okamoto et al., 2014), we speculate that
236 cyanobacterial BEVs enriched with electron acceptors could also serve as a direct energy source for
237 marine heterotrophic bacteria such as the *Alteromonadales*. Given the recent observation in
238 *Prochlorococcaceae* and *Synechococcaceae* of intercellular extensions (nanotubes), containing vesicle-
239 like structures (Angulo-Cánovas et al., 2024), it is also possible that such extracellular electron transfer
240 occurs through direct contact between cells. In the ocean, such extracellular electron transfer
241 interactions are more likely to take place among particle-attached bacteria in a spatially confined
242 environment, which is often characterized by anaerobic conditions (Bianchi et al., 2018). Although this
243 study focuses on free-living marine bacteria, it is likely that our pre-filtration step, which aimed to
244 remove the particulate size fraction, disrupted any such structures present in the sample. Parts of these
245 structures may have ended up in the bulk BEVs fraction.

246 An alternative mechanism cyanobacteria use to cope with oxidative stress is photoprotection (Mella-
247 Flores et al., 2012). Interestingly, while in the regions with elevated cyanobacterial activity we observed
248 phycobilisomes enriched in the cellular fraction, in the centre of the subtropical gyre they were strongly
249 enriched in the BEVs fraction (Fig. 6). One possible explanation is that the BEV-enriched
250 phycobilisomes are damaged proteins that were removed from the cell (Schwechheimer & Kuehn,
251 2015). However, the identification of protein modifications resulting from oxidation revealed that,
252 while cell-enriched phycobilisomes exhibited up to 5 modifications, BEV-enriched phycobilisomes
253 exhibited only 0-1 modifications (Table S5). Therefore, it is conceivable that the release of
254 phycobilisomes from cells into BEVs in response to light and UV stress could be a photoprotective
255 mechanism, reducing the amount of light energy absorbed by the cell by removing light-harvesting
256 compounds. Furthermore, given the large size of cyanobacterial phycobilisomes (6.2 MDa; Domínguez-
257 Martín et al., 2022) and the assumption that BEVs remain attached to the cell, these phycobilisome-
258 enriched BEVs could provide additional extracellular shading, reducing the amount of light that reaches
259 the cyanobacterial cell.

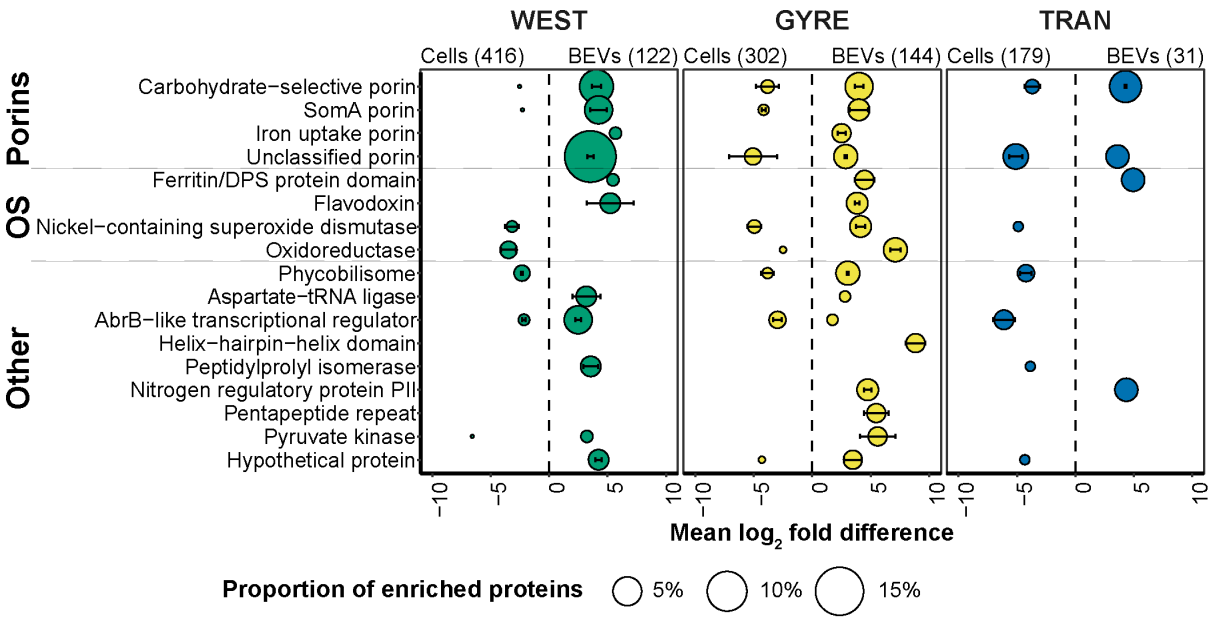



***Fig. 6 – Cyanobacterial proteins significantly enriched in BEVs and cellular fractions in different regions****. The mean $log_2$ fold difference was calculated for each functional protein group in each fraction. Positive $log_2$ fold difference represents significant enrichment in the BEVs fraction, negative mean $log_2$ fold difference represents significant enrichment in the cellular fraction. The total number of enriched proteins in the BEVs and the cellular fractions is provided in brackets. The proteins were grouped according to their proposed functions in BEVs (porins, oxidative stress proteins (OS) and other proteins). For visualisation purposes, only functional groups containing at least three enriched proteins in at least one region were included.*

**Marine BEVs as extracellular reserves of bio-available phosphate?**

The surface waters of the South Pacific subtropical gyre are one of the most oligotrophic regions in the global ocean (Morel et al., 2010). Among the bacterial lineages that successfully inhabit such limiting environment are members of the order *Pelagibacterales* (also known as SAR11 clade). Their small cell size and large periplasm, which contains many substrate-binding proteins with exceptionally high affinities (Clifton et al., 2024; Sowell et al., 2009), which facilitates their uptake of soluble substrates (e.g., phosphate, sulphate, amino acids, and sugars) from the environment (Bosdriesz et al., 2015). This enables SAR11 to dominate the bacterial communities in the ocean (R. M. Morris et al., 2002) and thrive in nutrient-depleted marine ecosystems (Giovannoni, 2017). We observed that in all regions around one third of all proteins enriched in the cellular fraction were periplasmic substrate-binding proteins annotated to bind amino acids, compared to around 10% in the BEVs fraction, which consisted of other periplasmic binding proteins, mostly with unknown substrates (Table S5). Against this backdrop, in the centre of the subtropical gyre we found significant enrichment of five different homologues of the SAR11 phosphate binding protein PstS (Fig. 7). This periplasmic binding protein has a unique structure that has been suggested to have high affinity for phosphate, as well as being capable of binding larger organic phosphorus molecules (W.-J. Zhu et al., 2025). We propose that outer-membrane porins, which were also observed among the SAR11-derived BEV-enriched proteins (Fig. 7), form channels that allow phosphate molecules to diffuse passively into the BEVs, as they would in intact cells. They become trapped there by binding to high-affinity PstS proteins, making the BEVs a potential phosphate reservoir. Moreover, the accumulation of phosphate in the periplasm is enhanced in the presence of a proton motive force (Kamennaya et al., 2020). Therefore, the observation of proteorhodopsins enriched in BEVs of SAR11 (Fig. 7) raises the possibility that light energy could, in some individual particles, further enhance the accumulation of phosphate inside BEVs in surface waters.

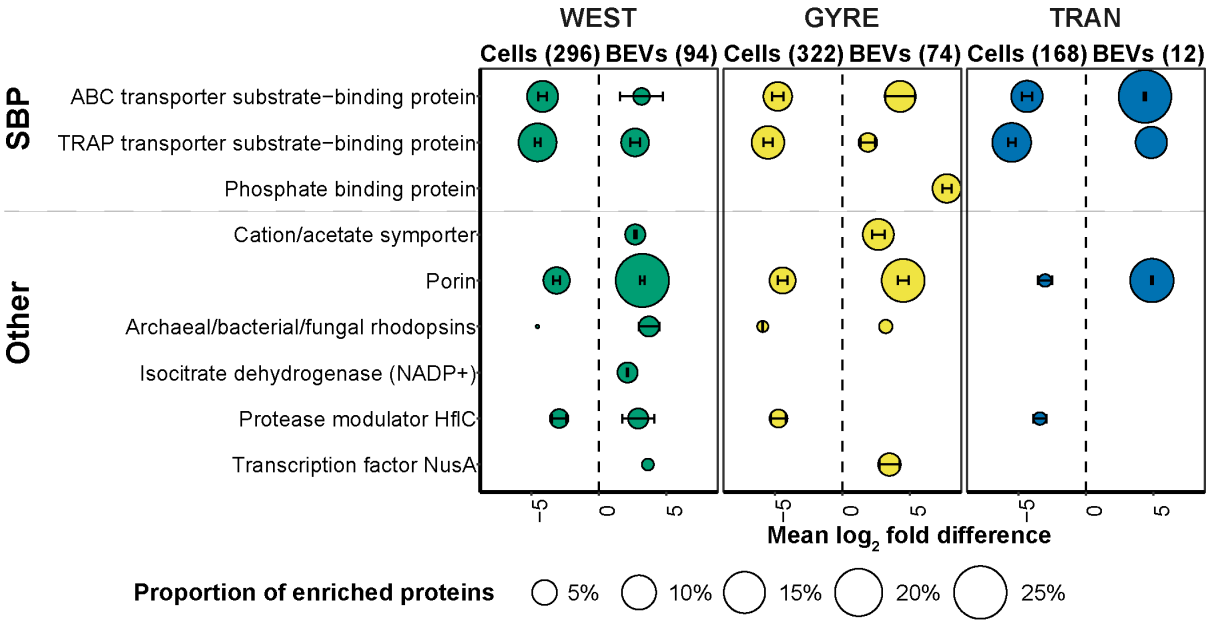



***Fig. 7 – Proteins of the SAR11 clade significantly enriched in BEVs and cellular fractions in different regions.*** *The mean $log_2$ fold difference was calculated for each functional protein group in each fraction. Positive mean $log_2$ fold difference represents significant enrichment in the BEVs fraction, negative mean $log_2$ fold difference represents significant enrichment in the cellular fraction. The total number of enriched proteins in the BEVs and the cellular fractions is provided in brackets. The proteins were grouped according to their proposed functions in BEVs (substrate-binding proteins (SPB) and other proteins). For visualisation purposes, only functional groups containing at least two enriched proteins in at least one region were included.*

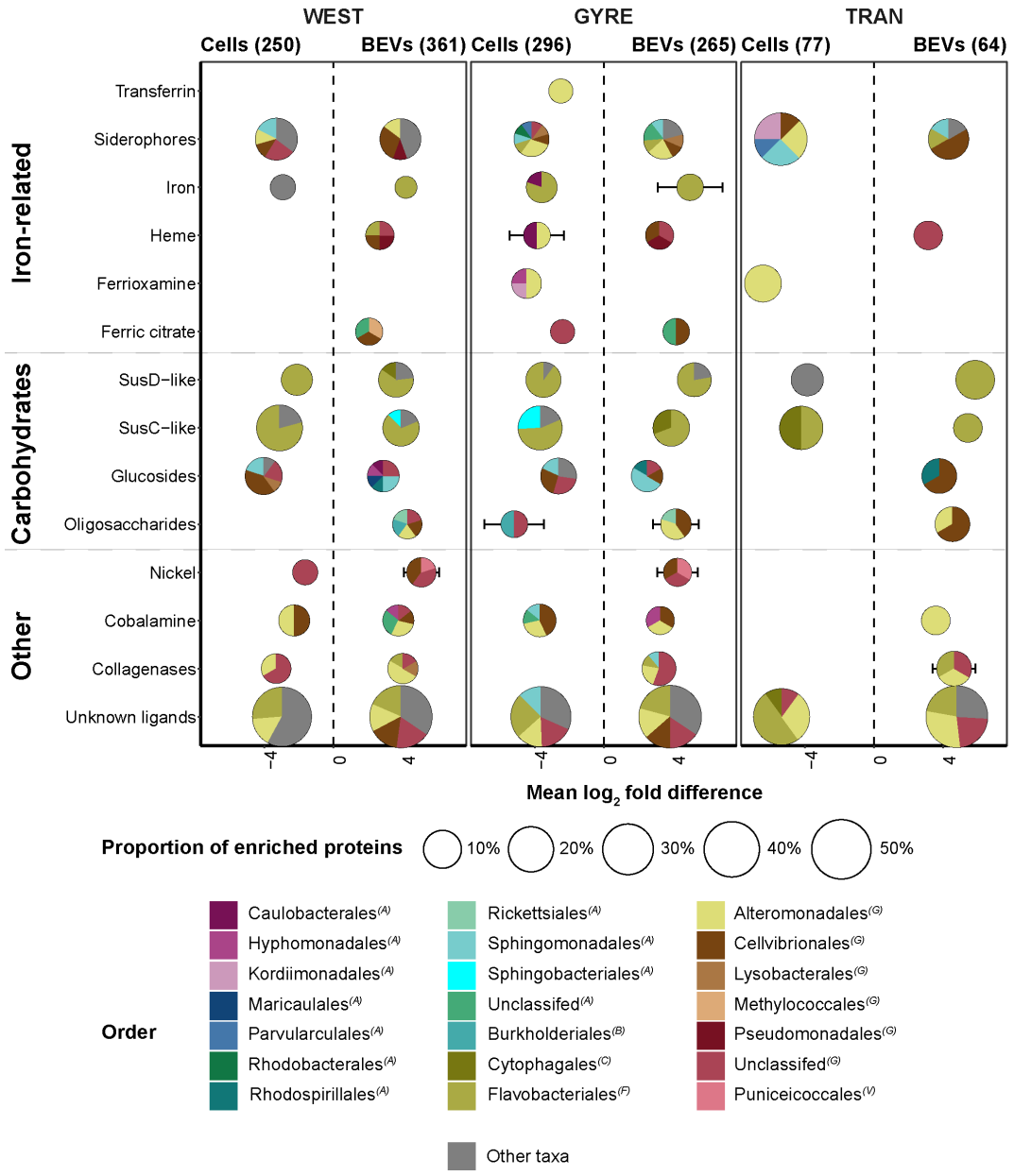
**Marine BEVs as passive scavengers of iron?**

Unlike the highly streamlined ecophysiology of SAR11, which relies mostly on passive diffusion from the environment through outer-membrane porins (Giovannoni, 2017), other heterotrophic marine bacteria actively acquire elements using specialised transport systems (Hopkinson & Barbeau, 2012; R. M. Morris et al., 2010; Tang et al., 2012). Our analysis revealed that one of the largest group of proteins significantly enriched in marine BEVs consisted of proteins related to the outer-membrane TonB-dependent transport system (Table S5). The TonB-dependent transport system is a highly diverse functional group of proteins that are involved in cellular uptake of a wide range of substrates, such as iron and iron-containing molecules (e.g., siderophores), as well as vitamins and various organic compounds (Braun, 2024), all of which were observed enriched on BEVs in our dataset.

For BEVs produced by 'blebbing' mechanisms, wherein pieces of the outermost cellular membrane detach from the cell to form the vesicle, their proteome is expected to be biased towards outer-membrane proteins (Kulp & Kuehn, 2010). Therefore, in order to determine whether TonB-related proteins are specifically enriched in BEVs, we separately tested the 3,700 outer-membrane proteins identified in our dataset for enrichment. We identified a total of 301 TonB-related proteins that were enriched in BEVs in one or more regions across the transect, compared to 229 TonB-related proteins enriched in the cellular fraction (out of a total 690 and 623 enriched outer-membrane proteins, respectively; Table S6). The majority of the BEV-enriched TonB-dependent proteins belonged to the one-component system of the outer-membrane receptor (OMR) family that perform both binding and transport of the substrate through the outer membrane. The enriched OMRs were primarily affiliated with the orders *Sphingomonadales* (class Alphaproteobacteria), *Alteromonadales* and *Cellvibrionales* (both class Gammaproteobacteria), and more than 20% of them were linked to the utilization of iron (Fig. 8 and Supplementary Text). Along with nitrate, iron was the main limiting micronutrient for surface water primary production across most of the transect (Liu et al., 2024; Yuan et al., 2025). TonB-dependent receptors are highly expressed in heterotrophic bacterial communities in the upper water column (< 100 m depth) (Bergauer et al., 2018). Their strong enrichment on BEVs has previously been

observed in cultures of related marine bacteria, including several isolates of *Alteromonadales* (Fadeev et al., 2023), an isolate of endosymbiotic marine *Cellvibrionales* (Gasser et al., 2024), and other marine Gammaproteobacteria (Dürwald et al., 2021; Frias et al., 2010). It has been also observed that iron limitation can lead to enhanced BEVs production in non-marine bacteria (Prados-Rosales et al., 2014) and preferential packaging of iron-related transporters into BEVs has been proposed to facilitate iron accumulation (Lin et al., 2017; Orench-Rivera & Kuehn, 2021; Wang et al., 2021). Taken together, these findings suggest that BEVs might play a role in extracellular scavenging of iron by marine bacteria.

To actively transport iron-containing molecules across the outer membrane, such TonB-dependent receptors require proton motive force from the inner membrane (Noinaj et al., 2010). Although BEVs are unlikely to have a proton motive force, these TonB-dependent receptors can still bind the substrates as was recently demonstrated in a culture of a pathogenic Gammaproteobacterium (Dhurve et al., 2022). Taking this into account, we propose that, in conditions of limited availability such as those observed in this study, marine BEVs may 'collect' iron in the environment. They could therefore be considered nanoscale 'hotspots' of bioavailable iron which could potentially provide a relatively concentrated source for marine bacteria.



343 ***Fig. 8 – TonB-dependent receptors significantly enriched in BEVs and cellular fractions in different regions.***
344 *The mean $log_2$ fold difference was calculated for each protein group in each fraction. Positive $log_2$ fold difference*
345 *represents significant enrichment in the BEVs fraction, negative $log_2$ fold difference represents significant*
346 *enrichment in the cellular fraction. The total number of enriched proteins in the BEVs and the cellular fractions*
347 *is provided in brackets. The proteins were grouped according to their predicated ligands. The pie charts show the*
348 *breakdown of protein taxonomic affiliations on order level within each group. The taxonomic class of each order*
349 *is given in parentheses: 'A' – Alphaproteobacteria, 'B' – Betaproteobacteria, 'C' – Cytophagia, 'CY' –*
350 *Cyanophyceae, 'F' – Flavobacteriia, 'G' – Gammaproteobacteria.*

351 **Marine BEVs as mediators of carbohydrate utilisation?**

352 The second largest functional group of TonB-dependent receptors enriched in BEVs was related to the
353 unique SusC-like proteins family (Fig. 8), which play an important role in carbohydrate utilisation by
354 marine Flavobacteria (i.e., order *Flavobacteriales*). Flavobacteria efficiently utilise various sugars
355 using a two component SusCD system, where SusC-like proteins are integral outer-membrane
356 transporters and SusD-like lipoproteins facilitate extracellular high-affinity binding of the sugars
357 (Glenwright et al., 2017). We found a total of 30 SusC-like proteins enriched in BEVs, compared to 55
358 SusC-like proteins enriched in the cellular fraction. In contrast, there were 27 SusD-like lipoproteins
359 enriched in BEVs, compared to 15 such proteins in the cellular fraction (Table S6). The stronger
360 enrichment of SusD-like lipoproteins in the BEV fraction compared to SusC suggests that flavobacterial
361 BEVs may only be able to bind to carbohydrate molecules, without the ability to transport them. This
362 phenomenon was previously observed in an isolate of marine *Flavobacteriales* (*Formosa* spp.; (Fischer
363 et al., 2019). Trapping carbohydrate molecules on the BEV membrane (or BEV getting attached to a
364 large carbohydrate) may facilitate their efficient digestion by hydrolytic enzymes, in a manner similar
365 to that observed for cellular outer membranes (Reintjes et al., 2017). While we lack such a mechanistic
366 understanding for marine BEVs, the presence of hydrolytic enzymes associated with BEVs and their
367 activity has been observed in several isolates of heterotrophic marine bacteria (Dürwald et al., 2021;
368 Fadeev et al., 2023; J. Li et al., 2016; Naval & Chandra, 2019).

369 Recent observations have shown that gut bacteria can actively alter the enzyme content of BEVs to
370 optimise carbohydrate utilisation (Sartorio et al., 2023), an analogous process may occur also in marine
371 bacteria. In our dataset, we identified an enrichment of a single glycoside hydrolase (family GH16) in
372 BEVs in the western region ($log_2$ fold difference of approximately 4), which was taxonomically
373 affiliated with *Flavobacteriales* (Table S6). The GH16 family contains a diversity of glycoside
374 hydrolases that are active on a wide variety of marine carbohydrate molecules (Viborg et al., 2019).
375 Conceptually, the association of enzymes with marine BEVs is advantageous because it allows them to
376 be delivered to the substrate in a more protected manner compared to being freely dispersed in the water
377 column, and may also allow for more efficient degradation in combination with the SusC-like and Sus-
378 D like proteins (Du Toit, 2023). When considered alongside other BEV-enriched proteins (Table S6),
379 our observations on marine BEVs are in line with previous findings regarding the composition of BEVs
380 in isolates of marine *Flavobacteriales* and *Alteromonadales* (Dürwald et al., 2021; Fischer et al., 2019).
381 In these studies, it was suggested that BEVs play a role in carbohydrate utilisation, based on their
382 transporter and hydrolytic enzyme content. Previous work has shown that marine bacteria release
383 hydrolytic enzymes into the extracellular milieu to facilitate carbohydrate utilisation during periods of
384 increased phytoplankton activity (Arnosti et al., 2021) - a phenomenon observed in the transitional zone
385 and the western regions of the transect. This suggests that some of the secretion occurs via marine
386 BEVs, potentially affecting degradation rates or access to the products.

387 **Is the production of marine BEVs a 'selfish' trait or a 'public good'?**

388 The organisms our data indicate to produce most of the BEVs can be found both planktonic (i.e., free-
389 living) and associated with particles (including phytoplankton)(Ivars-Martinez et al., 2008; Seymour et
390 al., 2017). In contrast to the relatively confined particle-associated environments (e.g., biofilms), it is
391 unlikely that BEVs released by a planktonic cell directly into seawater will be encountered again by the

same, or a highly similar, bacterial cell (Kashtan et al., 2014; Słomka et al., 2023). This led to the initial hypothesis that marine BEVs likely serve to dispose harmful elements or act as a defence mechanism against viral infection (Biller et al., 2014). However, it has been shown that in some cases the uptake of BEVs only occurs between closely related bacterial lineages (Wang et al., 2021), suggesting that there may be mechanisms that bias interactions between BEVs and recipient cells (Knoke et al., 2020). Thus, BEV production could represent a mechanism through which cells compete for nutrients in the marine environment, potentially favouring only closely related phylogenetic populations.

The environmental observations of natural bacterial communities in this study, alongside previous observations of marine bacterial cultures, may also suggest other scenarios. One alternative explanation is that marine BEVs fulfil their intended function while remaining within the diffusive boundary layer (Stocker & Seymour, 2012). Another possible scenario is that the BEVs remain attached to the cell. Some 'selfish' marine bacteria appear to have developed means of producing BEVs that are found in chains attached to the cell. This trait was previously observed in isolates of marine *Alteromonadales* and *Flavobacteriales* (Dürwald et al., 2021; Fischer et al., 2019). Chains of such outer membrane-attached BEVs significantly increase the surface area through which the cell interacts with its environment, facilitating the accumulation of elements necessary for life in the vicinity of the cell and their subsequent uptake. Taking into account the potential stability of marine BEVs for at least a number of days (Biller et al., 2014), they may continue to perform their function (e.g. accumulating phosphate or iron) even after detachment from the cell. Therefore, whether separated from cells or released directly into the environment, marine BEVs could also be considered a 'public good' that meets the various needs of the bacterial community.

# Conclusions

Here we have explored BEVs contents within highly diverse natural marine bacterial communities on an extensive oceanic scale in the South Pacific. We found that marine BEVs are ubiquitous in surface seawater and possess a substantial degree of protein diversity. The enrichment of functionally specific proteins in BEVs, which appears to be linked to the ecological conditions experienced by the local bacterial community, which suggests BEVs may contribute to marine biogeochemistry and microbial community metabolism. These findings reinforce the emerging hypothesis that BEVs play a role in nutrient and energy scavenging in the marine environment. Our study provides initial observations on the novel functional potential of marine BEVs, such as their ability to perform local enrichment of molecules necessary for life, which motivate future investigations into the underlying mechanisms. To develop a better understanding of the role of BEVs in the ecophysiology of marine bacteria, research into their uptake dynamics is urgently needed. This is a significant knowledge gap regarding the relationship between BEVs and bacterial cells, which could help us understand whether BEVs are a 'public good' or a competitive feature of marine bacterial communities. Based on the extensive oceanographic observations presented in this study, the next stage of the research requires the quantification of the contribution of BEVs to the distribution of essential life-sustaining elements (e.g. iron and phosphate) in the marine environment. This will shed light on their importance for microbial processes in the ocean.

# Materials and methods

**Sample collection**

The oceanographic sampling was conducted onboard RV Sonne (cruise SO289 representing section GP21 of the GEOTRACES programme) from 18 February to 8 April 2022 (Fig. 1A). Water samples with a total volume of 60 L were taken from a depth of ca. 6 m at each sampling station using the onboard water pump. To remove particulate matter and eukaryotic cells, water samples were pre-filtered at low pressure using a 3 µm pore size membrane filter (Merck Millipore, MA, USA). From the pre-filtered water sample, 1 mL was preserved with 4% glutaraldehyde (final concentration) and stored at -

80°C until flow cytometric analysis. The pre-filtered seawater sample was then filtered through a 142 mm diameter polycarbonate filter with a pore size of 0.22 µm (Merck Millipore, MA, USA), which were then stored at -80°C and later used for molecular analyses of the bacterial communities. The filtrate (< 0.22 µm) was concentrated ca. 60 times using a Vivaflow tangential flow filtration system (Sartorius, Germany) with a 100 kDa cut-off to 1 L and stored at -20°C until further BEVs purification in the laboratory.

**Phytoplankton pigments analysis**

Seawater samples (2-4 L) for diagnostic phytoplankton pigments were filtered onto MF300 glass fibre filters (ThermoFisher Scientific, MA, USA) and stored at -80°C until analysis. Pigments extraction and analysis followed the procedure described by (Browning et al., 2017). Briefly, upon return to a land-based laboratory, pigments were extracted in 90% acetone by homogenization of the filters using glass beads in a cell mill, centrifuged (10 min, 5200 rpm, 4 °C). The supernatant was filtered through 0.2 µm PTFE filters (VWR, PA, USA) and subsequently analysed by reverse-phase high performance liquid chromatography (HPLC) Dionex UltiMate 3000 LC system (ThermoFisher Scientific, MA, USA)(Van Heukelem & Thomas, 2001). Diagnostic pigments were converted to estimated contributions of different phytoplankton types using CHEMTAX (Mackey et al., 1996), with initial pigment ratios according to (DiTullio et al., 2003). Total chlorophyll-a derived from HPLC include both chlorophyll-a and divinyl chlorophyll-a, and is referred to as chlorophyll-a throughout this study.

**Bacterial cell counts**

A subsample of 100 µL of seawater from each sampling stations was stained with 1×SYBR Green I (Thermo Fisher Scientific, MA, USA) for 15 min at room temperature and then quantified using FACSAria Flow Cytometer (BD Biosciences, NJ, USA). Counts were processed using R package 'flowWorkspace' v4.18.0 (Finak & Jiang, 2022). Quality control of the FCS files was carried out using 'flow_auto_qc' function in R package 'flowAI' (Monaco et al., 2016). The stained cells were subject to logicle transformation and quantified using the 'gate_flowclust_2d' function in R package 'openCyto' (Finak et al., 2014), applied on the entire dataset (Fig. S5).

Cell abundances of *Prochlorococcus* and *Synechococcus* were reported by (Yuan et al., 2025) at locations different from those sampled in this study but from the same biogeochemical provinces. Therefore, the abundance of these cells was used to roughly estimate the contribution of cyanobacteria to the total bacterial abundance.

**BEVs isolation and purification**

A sub-sample of 100 mL of concentrated seawater was further concentrated about 30 times to a final volume of 3 mL using Vivaspin centrifugal filter units (Sartorius, Germany) with a 100 kDa cut-off. The entire concentrate was then loaded onto an iodixanol density gradient following a published protocol (Biller, Muñoz-Marín, et al., 2022), and centrifuged at 100,000×g at 4°C for 6 h. In the 15-30% and 35-40% fractions, the iodixanol solution was exchanged twice with 1×PBS using Vivaspin centrifugal filter units with a 100 kDa cut-off at 2000×g, and resuspended in 1 mL 1×PBS. From each purified fraction, 800 µL was used for molecular analyses and 200 µL for quantification of BEVs.

**Nanoparticle tracking analysis**

The abundance of BEVs was measured by nanoparticle tracking analysis (NTA) using a NanoSight NS300 instrument (Malvern Panalytycal, UK) equipped with a 488 nm laser. Each sample was injected using a syringe pump with a flow speed of 100 µL $min^{-1}$, and five videos of 60 sec were recorded. Videos were analysed using NanoSight NTA software v3.2. All particles in the size up to 300 nm in both the 15-30% and 35-40% fractions were included in the final abundance estimates. The flow cell was thoroughly flushed with Milli-Q water (Merck Millipore) between samples, and visually examined to exclude carry over. The performance of the instrument was routinely checked by measuring the concentration and size distribution of standardized 100 nm silica beads. To estimate the background, we quantified nanoparticles in Optiprep media that was subject to the same washing steps with 1×PBS as the BEVs samples, yielding concentrations of ca. $10^5$ particles $mL^{-1}$, with most particles being below 50 nm in size.

489 **Metagenomics workflow**
490 One third of the polycarbonate filters with a pore size of 0.22 µm were used for the extraction of
491 genomic DNA using the DNeasy PowerWater Kit (QIAGEN N.V., Germany) according to the
492 manufacturer's recommendations. The extracted DNA was subject to enzymatic shearing and then
493 shotgun sequencing, performed on two lanes of the NovaSeq S4 platform (Illumina, Inc., CA, USA)
494 at the Vienna Biocenter Core Facilities (https://www.viennabiocenter.org/vbcf/ next-generation-
495 sequencing/).

496 The sequences from all samples were subject to the snakemake (Köster & Rahmann, 2012)
497 metagenomic workflow in Anvi'o v8 (Eren et al., 2021), with recommended parameters. Briefly, the
498 raw FASTQ files were subject to quality control by 'illumina-utils' v2.13 (Eren et al., 2013) and
499 taxonomic classification using KrakenUniq v1.0.4 (F. P. Breitwieser et al., 2018) and analysed using R
500 package 'pavian' v1.2.1 (Florian P. Breitwieser & Salzberg, 2020). Then the reads were assembled
501 using MEGAHIT v1.2.9 (D. Li et al., 2015) and the gene calling was performed using Prodigal v2.6.3
502 (Hyatt et al., 2010).

503 Taxonomic affiliation of the protein coding genes was estimated using Kaiju v1.9.2 (Menzel et al.,
504 2016) against the NCBI RefSeq database (date: 2024-08-14), followed by classification of sequences
505 with unknown taxonomic affiliation against the NCBI nr database (date: 2024-08-25). Functional
506 annotation of the protein coding genes was carried out against InterPro v98 (Blum et al., 2025),
507 NCBIfam v17.0 (Wenjun Li et al., 2021), and Pfam v37 (Mistry et al., 2021) databases using
508 InterProScan v5.66 (Jones et al., 2014). The unidentified proteins sequences with no hits, were assigned
509 to their taxonomy and function using BLASTp (Camacho et al., 2009) best-hit approach against NCBI
510 RefSeq database (date: 2025-01-27). Protein sub-cellular localization predictions were established
511 using DeepLoc v2.0 (Thumuluri et al., 2022). Porins and TonB-dependent receptors were further
512 classified using PSIBLAST (Camacho et al., 2009) against the Transporter Classification Database
513 (TCDB) (Saier et al., 2006), best hit was defined based on identity > 30%, e-value < 0.001 and bit score
514 > 50.

515 To construct the database for downstream metaproteomic analysis, bacterial protein coding genes were
516 clustered at 99% similarity using CD-HIT v4.6.881 (Weizhong Li & Godzik, 2006).

517 **Proteomics workflow**
518 We used the remaining two thirds of the 0.22 µm pore-size polycarbonate filters for extraction of
519 cellular proteins. Protein extraction from the filter was performed as described elsewhere (Tinta et
520 al., 2020). Briefly, the filters were ground into small pieces with a sterile metal spatula after
521 submerging the tubes with the filters into liquid nitrogen. Filter pieces were resuspended in lysis
522 buffer (100mM Tris-HCl pH 7.4, 1% SDS, 150mM NaCl, 1mM DTT, 10mM EDTA) and cells were
523 lysed with five freeze-and-thaw cycles. After centrifugation (20,000x *g* at 4°C for 25 min) the
524 supernatant was transferred into a tube and proteins were co-precipitated with 0.015% deoxycholate
525 and 7% trichloroacetic acid (TCA) on ice for 1h and washed twice with ice-cold acetone. Dried
526 protein pellets were resuspended in 50 mM TEAB buffer (Millipore Sigma, Burlington, MA, USA)
527 and cysteines were reduced and alkylated with 10 mM DTT and 55 mM iodoacetamide (IAA),
528 respectively. To extract proteins associated with the BEVs, we added the sample reducing agent
529 NuPAGE (Invitrogen, Waltham, MA, USA) to 600 µL of purified BEVs at 1X final concentration.

530 All samples were re-precipitated using 9 times the sample volume of 96% EtOH at -20°C overnight.
531 Pellets were resuspended in 50 mM TEAB, followed by overnight in-solution trypsin (Roche, Basel,
532 Switzerland) digestion (1:100, w/w) at 37°C. TFA was added to the samples at 1% final
533 concentration to terminate trypsin digestion. Samples were desalted using Pierce C18 Tips
534 (ThermoFisher Scientific, MA, USA) according to the manufacturer's protocol. Prior to the LC-MS
535 analysis, digested peptides were dissolved in 0.1% formic acid and 2% acetonitrile.

536 Purified tryptic peptides were dissolved in 0.1% formic acid (FA) and measured using a nano-reversed-
537 phase high-performance liquid chromatography (RP-HPLC) system Dionex Ultimate 3000
538 (ThermoFisher Scientific, MA, USA), coupled with a benchtop Quadrupole Orbitrap mass spectrometer

Q-Exactive Plus (ThermoFisher Scientific, MA, USA). Peptides were separated on a PepMap RSLC C18 column (ThermoFisher Scientific, MA, USA) at 55°C with a flow rate of 300 nL/min. The two-hour segmented LC gradient ranged from 5% to 80% buffer B (79.9% acetonitrile, 0.1% FA). Mass spectra were acquired in positive ion mode using a top-15 data-dependent acquisition method. Full MS scans were performed at a resolution of 70,000 (*m/z* 200), followed by MS/MS scans at a resolution of 17,500. High-energy collisional dissociation (HCD) fragmentation was applied with a normalized collision energy (NCE) of 30%. Dynamic exclusion was set to 60 s.

The MS raw files of proteins extracted from the bacterial cells and the BEVs were processed using the Spectrum Files RC node in Proteome Discoverer v2.2.0.338 (ThermoFisher Scientific, MA, USA) and searched using SEQUEST-HT against the bacterial protein coding genes from the co-assembled metagenome. Search parameters were as follows: enzyme - trypsin, fragment mass tolerance - 0.8 Da, max. missed cleavages - 2, fixed modifications - carbamidomethyl (Cys), optional modifications - oxidation (Met). Percolator parameters were as follows: max. delta Cn: 0.6, max. rank: 0, validation based on q-value, false discovery rate (calculated by automatic decoy searches) 0.05. Abundance of each protein was estimated by summing the abundance of unique and razor peptides with at least 2 Peptide Spectrum Matches (PSMs). Only proteins with at least one unique peptide and at least two peptides in total (unique and/or razor peptides) were included in the statistical analyses.

**Statistical analyses**

Statistical analyses were done in R v4.4.2 (R Core Team 2022) using RStudio v2024.09.1 (RStudio Team 2019). Prior to statistical analyses, the protein abundance matrix was $\log_2$-transformed and median-centred. Protein enrichment analysis was performed using the R package 'DEqMS' v1.22.0 (Y. Zhu, 2018). Only those proteins were included that were observed in at least two samples in each fraction. The criteria for a significantly enriched protein were an absolute $\log_2$ fold change > 1 and adjusted p-value < 0.1. All figures were generated using the R package 'ggplot2' v3.5.1 (Villanueva & Chen, 2019).

**Data availability**

The metagenomic raw sequences have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under Project accession number PRJEB88044. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Deutsch et al., 2023) via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD062682.

Scripts for data processing and statistical analyses, as well as raw flow cytometry and NTA files, can be accessed via Zenodo (https://www.zenodo.org/) under doi: 10.5281/zenodo.15772578.

## Author contributions

EF performed fieldwork, the bioinformatic and statistical analyses, and wrote the manuscript. EF and GJH designed the project. EF, NO, TT performed the protein extraction and LAS carried out the LC-MS measurements and wrote the corresponding methods section. HL, ZY and TJB performed the phytoplankton pigment sampling and analyses. DS and SJB contributed to data interpretation and manuscript writing. All authors approved the submitted version of the article.

## Acknowledgments

We thank the captain, the crew and the scientific team of the SO289 research expedition ('S Pacific GEOTRACES', funded by the Bundesministerium für Bildung und Forschung (BMBF), Förderkennzeichen 03G0289NA). We thank A. Mutzberg, D. Jasinski, F. Evers, T. Schott, K. Nachtigall and T. Klüver for technical support. We thank Z. Steiner for his support during the expedition. M. Aubert for assistance with laboratory sample processing. EF acknowledges funding by the Austrian Science Fund (FWF) M2797-B. This work was also supported by the Austrian Science Fund (FWF) project I4978-B to GJH and by the Slovenian Research Agency (ARIS) project J7-2599 to TT.

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